August 2016

The Mechanism of Activation of Potassium Dependent and Potassium Independent Asparaginases from Common Bean (Phaseolus vulgaris)

Ebenezer Oluwafemi Ajewole
The University of Western Ontario

Supervisor
Dr. Frederic Marsolais
The University of Western Ontario

Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

© Ebenezer Oluwafemi Ajewole 2016

Follow this and additional works at: https://ir.lib.uwo.ca/etd
Part of the Biochemistry Commons

Recommended Citation
https://ir.lib.uwo.ca/etd/3872

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact tadam@uwo.ca.
Abstract

L-asparaginases play an important role in nitrogen mobilization in plants. This study investigated the biochemical and biophysical properties of potassium dependent (PvAspG1) and potassium independent (PvAspG-T2) L-asparaginases from *P. vulgaris*. Previous studies revealed that PvAspG1 requires potassium for catalytic activation and crystal structure analysis suggested that Ser-118 in the activation loop plays a critical role in alkali metal coordination. This amino acid residue is replaced by an isoleucine in PvAspG-T2. Reciprocal mutants of the enzymes were produced and the effect of the amino acid substitution on the kinetic parameters, secondary structure conformation, allosteric effector binding and pH profile were studied. Introduction of the serine residue conferred potassium dependent activity within PvAspG-T2. Potassium elicited a similar conformational change in PvAspG1 and PvAspG-T2-I117S, as determined by circular dichroism spectroscopy. However, no change in conformation was observed for PvAspG-T2 and PvAspG1-S118I. The presence of the serine residue was associated with a narrow pH profile, with a sharp peak in enzyme activity between pH 6.5-7 and pH 8.0, suggesting that potassium influences the ionization of catalytic residues in the active site. Together, these results indicate that Ser-118 of PvAspG1 is essential and sufficient for potassium activation of plant L-asparaginases.

Keywords

L-asparagine, L-asparaginase, N-terminal nucleophile hydrolase, potassium activation, conformational change, circular dichroism, isothermal titration calorimetry
Acknowledgments

I am grateful to God Almighty for being my source, for His grace alone has seen me this far. I would like to thank my supervisor Dr. Frederic Marsolais, for the privilege to work on this project. I sincerely appreciate your support, mentorship and open-door policy. Working with you has been a great and wonderful experience.

I greatly appreciate the advice and kind assistance of my co-supervisor, Dr. Robert Cumming. Thank you for your valuable suggestions and taking the time to review the proposal and thesis drafts. A special thank you to members of my advisory committee: Dr. Rima Menassa and Prof. Norman Huner, for their availability, kind suggestions, advices and guidance at all levels of the research project.

My experience in the Marsolais laboratory would not have been so great without the wonderful people I call friends and colleagues. First I would like to thank the lab technician, Agnieszka Pajak for technical support. I thank Dr. Liliana Santamaria-Kisiel for her expert advice on the circular dichroism and isothermal titration calorimetry techniques. Many thanks to the present and past lab mates for their kind help, educative discussions and advice along the way like, Dr. Marwan Diapari, Sudhakar Pandurangan, Jaya Joshi, Shrikaar Kambhampati, Jeffery Palmer, Cecilia Viscarra, Gabrielle Song, Katie Clarke, thanks for making the lab an interesting place to work. I would also like to appreciate the help of Lee-Ann Briere, the facility manager at the Bimolecular and Interaction and Conformation Facility (BICF), for her expertise and help on the circular dichroism and isothermal titration calorimetry experiments. Many thanks to Dr. Justin Renaud for his help on ESI-MS analysis.

My gratitude also goes to the management, staff and friends at the London Research and Development Center - Agriculture and Agri-Food Canada, for providing a supportive and stimulating work environment. I thank Western University and the Biology department for the opportunity and support throughout the completion of my degree.

Finally, I wish to thank my family and friends for their support and prayers.
Table of contents

Abstract ........................................................................................................................................ i
Acknowledgments ....................................................................................................................... ii
Table of contents ........................................................................................................................ iv
List of Tables ............................................................................................................................... vii
List of Figures ............................................................................................................................. viii
List of Abbreviations ................................................................................................................... x

Chapter 1: Introduction .................................................................................................................. 1

1.1 Background .............................................................................................................................. 1
1.2 L- Asparagine .......................................................................................................................... 2
1.3 Regulation and Function of L-Asparaginase ........................................................................... 5
1.4 Plant L-Asparaginase Structure .............................................................................................. 9
1.5 Hypothesis .............................................................................................................................. 14
1.6 Objectives ................................................................................................................................ 14

Chapter 2: Materials and Methods ............................................................................................... 16

2.1 Cloning of PvAspG1 and PvAspG-T2 from P. vulgaris seeds ................................................. 16
2.2 Site Directed Mutagenesis ....................................................................................................... 17
2.3 Production and Purification of His-tagged Proteins ................................................................. 18
2.4 Protein Quantification and SDS-PAGE Analysis of Recombinant L-Asparaginases ............... 19
2.5 Estimation of Protein Molecular Weight Subunits by ESI-MS ................................................ 19
2.6 Desalting of Asparaginase and Purification of Asparagine ..................................................... 19
2.7 Enzymatic Assays ................................................................................................................... 20
2.7.1 Determination of Kinetic Parameters of L-asparaginase for L-Asparagine ....... 20
2.7.2 Determination of L-asparaginase pH Profile ..................................................... 21
2.7.3 Determination of Kinetic Parameters of L-asparaginase for Potassium ........... 21
2.8 Isothermal Titration Calorimetry ........................................................................ 21
2.9 Circular dichroism spectrometry ......................................................................... 22
2.9.1 Effect of Point Mutation and Potassium on the Secondary Structures of  P. vulgaris L-Asparaginase ...................................................................................... 23
2.9.2 Effect of Point Mutation and Potassium on the Stability of P. vulgaris L-Asparaginase ........................................................................................................... 23

Chapter 3: Results ........................................................................................................ 25
3.1 Ser-118 of PvAspG1 is Necessary for Potassium Activation .............................. 25
3.2 Ser-118 is Required for Potassium Binding as Evidenced by Isothermal Titration Calorimetry ........................................................................................................... 31
3.3 Binding of Potassium is Associated with Similar Conformational Changes .... 37
3.4 Point Mutation and Potassium does not Affect Plant L-Asparaginase Stability .... 41
3.5 Potassium Activation of PvAspG1 and PvAspG-T2-I117S is Associated with a Narrow Optimal pH Profile ................................................................. Error! Bookmark not defined.

Chapter 4: Discussion .................................................................................................. 46
4.1 Research Overview ............................................................................................... 46
4.1.1 Structure – Function Architecture of Potassium Binding in Plant L-Asparaginase .................................................................................................................. 47
4.1.2 Mechanism of Potassium Selectivity of Plant L-Asparaginase and other Potassium Activated Enzymes ........................................................................... 50
4.1.3 Potassium and Ser-Ile-118 Substitution does not Improve P. vulgaris L-asparaginase Stability ................................................................................................. 52
4.2 Plant L-asparaginase: A potential enzyme with anti-leukemic properties............ 53

Chapter 5: Conclusion........................................................................................................ 55

References.........................................................................................................................57

Curriculum Vitae .............................................................................................................. 65
List of Tables

Table 1 Apparent kinetic parameters of recombinant asparaginases in the presence or absence of 50 mM KCl .......................................................... 33

Table 2 Kinetic parameters of recombinant asparaginases for potassium .................................. 34

Table 3 Thermodynamic parameters of potassium binding to P. vulgaris L-asparaginases determined by ITC .................................................................................. 34

Table 4 Percentage estimation of the secondary structure conformation of L-asparaginases in the presence or absence of 10 mM KCl .................................................. 40

Table 5 Unfolding temperature of P. vulgaris L-asparaginases in the presence and absence of potassium .................................................................................. 42
List of Figures

Figure 1.1 Schematic pathways of asparagine metabolic pathways. .............................. 4
Figure 1.2 Phylogenetic tree of selected plant L-asparaginases .................................. 10
Figure 1.3 Amino acid sequence alignment of PvAspG1 and PvAspG-T2.................. 11
Figure 1.4 A cartoon representation of the catalytic apparatus of PvAspG1............. 15
Figure 3.1 SDS-PAGE of wild-type and mutant asparaginases. ............................... 28
Figure 3.2 ESI-MS analysis of recombinant PvAspG1 showing the molecular weight of α subunit of PvAspG1.......................................................... 29
Figure 3.3 ESI-MS analysis of recombinant PvAspG1 showing the molecular weight of β subunit of PvAspG1.......................................................... 29
Figure 3.4 ESI-MS analysis of recombinant PvAspG1 showing the molecular weight of α subunit of PvAspG-T2.......................................................... 30
Figure 3.5 ESI-MS analysis of recombinant PvAspG1 showing the molecular weight of β subunit of PvAspG-T2.......................................................... 30
Figure 3.6 Representative ITC data for potassium binding to PvAspG1(A) and PvAspG1-S118I (B).......................................................... 35
Figure 3.7 Representative ITC data for potassium binding to PvASP-G-T2 (A) and PvAspG-T2I117S (B). .......................................................... 36
Figure 3.8 Overlay Circular dichroism (CD) spectra of PvAspG1 in the presence and absence of 10 mM KCl .......................................................... 38
Figure 3.9 Overlay Circular dichroism (CD) spectra of PvAspG1S118I in the presence and absence of 10 mM KCl .......................................................... 38
Figure 3.10 Overlay Circular dichroism (CD) spectra of PvAspG-T2 in the presence and absence of 10 mM KCl ......................................................... 39
Figure 3.11 Overlay Circular dichroism (CD) spectra of PvAspG-T2I117S in the presence and absence of 10 mM KCl ......................................................... 39
Figure 3.12 Thermal unfolding of plant L-asparaginases ............................................... 42
Figure 3.13 of PvAspG1 activity across a pH spectrum ................................................. 44
Figure 3.14 PvAspG1S118I activity across a pH spectrum ............................................. 44
Figure 3.15 PvAspG-T2 activity across a pH spectrum ................................................. 45
Figure PvAspG-T2I117S activity across a pH spectrum .............................................. 45
Figure 4.1 The activation loop of PvAspG1 binding to potassium ............................... 54
List of Abbreviations

*Standard SI units not listed

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AspAT</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>ASPG</td>
<td>Asparaginase</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism spectroscopy</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray ionization- mass spectrometry</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>GS/GOGAT</td>
<td>Glutamine synthetase / glutamine: 2-oxoglutamate aminotransferase</td>
</tr>
<tr>
<td>Ile</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-1-thigalactopyranoside</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>k_{cat}</td>
<td>Apparent unimolecular rate constant</td>
</tr>
<tr>
<td>k_{cat}/K_{m}</td>
<td>Catalytic efficiency</td>
</tr>
<tr>
<td>K_{m}</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MUSCLE</td>
<td>Multiple Sequence Comparison by log-expectation</td>
</tr>
<tr>
<td>NAD^{+}</td>
<td>β-Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NADH</td>
<td>β-Nicotinamide adenine dinucleotide, reduced</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>Ntn</td>
<td>N-terminal nucleophile</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate - polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-violet</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>Maximum reaction rate</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Background

Common bean (*Phaseolus vulgaris* L.) is a member of the *Leguminosae* family and is a major grain legume cultivated worldwide for both human and livestock consumption. *P. vulgaris* originated from Central and South America, and was first cultivated in Peru and Mexico in 6000 BC (Kaplan et al., 1973). It is now cultivated as a major food crop in many tropical, and temperate areas of Americas, Africa, Europe and Asia. The products of common bean include leaves, pods, immature and dry seeds. The dry seeds are the major economic component of the bean plant being produced all over the world, except in the Antarctica, because of its high dietary protein content (Gepts, 1998). The world annual production of common bean is estimated at 23 million tonnes in 2012 with total production value of 577 million USD (Faostat, 2013).

Common bean has been identified as the world’s most important legume for human consumption due to its high protein content (Beebe et al., 2000). It contains about 21 to 25% protein by weight, supplies good low fat protein for consumption, serving as a cheaper source of protein compared to animal sources (Geil and Anderson, 1994). Protein biosynthesis in plants involve a set of biochemical processes by which amino acids are produced from important elements such as carbon, oxygen, hydrogen, sulphur, and nitrogen through series of metabolic processes. Nitrogen is one of the major elements required during amino acid biosynthesis in plants. The fundamental challenge that higher plants encounter during this process is the availability and absorption of nitrogen in its usable form. Nitrogen is an essential element for plant growth and development, and majorly contributes to total plant biomass and productivity (Gaufichon et al., 2015).

Nitrogen is taken up by plants from the soil in form of nitrate (NO$_3^-$) or ammonium (NH$_4^+$) or in some species (e.g., legumes) by the direct fixation of atmospheric N$_2$ in association with bacteria. Nitrogen assimilation involves the action of the GS/GOGAT
cycle whereby ammonium is incorporated into carbon skeletons to produce glutamine by the glutamine synthetase (GS), which is followed by the transfer of the glutamine-amide nitrogen to 2-oxoglutarate by glutamate synthase (GOGAT) to produce glutamate. Asparagine is produced by transfer of the amide group of glutamine to aspartate, catalyzed by asparagine synthetase. Aspartate is produced by transamination of glutamate catalyzed by aspartate aminotransferase, or by hydrolysis of the amide group of asparagine catalyzed by asparaginase (Figure 1.1). Asparagine has been identified as a major intercellular nitrogen metabolite in the xylem and phloem tissues of plants necessary in storage and transport for protein biosynthesis (Sieciechowicz et al., 1988; Lea et al., 2007). Asparagine, due to its high carbon-nitrogen (C/N) ratio and stability, is the major amino acid reserved and transported in the xylem from roots to leaves of most leguminous plants (Gaufichon et al., 2010).

In plants, the major routes of asparagine catabolism involve transamination and deamidation. Transamination is commonly observed in mature plants and it involves the incorporation of nitrogen for the synthesis of glycine and serine, both of which are intermediates of the photorespiratory pathway (Sieciechowicz et al., 1988). During photorespiration in fully expanded leaves, asparagine aminotransferase catalyzes the release of amino nitrogen from transported asparagine in the peroxisome; the nitrogen enters into the photorespiratory pathway to balance the removal of glycine and serine during the process of photorespiration (Ta et al., 1985). On the other hand, deamidation involves the utilization of carbon and nitrogen from asparagine to synthesize aspartate and ammonia (Gaufichon et al., 2015). The deamidation reaction is mostly common in the developing tissues and it is catalyzed by L-asparaginase (Sieciechowicz et al., 1988; Credali et al., 2013).

1.2 L-Asparagine

L-Asparagine, one of the 20 protein amino acids, is a non-essential amino acid in the diet of humans that is largely distributed in common bean and other plants. It plays an important role in the production of ammonia which readily makes nitrogen available in plants for protein biosynthesis. The deamidation of asparagine yields ammonia and
aspartate, which is necessary for further transformation to other amino acids. Asparagine was first isolated from *Asparagus sativus* in 1806 to become the first amino acid to be isolated from its natural source (Vauquelin and Robiquet, 1806). It was identified to play a key role in the storage and transport of nitrogen in plants. Unlike other nitrogen based compounds such as ureides and glutamine, asparagine is a more soluble and stable molecule with higher N:C ratio of 2:4 which makes it a highly efficient nitrogen storage and transport compound, especially in leguminous plants (Sieciechowicz et al., 1988).

Asparagine is the major transport compound in xylem and phloem tissues of leguminous plants. Previous studies showed that asparagine accounts for over 45 – 50 % of total amino acids in the xylem tissues and between 20 – 30 % in the phloem tissues of leguminous plants such as *Lupinus luteus*, *Lotus japonicus*, *Medicago truncatula*, *Vigna unguiculata* and *Phaseolus vulgaris* (Pate et al., 1979; Atkins et al., 1983). Asparagine and other amides such as glutamine are produced upon hydrolysis of storage proteins during seed germination, or during protein degradation due to leaf senescence, and are transported to the developing tissues of plants through the phloem (Sieciechowicz et al., 1988).

In *P. vulgaris*, asparagine plays a crucial role in the release and transport of nitrogen to sink tissues. The breakdown of asparagine into aspartate and ammonia by asparaginase is important in protein biosynthesis in common bean, especially during seed formation (Haroun et al., 2010). A strong positive correlation has been previously established between free asparagine levels and storage protein content (Hernández-Sebastià et al., 2005; Pandurangan et al., 2012). In the developing cotyledon of soybean seeds, asparagine represents up to 50% of total free amino acid which serve as nitrogen sources for storage protein synthesis (Hernández-Sebastià et al., 2005).
Figure 1.1 Schematic pathways of asparagine metabolic pathways.

The amide group of asparagine is released by asparaginase (ASPG), yielding ammonium and aspartate; the aspartate undergoes further transamination to produce other amino acids while the ammonium is utilized during protein biosynthesis. Blue circles represent carbon atoms, Glutamine synthetase (GS), Asparagine synthetase (AS), Asparagine aminotransferase (AsnAT). Adapted from (Gaufichon et al., 2015).
1.3 Regulation and Function of L-Asparaginase

L-Asparaginase (EC 3.5.1.1) is the enzyme responsible for the catalytic deamidation of asparagine into aspartate and ammonia. It is present in most organisms from bacteria, plants and animals with several roles which include asparagine degradation, protein biosynthesis and nitrogen remobilization. L-asparaginases have been characterized and classified into several families using biochemical properties, amino acid sequences and evolutionary sources. The two major and best characterized families include the bacterial- and plant-type asparaginases (Borek and Jaskólski, 2001).

Asparaginase has been isolated from a wide range of sources: bacteria, fungi, plants and animal cells, and its activities have been studied for over 30 years (Campbell et al., 1967). There has been keen interest in the isolation, purification and characterization of the bacterial type asparaginases, because they have been identified as an important antineoplastic agent for acute lymphoblastic leukemia (Gallagher et al., 1989; Shrivastava et al., 2012; Ali et al., 2016). Michalska and Jaskolski (2006) classified the bacterial-type asparaginase to subtype I and subtype II, based on their substrate affinity and cellular localization. The subtype I has lower affinity for L-asparagine and it is abundant in the cytosol, while the subtype II demonstrates a much higher affinity for L-asparagine and is mostly found in the periplasm (Swain et al., 1993; Michalska and Jaskólski, 2006). Bacterial-type asparaginase has been isolated mostly from Escherichia coli (EcAII) and Erwinia carotova (ErA).

Asparaginase isolated from E. coli, EcAII, is the first L-asparaginase with antileukemic properties to be used clinically in the treatment of acute lymphoblastic leukemia (ALL) (Mashburn and Wriston, 1964). It is localized in the periplasm and effective against leukemic cells. Leukemic cells grow by feeding on exogenous asparagine in the blood vascular system, because they are unable to synthesize their own L-asparagine. EcAII has a high L-asparagine affinity (K_m ~ 10^{-5} M) (Kozak and Jaskólski, 2000), and catalyzes the breakdown of L-asparagine into L-aspartic and ammonia in the blood vascular system, hence depriving the leukemic cells of exogenous
L-asparagine required by the cancerous cells to survive (Ho et al., 1970; Kelo et al., 2009).

The cytosolic asparaginase isoform from *E. coli* (EcAI) is not an effective against cancer due to its lower L-asparagine affinity ($K_m = 3.5 \times 10^{-3}$ M) compared to its periplasmic isoform EcAII (Willis and Woolfolk, 1974). EcAI and EcAII maintain a high sequence similarity but they are structurally different. In structure, EcAI exist as a dimer of two intimate dimers (Yun et al., 2007), while EcAII is a tetramer of four identical subunits (Michalska and Jaskolski, 2006; Huang et al., 2014). It is however unclear if the variation in substrate affinity for L-asparagine in EcAI and EcAII occurs as a result of the differences in their functional oligomeric form or localization.

On the other hand, plant-type asparaginase plays a critical role in nitrogen remobilization in plants. Plants normally acquire nitrogen in the form of nitrate ($\text{NO}_3^-$) or ammonium ($\text{NH}_4^+$) or by direct fixation in legumes with bacteria association. The nitrogen taken up by plant can either be reduced immediately and stored in the roots or translocated to the shoots for further metabolism and storage in the form of asparagine. During plant metabolism, the breakdown and transport of asparagine to make nitrogen available for plants requires L-asparaginase activity.

L-asparaginase activity has been previously reported to play an important role in determining the concentration of storage protein in soybean seeds (Pandurangan et al., 2012). The high free asparagine level present in the developing cotyledons of soybean seeds is metabolized by L-asparaginase, which releases nitrogen that is utilized to synthesize storage protein. The localization of asparagine in the developing cotyledon suggests that it may be a physiological marker or metabolite signal for the seed cotyledon capacity to utilize nitrogen for storage protein synthesis. The effect of lack of L-asparaginase activity was studied in *Arabidopsis thaliana* mutants deficient in asparaginases (Ivanov et al., 2012). Mutants lacking asparaginases exhibited reduced root growth and enhanced root hair inhibition due to increased accumulation of asparagine in the root. This unusual phenotype of root growth inhibition suggested a blockage in asparagine catabolism due to L-asparaginase activity deficiency in the mutant plants.
Generally in legumes, L-asparaginase is recognized to be the major enzyme metabolizing transported asparagine in tissues demanding high amounts of N, such as young leaves and developing seeds (Sieciechowicz et al., 1988; Lea et al., 2007). It however plays a primary role in reallocating transported nitrogen for the synthesis of other amino acids by deamidation of L-asparagine.

Plant L-asparaginase activity has been investigated in soybean (Streeter, 1977), lupin (Lough et al., 1992; Lough et al., 1992), and in pea (Sodek et al., 1980). Sodek et al., (1980) in their study on the developing seeds of pea (*Pisum sativum*), identified and classified plant L-asparaginase into two subclasses, potassium dependent and potassium independent asparaginase (Sodek et al., 1980). The classification is based on the relative requirement of plant L-asparaginase for potassium (K⁺) for catalytic activation. The potassium dependent L-asparaginase requires potassium for activation, while the catalytic activity of potassium independent asparaginase is insensitive to the presence of potassium. The two plant-type asparaginases have a high sequence similarity of 60–70% with varying degrees of catalytic activation and substrate specificities (Bruneau et al., 2006; Michalska and Jaskolski, 2006).

Previous studies demonstrated that the potassium dependent asparaginase is strictly specific for asparagine as substrate, while the K⁺ independent isozyme is catalytically active towards asparagine and isoaspartyl substrates (Bruneau et al., 2006; Gabriel et al., 2012). L-asparaginases from the model plant *Arabidopsis thaliana* showed different substrate preference and activity level. The potassium dependent asparaginase (At3g16150) strictly prefers L-asparagine as a substrate when compared to potassium independent asparaginase (At5g08100), which is catalytically active towards both L-asparagine and β-aspartyl-His (Bruneau et al., 2006). The potassium dependent asparaginase in *A. thaliana* (At3g16150) was also reported to have a higher catalytic efficiency (80-fold) with L-asparagine than the potassium independent asparaginase (At5g08100) (Bruneau et al., 2006), which suggests that regardless of their high sequence similarities there is a significant difference in the catalytic apparatus of the isozymes.
A sequence alignment and phylogenetic analysis revealed that the potassium dependent and potassium independent asparaginases from *P. vulgaris*, *Glycine max*, *Medicago truncatula*, *Brassica oleraceae*, *Arabidopsis thaliana* and *Oryza sativa* belong to two evolutionarily distinct subfamilies of plant L-asparaginases (Figure 1.2). This clear divergence in the evolutionary origin of plant L-asparaginases has previously been reported (Bruneau et al., 2006).

In addition to the catalytic deamidation function of L-asparagine by the plant L-asparaginases, the potassium independent L-asparaginases are also active in the splitting of isoaspartyl peptide bonds (Michalska et al., 2006). The isoaspartyl residues are usually formed by the transfer of the peptide backbone to the side chain of asparagine or aspartate; this however results into the formation of aberrant β-peptides instead of the proper α-peptide bond (Geiger and Clarke, 1987; Stephenson and Clarke, 1989). This aberration consequently leads to structural alteration and dysfunction of the storage protein, especially in dry seeds. The potassium independent L-asparaginase plays a crucial role as a repair enzyme in the elimination of the damaged toxic proteins, thereby improving seed viability and protein quality.

One major distinctive feature of plant L-asparaginase is the ability to undergo autocatalytic cleavage from an inactive precursor to functional subunits, known as α and β subunits. All enzymes that undergo this type of auto-processing are classified as N-terminal nucleophile (Ntn) hydrolase (Brannigan et al., 1995). During the auto-proteolytic activation step, enzymes in this family liberate an N-terminal nucleophilic residue, threonine, serine or cysteine from a non-functional precursor. Other examples of enzymes in this Ntn superfamily includes aspartylglucosaminidases (Guo et al., 1998; Wang and Guo, 2010), taspase1 (Oinonen et al., 1995; Khan et al., 2005), (Michalska and Jaskolski, 2006).

Ntn-hydrolases are expressed as inactive precursors; the functional unit of these enzymes is their heterodimer which results following an activation step. The maturation of the inactive precursor usually occurs as a result of the obligatory liberation of an N-terminal pro-peptide residue from the precursor polypeptide which gives rise to two
subunits (α and β). Furthermore, all Ntn-hydrolases basically share common structural properties. Mostly, in their mature form they are folded as either dimers of αβ heterodimers or αββα heterotetramers (Michalska and Jaskolski, 2006; Michalska et al., 2008). The functional unit of Ntn-hydrolases exists in different oligomeric forms, ranging from a simple (αβ) heterodimer in penicillin G acylase (Duggleby et al., 1995), to a multiple (αββα)/(αβ)2 in P. vulgaris (Bejger et al., 2014), and a more complex form of about 14 subunits in the proteasome (Lowe et al., 1995).

Enzymes in the Ntn superfamily are generally involved in catalyzing the hydrolysis of L-asparagine/L-aspartate-derived substrates, and mostly utilize threonine (Thr) as their nucleophilic residue. In Lupinus luteus L-asparaginase, Thr179 was identified as the nucleophilic residue when the protein was activated following autocatalytic cleavage into the α and β subunits (Borek et al., 2004). The potassium dependent and potassium independent L-asparaginases in A. thaliana were described to have a similar subunit structure and conserved auto-proteolytic cleavage site with the corresponding nucleophilic residue Thr183 (Bruneau et al., 2006). In Phaseolus vulgaris, the corresponding Thr 196 is the nucleophilic residue in the potassium dependent and independent asparaginase (Bejger et al., 2014).

1.4 Plant L-Asparaginase Structure

The plant-type asparaginases are different in crystal structure and have a different evolutionary origin from the bacterial L-asparaginases (Michalska and Jaskolski, 2006). The crystal structure of EcAIII (a homolog of plant-L-asparaginase), was the first structure of a plant-type L-asparaginase to be determined (Borek and Jaskólski, 2000). It is an (αβ)2 oligomeric protein structurally arranged as a dimer of two heterodimers (α and β subunits) which result from autocatalytic cleavage of the inactive precursor. The αβ subunits consist of two antiparallel β-sheets that are flanked on both sides by layers of α-helices.
Figure 1.2 Phylogenetic tree of selected plant L-asparaginases

The tree shows that $K^+$-dependent and $K^+$-independent L-asparaginases belong to two evolutionarily distinct subfamilies. Peptide sequence of the selected plant species were retrieved from the NCBI database. Sequence alignment was done by MUSCLE and the tree was constructed using the maximum likelihood method on the JTT matrix-based model of MEGA6 (Jones et al., 1992; Tamura et al., 2013). The selected plant species include *Phaseolus vulgaris*, *Glycine max*, *Medicago truncatula*, *Brassica oleraceae*, *Arabidopsis thaliana* and *Oryza sativa*. The human isoaspartyl peptidase/L-asparaginase (NP_079356.3) was used as the outgroup.
Figure 1.3 Amino acid sequence alignment of PvAspG1 and PvAspG-T2.

Alignment showing the amino acid residues that are similar (filled) or conserved (boxed, unfilled), or dissimilar. The activation loop that binds potassium is formed by residues at positions Val-111 - Ser-118 (Black arrows). In the activation loops, PvAspG1 and PvAspG-T2 differ at position 118, bearing a serine or isoleucine, respectively. Thr 196 (Bold black X) is the nucleophilic residue.
The nucleophilic residue Thr-179 is positioned in the middle of the N-terminal β-strand of the β-subunit. Close to the catalytic apparatus is a sodium cation coordination sphere located in each α-subunit of the heterodimer (Borek and Jaskólski, 2000). In *L. luteus* L-asparaginase (LIA), like in its bacterial homolog (EcAIII), the quaternary structure is a dimer of heterodimers, \((αβ)_2\) with a \(αββα\) topology whereby each heterodimer consists of two \(α\)–subunits between amino acid residues 1 – 192, and two \(β\)-subunits with amino acid residues 193 – 325. The subunits were formed following maturation of the inactive precursor protein by auto-proteolytic processing (Michalska et al., 2006).

In LIA however as reported by Michalska et al., (2006), there is a significant difference between regions Ala-122 – Phe-123 when compared to its bacterial homolog, EcAIII, in the region Ile-123 – Gly-124. The Phe-123 position in particular is specific and required by the LIA to adopt a unique conformation necessary for interactions with the hydrophobic side chains. The metal coordinating sphere in LIA was revealed to be composed of six main-chain carbonyl groups from Leu-59, Glu-60, Ile-62, Phe-65, Ala-67 and Ile-69. This hexahedral coordination geometry is similar to that observed in EcAIII, except that there is a slight shift in the position of the first residue of the coordination sphere, Leu-59 (Michalska et al., 2006). The nucleophilic residue of LIA is Thr-193; this position marks also the active site of the enzyme. It was also observed that Arg-221 is the residue that determines the enzyme specificity for substrates by forming a salt-bridge docking the α-carboxylate group of the aspartate ligand. The distance between this residue and the nucleophilic threonine determines the length of the substrate.

A recent crystallographic study of the *Phaseolus vulgaris* \(K^+\) dependent L-asparaginase, PvAspG1, provided insight into the mechanism of potassium activation (Bejger et al., 2014). There are two alkali metal binding sites in each α subunit, located in highly characteristic metal coordination loops. One of them is structural and is referred to as the stabilization loop. It is also present, as a sodium binding loop, in the crystal structure of \(K^+\) independent asparaginase from *Lupinus luteus* (Michalska et al., 2006). The second site is referred to as the activation loop and it is unique to \(K^+\) dependent
asparaginases. The activation loop of PvAspG\textsubscript{1} is formed by eight residues, Val-111, Met-112, Asp-113, Lys-114, Ser-115, Pro-116, His-117, and Ser-118, forming a right-handed turn around the centrally positioned potassium ion. The coordination sphere is formed by four main-chain carbonyl groups of the loop residues, Val\textsubscript{111}, Met\textsubscript{112}, Ser\textsubscript{115} and His\textsubscript{117}, and two water molecules, positioned in such a way that one of them is held in place by the side chain of Ser-118 (Bejger et al., 2014).

Bejger et al., (2006) reported the crystal structures to maintain a catalytic switch mechanism, deactivated (switched OFF) when sodium binds in place of potassium in the activation loop. Sodium binding results in a conformational change of the activation loop which affects the position of the side chains of three key residues: His-117, which is part of the activation loop in subunit \( \alpha \), and Arg-224 and Glu-250 present in the \( \beta \)-subunit of the other heterodimer as shown in figure 1.4. When potassium is present in the activation loop, the side chain of Arg-224 holds the L-aspartate/L-asparagine product/substrate in place in the active site through a fork of hydrogen bonds in a salt bridge between the N atoms of the Arg-224 guanidium group and the \( \alpha \)-carboxylate group of the ligand. Glu-250 stabilizes this conformation of the active site by forming hydrogen bonds with the side chains of Arg-224 and His-117. Binding of sodium into the activation loop results in a conformational change whereby the side chain of His-117 swings deeper into the protein core, pushing Arg-224 to rotate away from the active site. The side chain of Glu-250 also moves away from the active site, no longer interacting with the other two residues. It was suggested in the above study, that it is Ser-118 that is responsible for the alkali metal coordination in the activation loop, and ultimately for the operation of the catalytic switch (Bejger et al., 2014).

In the present work, a cDNA coding for a K\textsuperscript{+} independent asparaginase, PvAspG\textsubscript{T2}, was isolated from \( P. vulgaris \). A comparison of deduced amino acid sequences confirmed the unique 118 position in the activation loop of PvAspG\textsubscript{1}, differentiating it from PvAspG\textsubscript{T2}. Sequence alignment of PvAspG\textsubscript{1} and PvAspG\textsubscript{T2} (Figure 1.3), generated using Clustal \textit{W} software for multiple sequence alignments showed that the Ser-118 in PvAspG\textsubscript{1} is replaced by Isoleucine in the PvASPG-T2 while other amino acid
residues remain the same or conserved. The function of this residue was characterized by reciprocal mutations using enzymatic and biophysical methods. The results demonstrate that Ser-118 is indeed essential for potassium binding and catalytic activation of PvAspG1. While a catalytic switch mechanism involving potassium or sodium binding has been discovered in the above study, the structural determinants responsible for potassium activation in the plant $K^+$ dependent asparaginases remain to be unequivocally identified.

1.5 Hypothesis

This study sought to understand the mechanism of potassium activation of the plant L-asparaginases. I hypothesized that Ser-118 is critical in coordinating potassium for activation in PvAspG1, and the mutation of serine and isoleucine in the 118 position of the activation loops would affect the activity, conformation and potassium binding property of the asparaginases.

1.6 Objectives

The objectives of this research are to determine if the dependence of asparaginase on potassium can be altered by site directed mutagenesis and to investigate the effect of the point mutation on the enzymes.

1. The reciprocal mutants of PvAspG1 and PvASPG-T2 were engineered by substituting serine and isoleucine in the 118 position.

2. Investigated the catalytic activities of PvAspG1, PvAspG-T2 and their reciprocal mutants in the presence and absence of potassium by enzyme assay.

3. Examined the effect of potassium and point mutation on the conformation, stability and thermodynamics of the enzymes, using circular dichroism and isothermal titration calorimetry.
Figure 1.4 A three-dimensional representation of the catalytic apparatus of PvAspG1.

The catalytic switch area of PvAspG1 in the OFF state (chain B, dark violet; chain C, light violet) with Na (light violet sphere) in the activation loop (PDB: 4PV3), is superimposed with the same area in the ON state (chain B, dark green; chain C, light green) with potassium (light green sphere) in the activation loop (PDB: 4PU6), including the molecule of L-Asp (grey ball-and-stick model) in the active site. The nucleophilic Thr-196 residue of subunit β (chain B) is also indicated (Bejger et al., 2014).
Chapter 2: Materials and Methods

2.1 Cloning of PvAspG1 and PvAspG-T2 from *P. vulgaris* seeds

A cDNA containing the full-length coding sequence of PvAspG-T2 was cloned by reverse transcription PCR from developing seeds of the *P. vulgaris* line BAT93 as previously described (Bejger et al., 2014). The isolated cDNA aligned with the sequence of the transcript with accession number Phvul.001g025000.1 in the reference Andean common bean genome (Schmutz et al., 2014), except that His is encoded at position 24 instead of Gln and Lys at position 174 instead of Arg. In the sequence from the recently reported BAT93 genome, only the change at position 24 is present (Vlasova et al., 2016).

However, the two polymorphisms were confirmed by cloning the cDNAs from two other Mesoamerican common bean genotypes, SARC1 (Osborn et al., 2003) and 1533-15 (Beninger et al., 2005). The cDNA was amplified using the following primers: forward, 5'-CTACGGATCCATGGTGGCCATAGCTC-3' (translation initiation codon underlined); and reverse, 5'-GTAGGTCGACTCAGTCAATTTTGGCAGAAGG-3'. These primers were designed to introduce *Bam*HI and *Sal*I restriction sites at the respective 5' and 3' ends of the cDNA. The PCR product was cloned into the pCR BluntII TOPO vector, transformed in *Escherichia coli* TOP10 cells (Thermo Fisher Scientific), and grown in Luria-Bertani (LB) medium containing 50 μg/ml kanamycin. The inserts were verified by sequencing either at the DNA sequencing facility of Robarts Research Institute (London, ON) or using a 3130xl Genetic Analyzer (Thermo Fisher Scientific).

Plasmid DNA was digested with *Bam*HI and *Sal*I. The excised fragment was subcloned at the corresponding sites of the polylinkers of the bacterial expression vectors pQE30 (Qiagen, Toronto, ON) and pProExHTb (Thermo Fisher Scientific). The expression constructs were transformed in *E. coli* XL10-Gold cells (Agilent Technologies, Mississauga, ON). Cloning of the PvAspG1 cDNA into the pProExHTB plasmid has been described previously (Bejger et al., 2014). The PvAspG1 cDNA was also cloned as described above into the pQE30 vector. The cDNA was amplified with the following primers: forward, 5'-AATTGGGTACCATGGGAGGTGGGCAATTGC-3'; and
reverse, 5'-AATTGTCGACTTAATCCAAAATTGCAACCT-3'. These primers were designed to introduce KpnI and SalI restriction sites at the respective 5' and 3' ends of the cDNA. Deduced amino acid sequences were aligned using Clustal W (Larkin et al., 2007) and the alignment displayed with ESPript (Robert and Gouet, 2014).

2.2 Site Directed Mutagenesis

The sequence alignment of the *P. vulgaris* asparaginases shows that Ser-118 which was confirmed to be closely associated in coordinating potassium for activation in the potassium dependent asparaginase, PvAspG1 (Bejger et al., 2014), is substituted by isoleucine in the potassium independent asparaginase, PvAspG-T2. To understand the potassium-amino acid relationship, mutants were created with reciprocal replacement of the amino acid residues using the site directed mutagenesis method as previously described (Gabriel et al., 2012). Mutagenic primers specific for the desired point mutation were generated using the QuikChange Primer Design Program (Agilent, Mississauga, ON). PvAspG1 and PvAspG-T2 mutant cDNAs, PvAspG1-S118I and PvAspG-T2-I117S, respectively, were synthesized and the mutagenic primers used were: PvAspG1 forward and reverse, 5'-GCCGGAGAAGGCGATGTATATATGTGGGGATTTGTCCATA-3' and 5'-TATGGACAAATCCCCACATATACATCGCCTTCTCCGGGC-3'; and PvAspG-T2 forward and reverse, 5'-TCCATCGAAAGCGAGATAAGAGTGAGGCGTTTTCTCCATG-3' and 5'-CATGGAGAAAACGCTCAGTATCTCTAGTCTCGTTTCGATGGA-3'. Mutagenesis was performed with QuikChange II Site-Directed Mutagenesis kit (Agilent) using the cDNAs cloned in pProExHTb. Plasmid of different concentrations ranging between 5ng – 50ng were used as template for the mutagenesis reaction mixture.

The synthesized mutant strands were treated with DpnI to digest parental methylated DNA before transformation into super competent cells for nick repair. Reaction mixtures were transformed into XL10-Gold cells, plated on LB-agar and grown at 37 °C for 12 hours, after which clones were isolated, plasmid extracted and the
mutations and integrity of the remainder of the coding sequences verified by sequencing. Glycerol stocks were prepared and stored at -80 °C for future use.

2.3 Production and Purification of His-tagged Proteins

The wild-type and mutant recombinant proteins were purified by His-tagged affinity chromatography as described (Bruneau et al., 2006; Gabriel et al., 2012), with modifications. Wild-type constructs in pQE30 were used for expression. Colonies were isolated from LB-agar plate and grown overnight in 5 ml LB media with 100mg/ml ampicillin at 37 °C, as mini-culture. Expansion was done by transferring the 5 ml mini-culture into 500 ml NZY media with ampicillin (100mg/ml) in a 2 l Erlenmeyer flask and grown at 37 °C for approximately 3-4 hours until cell optical density at 600 nm reached 0.6. The gene transcription was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), the culture and allowed to grow for 16 hours at room temperature with shaking. The cells were pelletized by centrifugation at 6000 × g for 15 minutes and lysed with lysis buffer (50 mM Na₂HPO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0) and incubated with lysozyme for 30 minutes. Disruption of bacterial cells was done using high pressure French Cell Press and lysate was collected and centrifuged at 17,000 × g for 30 minutes. The clear supernatant was incubated for 2 hours at 4 °C in nickel-nitrilotriacetic acid Ni-NTA-agarose (Qiagen) for affinity chromatography purification, using 300 µl Ni-NTA-agarose per 1 mg of wet pellet.

The Ni-NTA agarose beads bind tightly to the 6× histidine-tag on the recombinant protein. After incubation, the Ni-NTA-supernatant was centrifuged at 2000 × g and pellets were extensively washed with wash buffer (50 mM Na₂HPO₄, 300 mM NaCl, 40 mM imidazole, pH 8.0). Pure recombinant protein was eluted with the elution buffer (50 mM Na₂HPO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). The purified samples underwent buffer exchange using the Amicon Ultra-15 filter device (Millipore, Etobicoke, ON) into the appropriate buffer, either 50 mM Tris-HCl pH 7.5 or 5 mM MOPS–NH₄OH pH 7.5 for biochemical or biophysical experiments, respectively.
2.4 Protein Quantification and SDS-PAGE Analysis of Recombinant L-Asparaginases

The protein quantification was performed using the Bio-Rad Protein Assay solution (Mississauga, ON), and bovine serum albumin (BSA) as standard using the spectrophotometer at 595 nm as previously described (Bruneau et al., 2006). Purified protein was analyzed by sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) using a 12% gel, and the protein bands were visualized by Coomassie staining as described (Laemmli, 1970).

2.5 Estimation of Protein Molecular Weight Subunits by ESI-MS

The molecular weights of each of the resulting subunits (α and β subunits) following autocatalytic activation of the L-asparaginases were determined by electrospray ionization – mass spectrometry (ESI-MS) using an orbitrap mass spectrometer (Agilent technologies, ON). Protein samples were prepared following purification and buffer exchanged into 20 mM ammonium acetate, pH 7.5, using a PD-10 column. A concentration of 100 µM of recombinant protein was flow injected and chromatographed using the HPLC C8 reversed phase column (Agilent Technologies, ON). The electrospray conditions were as described (Bruneau et al., 2006). Raw full MS files were converted to m/z format with proteoWizard software (Chambers et al., 2012), and deconvoluted using an in house python script. The resulting spectra were analyzed for peak separation for each of the subunits present in the protein sample.

2.6 Desalting of Asparaginase and Purification of Asparagine

Extensive desalting of the asparaginases was done in order to exclude any metal ions from the proteins. Centricon YM-30 column (Millipore) was used and the desalted protein was recovered over four cycles in 50 mM Tris-HCl buffer, pH 7.4 which was re-concentrated in an Amicon Ultra-15 filter column (Millipore,). Protein quantification was repeated after extensive desalting before enzymatic assays were performed. In order to remove all traces of aspartate, asparagine was purified using anion exchange chromatography (Prusiner and Milner, 1970). An asparagine stock solution of 200 mM
was passed through a 10 ml Poly-Prep chromatography column (Bio-Rad) filled with Dowex resin (1 × 8 strongly basic, 200-400 mesh resin, Sigma-Aldrich, St. Louis, MO). The anion resin binds to any contaminating aspartate while freely allowing the passage of pure asparagine. Purified asparagine was collected in a 50 ml tube and stored for use at 4 °C. Asparagine was freshly prepared every 2 weeks to prevent asparagine degradation to aspartate prior to enzymatic assay.

2.7 Enzymatic Assays

For the determination of enzymatic activities, NADH coupled enzyme assays were performed as previously described (Bruneau et al., 2006; Gabriel et al., 2012). This method utilizes the characteristic absorbance of NADH at 339 nm. It basically involves the transamination of aspartate (hydrolytic product of asparagine) by glutamate-oxaloacetate transaminase (GOT) in the presence of α-ketoglutarate (α-KG); which when coupled with NADH gets oxidized to NAD⁺ when malate dehydrogenase (MDH) reduces oxaloacetate to malate. Only NADH and not NAD⁺ absorb light at the wavelength of 339 nm. The depletion of NADH in the assay was spectrophotometrically followed at 339 nm using a PowerWave XS plate reader with Gen5.5 software (BioTeK Instruments, Winooski, VT) and the number of moles of NADH depleted over the course of the reaction was determined using the Beer-Lambert’s law of absorbance (\(A = ecl\); where \(A\) = Absorbance, \(e\) = extinction coefficients of NADH, \(l\) = path length).

2.7.1 Determination of Kinetic Parameters of L-asparaginase for L-Asparagine

Spectrophotometric analysis using the NADH coupled enzyme assay was used to determine the activities and kinetic parameters of \(P. vulgaris\) L-asparaginase with L-asparagine as substrate. The assay was composed of varying concentrations of L-asparagine (0 -20 mM), 2 units MDH, 0.5 units GOT, 1.5 mM α-KG, 0.3 mM NADH and appropriate concentrations of purified recombinant L-asparaginase. The blank was composed of all other constituents except the purified recombinant L-asparaginase. The assays were carried out in a 200 mM Tris-HCl buffer, pH 7.5, with and without 50 mM potassium, at 25 °C for a total period of 30 minutes with absorbance readings taken at 5
minutes interval. The apparent kinetic parameters ($K_m$ and $V_{max}$) were determined using the Michaelis-Menten transformation in the Graph Pad Prism 5 software (San Diego, CA).

2.7.2 Determination of L-asparaginase pH Profile

In order to determine the pH profile of the asparaginases, different biological buffers were used within their useful ranges to determine the response and activities of plant L-asparaginase at different pH. The pH buffers used were 2-(N-morpholino)ethanesulfonic acid; MES (5.5-6.7), 2-Bis(2-hydroxyethyl)amino-2-(hydroxymethyl)propane-1,3-diol; Bis-Tris-HCl (5.8-7.2), 2-Amino-2-hydroxymethyl-propane-1,3-diol; TRIZMA (7.0 – 9.0), and N-Cyclohexyl-2-aminoethanesulfonic acid; CHES (8.6 – 10). The assay was similar as above except for the buffers. The pH profile was visualized with Sigma Plot 12.5 (Systat Software, San Jose, CA).

2.7.3 Determination of Kinetic Parameters of L-asparaginase for Potassium

The same NADH coupled enzyme assay method was followed to determine the kinetic parameters of plant L-asparaginases for potassium. In addition to the assay set up described above, different potassium concentrations between 0 mM – 50 mM were assayed with appropriate purified L-asparaginase concentration to determine the effect of potassium affinity on L-asparaginase activation.

2.8 Isothermal Titration Calorimetry

The association constants ($K_a$), stoichiometry of interactions ($N$) and changes in enthalpy ($\Delta H$) and entropy ($\Delta S$) of potassium binding by L-asparaginase were determined by titrating potassium (in the form of KCl solution) against each enzyme solution and measuring the heat of the reaction. Experiments were performed using a MicroCal VP-ITC microcalorimeter (Malvern Instruments, Westborough, MA). After desalting, the protein sample was further extensively dialysed at 4 °C, to remove metal ions that might be present in the sample. The KCl solution was prepared using the last dialysis buffer consisting of 5 mM 3-(N-morpholino)propanesulfonic acid (MOPS-NH₄OH) pH 7.5.
Prior to titration, the protein, potassium and buffer solutions were extensively degassed using a ThermoVac station. The titration consisted of 10-µl injections every 5 min into the protein sample at 25 °C. The K\(^+\) concentration in the syringe was ~10-15 times the concentration of asparaginase in the sample cell (20-35 mM KCl was titrated against 1.2-2.0 mM asparaginase). Blank runs (buffer alone) were performed and subtracted from the productive runs. All titration data were fitted to a one-site model using MicroCal ITC-Origin Software (Malvern). The dissociation constants (\(K_d\)) were calculated as the reciprocal of \(K_A\) and the Gibbs free energy of binding (\(\Delta G\)) was determined as \(\Delta G = \Delta H - T\Delta S\).

The exact amount of potassium ion from the KCl solution was determined at 769.8 nm by inductively coupled plasma-optical emission spectrometry elemental analysis at A & L Canada Laboratories, London, ON. The calibration range of the potassium standards was 0 to 500 ppm and appropriate dilutions of the samples were made to stay within the 0-500 ppm range. The instrument (iCAP 6300 DUO, Thermo Fisher Scientific) was first calibrated by running a series of standards containing known concentrations of potassium. The calibration curve was used to evaluate the response of the samples. The potassium concentrations of the samples were multiplied by the dilution factors to yield the final reported concentrations.

2.9 Circular dichroism spectrometry

Circular dichroism (CD) is an important and sensitive technique for the determination of protein secondary structure and folding properties. It basically involves the differential absorption of circularly polarized light by chromophores such as proteins and nucleic acids. Optically active chiral molecules possess distinct characteristics for the absorption of circularly polarized light. CD measures the difference in the absorption of left-handed and right-handed polarized light. The difference in the light absorbed is the conformational property of the molecule under study, which is interpreted in terms of molar ellipticity. This technique was used to determine folding properties and the effect of point mutation and potassium on the secondary structures of \(P.\ vulgaris\) L-asparaginases.
2.9.1 Effect of Point Mutation and Potassium on the Secondary Structures of *P. vulgaris* L-Asparaginase

Protein samples, after purification and extensive desalting, were concentrated using an Amicon Ultra-15 filter device into 5 mM MOPS-NH$_4$OH pH 7.5 and dialyzed twice for 2 hours and once overnight at 4 °C to further remove any interfering metal ions. The experiment was performed for each of the proteins at a final concentration of 0.4-0.5 mg/ml in the presence or absence of 10 mM KCl.

CD spectra were measured with a water-bath-equipped Jasco Model J-810 spectropolarimeter (Easton, MD) using a 1 mm path length quartz cell. Blank runs were performed (using buffer with or without potassium) and the signals were subtracted from the sample runs. The CD spectra generated were in each case the average of five scans obtained at 25 °C with a scan speed of 100 nm/min, at 1 nm spectral bandwidth and a response time of 1 s. Data were collected at 0.1 nm intervals. The CD spectra in the range of 190–250 nm were acquired and analyzed for fractional content of secondary structures in CDPRO using the CDSSTR algorithm with reference data set 4 (SP43) for globular proteins (Provencher and Glockner, 1981; Manavalan and Johnson, 1987; Sreerama and Woody, 1993, 2000). The spectra were visualized using Sigma Plot 12.5 (Systat Software, San Jose, CA).

2.9.2 Effect of Point Mutation and Potassium on the Stability of *P. vulgaris* L-Asparaginase

The effect of point mutation and potassium on the stability and unfolding of asparaginase was also investigated. Protein denaturation was followed in the CD at wavelength 222 nm while increasing temperature between 5 – 95 °C with the following instrument settings; temperature slope, 2 °C/min; response, 0.5 s; 1 mm bandwidth and data pitch of 0.5 °C. The resulting unfolding spectra were processed using SigmaPlot 12.5 software, while other experimental conditions were the same as discussed above. After denaturation, the melting curves were plotted in terms of fraction unfolded and temperature. The melting temperature was determined using a non-linear regression by fitting the experimental points to the Boltzmann sigmoidal fit function of Graph Pad...
Prism (Miles et al., 2011). The Boltzmann sigmoidal relation describes protein unfolding transition as a function of temperature. The melting temperature ($T_m$) is the temperature of the midpoint of the transition from folded to unfolded states.
Chapter 3: Results

3.1 Ser-118 of PvAspG1 is Necessary for Potassium Activation

The deduced amino acid sequences of the potassium dependent asparaginase, PvAspG1, and potassium independent asparaginase, PvAspG-T2 were aligned as shown in Figure 1.3. All of the amino acid residues in the activation loop are either identical or conserved between the two enzymes, e.g. Asp-113 is replaced by glutamate and Ser-115 by threonine, except for Ser-118 which is replaced by Ile-117 in PvAspG-T2. To understand the structural basis of potassium binding and catalytic activation in plant potassium dependent asparaginases, reciprocal mutants of PvAspG1 and PvAspG-T2 were constructed using site-directed mutagenesis, by substituting Ser-118 with isoleucine in PvAspG1, and Ile-117 with serine in PvAspG-T2. The presence, location of the mutation and the integrity of the non-mutated cDNA was confirmed by DNA sequencing, and the mutant asparaginase was expressed in E. coli. The recombinant wild-type and mutant enzymes were purified from E. coli as N-terminal His-tagged proteins by affinity chromatography.

SDS-PAGE of the purified enzymes confirmed the autocatalytic cleavage of the polypeptide precursors into α- and β-subunits with approximate molecular weight of 23 and 14 kDa, respectively, as expected for members of the Ntn hydrolase superfamily (Figure 3.1). The exact molecular mass of each subunit after autocatalytic cleavage was confirmed using electrospray ionization – mass spectrometry (ESI-MS). For PvAspG1, the molecular mass of α and β subunits are 24647.9 Da and 13609.5 Da respectively, while the molecular weights of the α and β of PvAspG-T2 are 22857.3 Da and 13610 Da respectively (Figures 3.2-3.5). The spectra for PvAsPG-T2 α – subunit (Figure 3.4) showed double peak with a difference of 16 Da between the two peaks. This could be as a result of a possible oxidation of methionine, that is, one methionine having additional oxygen (Chen and Cook, 2007).
The theoretical molecular mass of the PvAspG1 and PvAspG-T2 predicted using the amino acid sequence is slightly lower than the experimental values. For PvAspG1, the observed molecular mass of the α and β subunits are 23434.41 Da and 13610.61 Da respectively, while PvAspG-T2 molecular mass is 22750.58 Da and 13766.70 Da. The differences in the molecular mass between the experimental and sequence-predicted values of the α – subunits are accounted for by the extra N-terminal amino acids of histidine tags, spacers and TEV recognition sequences, included for purification and cleaving purposes.

The dependence of the catalytic activation of the mature asparaginases on potassium was investigated by determining apparent kinetic parameters in the presence or absence of potassium. The catalytic activity of PvAspG1 with asparagine was influenced by the presence of potassium. There was an approximately 7-fold increase in the apparent $V_{\text{max}}$ and 2-fold decrease in the apparent $K_m$ of PvAspG1 using asparagine as a substrate when 50 mM KCl was included in the assay. This resulted in a 12-fold increase of the catalytic efficiency in the presence of potassium (Table 1). These kinetic parameters are similar to those reported earlier (Bejger et al., 2014). As expected, the catalytic activity of the potassium independent asparaginase, PvAspG-T2, was not affected by potassium. The apparent kinetic parameters ($K_m$ and $V_{\text{max}}$) of PvAspG-T2 remained similar when 50 mM KCl was included in the assay.

Remarkably, the S118I mutant of PvAspG1 was no longer activated by potassium. Its apparent kinetic parameters were similar in the presence or absence of potassium. By contrast, the catalytic activity of the I117S mutant of PvAspG-T2 was responsive to the addition of potassium. The $K_m$ for asparagine decreased by close to 4-fold, with a 12-fold increase in $V_{\text{max}}$. As a result, the catalytic efficiency of the mutant enzyme was increased 45 times in the presence of potassium. These results indicate that Ser-118 is necessary for potassium activation of PvAspG1.

Furthermore, introduction of the serine residue is sufficient to make PvAspG-T2 activated by potassium. Interestingly, the presence of serine is associated with a higher value of $K_m$ in the absence of potassium in both PvAspG1 and PvAspG-T2-I117S, which
decreases following the addition of this cation. To determine the affinity of the plant L-asparaginases for potassium, different potassium concentrations were assayed using 10 mM asparagine as substrate. The kinetic parameters of the plant L-asparaginases for potassium were determined using potassium concentrations between 0 mM – 50 mM (Table 2). The apparent $K_m$ value for potassium was equal to 0.195 ± 0.007 mM for PvAspG1 and 0.395 ± 0.006 mM for PvAspG-T2-I117S (average ± SEM, n = 3), revealing a comparable affinity for the ligand in the two enzymes, whereas PvAspG-T2 and PvAspG1-S118I showed no activation (Table 2).
Figure 3.1 SDS-PAGE analysis of wild-type and mutant asparaginases.

Purified asparaginases were resolved by 12% SDS-PAGE and visualized by Coomassie blue staining. The position of the molecular weight markers is indicated on the left. P, α and β indicate polypeptide precursors, α- and β-subunit, respectively.
Figure 3.2 ESI-MS analysis of recombinant PvAspG1 showing the molecular weight of the α subunit, (% represents percentage relative abundance and Da represents molecular mass in Dalton).

Figure 3.3 ESI-MS analysis of recombinant PvAspG1 showing the molecular weight of the β subunit, (% represents percentage relative abundance and Da represents molecular mass in Dalton).
Figure 3.4  ESI-MS analysis of recombinant PvAspG-T2 showing the molecular weight of the α subunit, (% represents percentage relative abundance and Da represents molecular mass in Dalton). Double peak with a difference of 16 Da shows possible methionine oxidation.

Figure 3.5  ESI-MS analysis of recombinant PvAspG-T2 showing the molecular weight of the β subunit, (% represents percentage relative abundance and Da represents molecular mass in Dalton).
3.2 Ser118 is Required for Potassium Binding as Evidenced by Isothermal Titration Calorimetry

To determine whether the lack of catalytic activity is associated with a loss of potassium binding, the thermodynamic parameters of recombinant enzymes were investigated by isothermal titration calorimetry (ITC). The enzymes were extensively desalted into 5 mM MOPS-NH4OH pH 7.5, and titrated with KCl. Figures 3.6 and 3.7 shows the binding isotherms when potassium was injected into L-asparaginase inside the cell. ITC measures the heat that is absorbed or generated in the cell as a result of the interaction between the protein and ligand and the heat generated is represented by the peaks on the isotherm. Although saturation of the binding was not achievable in this experiment, the interaction between L-asparaginase and potassium was evident with this technique, and the thermodynamic parameters were partially studied (Table 3, Figures 3.6 and 3.7). The binding was exothermic, with a negative change in Gibbs free energy of binding (ΔG). The binding was spontaneous, as indicated by the positive change in entropy (ΔS).

The affinity was as expected for plant asparaginases, with a dissociation constant (K_d) in the low mM range. The experiment was repeated several times under different conditions, but saturation of the binding experiment could not be achieved. Therefore the thermodynamic parameters and binding isotherms reported here can only be interpreted as potentials of L-asparaginase binding to potassium and not the complete thermodynamics of binding, since saturation could not be reached. The reason for the inability to reach saturation was due to the low L-asparaginase affinity, in terms of dissociation constant. Plant L-asparaginases are known to have a low dissociation constant (K_d) in the millimolar range. K_d values in the millimolar range are however below the detection limit of the isothermal titration calorimetry technique. Another possible reason for the inability to reach saturation of the L-asparaginase-potassium binding could be that the enzyme’s active sites are already occupied by metal cation
residues during the purification procedure. Although several desalting procedures were followed and samples were extensively dialyzed to ensure removal of residual ions.

The binding of potassium to PvAspG1-S118I and PvAspG-T2 was not detectable using ITC. However, the thermodynamic parameters of potassium binding to PvAspG-T2-I117S were relatively similar to those for PvAspG1. The affinity of binding was slightly reduced, with a 2-fold increase in $K_d$. These results indicate that Ser-118 is required for potassium binding in PvAspG1. Furthermore, introducing the serine residue makes PvAspG-T2 capable of potassium binding, with similar thermodynamic properties.
Table 1 Apparent kinetic parameters of recombinant asparaginases in the presence or absence of 50 mM KCl.

Assays were performed in 200 mM Tris-HCl pH 7.5 buffer at 25 °C with asparagine concentration ranging between 0-10 mM. Values are the average ± SEM. This experiment was repeated three times with similar results.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Treatment</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (mM)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; (×10&lt;sup&gt;-8&lt;/sup&gt;)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt; (×10&lt;sup&gt;-8&lt;/sup&gt;)</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PvAspG1</td>
<td>With K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3.43 ± 0.09</td>
<td>50.0 ± 1.2</td>
<td>14.7 ± 0.1</td>
<td>37.4 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Without K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>6.16 ± 0.13</td>
<td>7.27 ± 0.63</td>
<td>1.18 ± 0.08</td>
<td>4.49 ± 0.23</td>
</tr>
<tr>
<td>PvAspG1-S118I</td>
<td>With K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3.72 ± 0.49</td>
<td>5.06 ± 0.61</td>
<td>1.38 ± 0.09</td>
<td>3.75 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>Without K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3.69 ± 0.47</td>
<td>5.45 ± 0.48</td>
<td>1.55 ± 0.31</td>
<td>4.03 ± 0.21</td>
</tr>
<tr>
<td>PvAspG-T2</td>
<td>With K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3.77 ± 0.10</td>
<td>3.71 ± 0.19</td>
<td>0.99 ± 0.01</td>
<td>2.75 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>Without K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>3.76 ± 0.38</td>
<td>3.09 ± 0.49</td>
<td>0.83 ± 0.05</td>
<td>2.29 ± 0.21</td>
</tr>
<tr>
<td>PvAspG-T2-I117S</td>
<td>With K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.61 ± 0.04</td>
<td>45.2 ± 0.6</td>
<td>28.1 ± 0.9</td>
<td>27.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Without K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>5.86 ± 0.41</td>
<td>3.72 ± 0.16</td>
<td>0.63 ± 0.04</td>
<td>2.76 ± 0.06</td>
</tr>
</tbody>
</table>
**Table 2 Kinetic parameters of recombinant asparaginases for potassium**

Assays were performed with 10mM asparagine prepared in 200 mM Tris-HCl pH 7.5 buffer at 25 °C with potassium concentration ranging between 0 mM – 50 mM. Values are the average ± SEM. This experiment was repeated three times with similar results.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ ($\times 10^{-8}$ katal/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PvAspG1</td>
<td>0.195 ± 0.007</td>
<td>49.1 ± 0.06</td>
</tr>
<tr>
<td>PvAspG-T2-I117S</td>
<td>0.395 ± 0.006</td>
<td>47.9 ± 0.1</td>
</tr>
</tbody>
</table>

**Table 3 Thermodynamic parameters of potassium binding to P. vulgaris L-asparaginases determined by ITC**

The model is one-set (or $N$ identical sites) of binding sites. $N$: stoichiometry of binding; $K_A$: association constant; $K_d$: dissociation constant; $\Delta H$: change in enthalpy; $\Delta S$: change in entropy; $\Delta G$: change in Gibbs free energy.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$N$</th>
<th>$K_A$ (mM$^{-1}$)</th>
<th>$K_d$ (mM)</th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
<th>$\Delta S$ (kcal K$^{-1}$ mol$^{-1}$)</th>
<th>$\Delta G$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PvAspG1</td>
<td>2.22</td>
<td>718 ± 81</td>
<td>1.43 ± 0.04</td>
<td>-7.58 ± 0.56</td>
<td>10.5</td>
<td>-10.71</td>
</tr>
<tr>
<td>PvAspG-T2-I117S</td>
<td>2.48</td>
<td>367 ± 16</td>
<td>2.72 ± 0.06</td>
<td>-19.4 ± 0.37</td>
<td>5.23</td>
<td>-20.96</td>
</tr>
<tr>
<td>I117S</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.6 Representative ITC data for potassium binding to PvAspG1 (A) and PvAspG1-S118I (B).

This experiment was repeated four times under different conditions with similar results. (A) Binding isotherm showing direct ITC titration of KCl in the ITC syringe into PvAspG1 in the sample cell. (B) Binding isotherm showing direct ITC titration of KCl into PvAspG1-S118I in the sample cell. The protein solutions were prepared and extensively dialyzed against 5 mM MOPS-NH₄OH pH 7.5, and the KCl solution was prepared using the final dialysis buffer, 5 mM MOPS-NH₄OH pH 7.5. The upper panels show the observed heat for each injection of KCl after base-line correction. The lower panels plot the binding enthalpies versus the potassium: protein molar ratio. The data was best fitted to the one set of site binding model. The best-fit parameters are listed in Table 3.
Figure 3.7 Representative ITC data for potassium binding to PvASPG-T2 (A) and PvAspG-T2-I117S (B).

These experiments were repeated four times under different conditions with similar results. (A) Binding isotherm showing direct ITC titration of KCl in the ITC syringe into PvAspG-T2 in the sample cell. (B) Binding isotherm showing direct ITC titration of KCl into PvAspG-T2-I117S in the sample cell. The protein solutions were prepared and extensive dialyzed against 5 mM MOPS-NH$_4$OH pH 7.5, and the KCl solution was prepared using the final dialysis buffer, 5 mM MOPS-NH$_4$OH pH 7.5. The upper panels show the observed heat for each injection of KCl after base-line correction. The lower panels plot the binding enthalpies versus the potassium: protein molar ratio. The data was best fitted to the one set of site binding model. The best-fit parameters are listed in Table 3.
3.3 Binding of Potassium is Associated with Similar Conformational Changes

Circular dichroism spectroscopy is a sensitive technique for evaluating the changes in secondary structure conformation upon ligand binding. CD is also important in estimating the effect of mutations, change in temperature or pH on the secondary structure of proteins by estimating the percentage of α helices, β sheets, β turns, and random coils. To determine whether the binding of potassium is associated with conformational changes in *P. vulgaris* asparaginases, the secondary structure conformation of the wild-type and mutant enzymes was investigated in the presence or absence of 10 mM KCl using CD. As for the ITC experiments, the protein was extensively desalted in 5 mM MOPS-NH$_4$OH pH 7.5, to exclude metal cations prior to the assays. Figures 3.8-3.11 shows the overlay of the far-UV CD spectra of each enzyme in the presence and absence of potassium. The CD spectra were processed using the CDSSTR algorithm to determine the percentage content of α helices, β sheets, turns, and unordered structures of the enzymes (Table 4). Similar analyses with two other algorithms, CONTINLL and SELCON3, produced very similar results.

For PvAspG1, in the absence of potassium, the CD data analyzed with CDSSTR indicated that the protein is predominantly α-helical. In the presence of potassium, the proportion of secondary structure elements was changed, with a close to 2-fold decrease in α-helices. This change suggests a conformational change upon potassium binding. The PvAspG1-S118I mutant had a lower percentage of α-helices than the wild-type enzyme. Notably, no major changes in the secondary structure of this mutant enzyme were observed upon addition of KCl. A similar result was observed with PvAspG-T2. Interestingly, PvAspG-T2 and PvAspG1-S118I shared a similar percentage of α-helices. In the absence of potassium, the secondary structure of PvAspG-T2-I117S was similar to that of PvAspG1. Upon the addition of potassium, the number of α-helices in this mutant enzyme was similarly reduced, by a factor close to 2. The data indicate that the introduction of the serine residue at position 117 in PvAspG-T2 confers similar CD spectral properties as in PvAspG1, both in the presence and absence of potassium.
Figure 3.8. Overlay of circular dichroism (CD) spectra of PvAspG1 in the presence or absence of 10 mM KCl.

Figure 3.9. Overlay of circular dichroism (CD) spectra of PvAspG1-S118I in the presence or absence of 10 mM KCl.
Figure 3.10. Overlay of circular dichroism (CD) spectra of PvAspG-T2 in the presence or absence of 10 mM KCl.

Figure 3.11. Overlay of circular dichroism (CD) spectra of PvAspG-T2-I117S in the presence or absence of 10 mM KCl.
Table 4. Percentage estimation of the secondary structure conformation of L-asparaginases in the presence or absence of 10 mM KCl.

The CD spectral data were processed using the CDSSTR algorithm. \( NRMSD \) represents the normalized mean root square differences between the empirical data and the back-calculated spectra produced from the predicted secondary structures (Mao et al., 1982).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Treatment</th>
<th>( \alpha )-Helices</th>
<th>( \beta )-Sheets</th>
<th>Turns</th>
<th>Unordered</th>
<th>( NRMSD )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PvAspG1</td>
<td>With ( K^+ )</td>
<td>26.2</td>
<td>29.6</td>
<td>18</td>
<td>26.2</td>
<td>0.086</td>
</tr>
<tr>
<td></td>
<td>Without ( K^+ )</td>
<td>44.8</td>
<td>20.5</td>
<td>13</td>
<td>21.2</td>
<td>0.001</td>
</tr>
<tr>
<td>PvAspG1-S118I</td>
<td>With ( K^+ )</td>
<td>17.8</td>
<td>22.5</td>
<td>25</td>
<td>34.7</td>
<td>0.015</td>
</tr>
<tr>
<td></td>
<td>Without ( K^+ )</td>
<td>18</td>
<td>24.5</td>
<td>32.6</td>
<td>24.9</td>
<td>0.048</td>
</tr>
<tr>
<td>PvAspG-T2</td>
<td>With ( K^+ )</td>
<td>19</td>
<td>33.3</td>
<td>21.9</td>
<td>25.8</td>
<td>0.056</td>
</tr>
<tr>
<td></td>
<td>Without ( K^+ )</td>
<td>17.2</td>
<td>31.5</td>
<td>22.7</td>
<td>28.3</td>
<td>0.001</td>
</tr>
<tr>
<td>PvAspG-T2-I117S</td>
<td>With ( K^+ )</td>
<td>18</td>
<td>25.7</td>
<td>22.4</td>
<td>33.9</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>Without ( K^+ )</td>
<td>34.6</td>
<td>22.3</td>
<td>12.1</td>
<td>31</td>
<td>0.008</td>
</tr>
</tbody>
</table>
3.4 Point Mutation and Potassium do not Affect Plant L-Asparaginase Stability

The circular dichroism (CD) technique was used to study the effect of a point mutation and presence of potassium on the stability of plant L-asparaginases. Melting curves were generated from the fraction unfolded ellipticity at 222 nm over a temperature range of 5 °C-95 °C. The melting temperature ($T_m$), which indicates the temperature at which the protein transitions from folded to unfolded stage, was obtained by fitting the melting curves generated to Boltzmann sigmoidal function in the GraphPad Prism 5. All the wild type and mutant enzymes unfolded at approximately the same temperature in the range of 46.0 – 50.0 °C, both in the presence and absence of potassium (Table 5, Figure 3.12).

The mutant asparaginases, PvAspG1-S118I and PvAspG-T2I118S showed only slightly higher unfolding temperatures in the presence of potassium than in the absence of potassium, while the wild type asparaginases, PvAspG1 and PvAspG-T2 maintained steady unfolding temperatures both in the presence and absence of potassium. The thermostability studies using CD revealed that potassium and point mutation of serine and isoleucine in the position 118 and 117 of the PvAspG1 and PvAspG-T2, respectively, do not contribute a major effect on the stability of plant L-asparaginases.

3.5 Potassium Activation of PvAspG1 and PvAspG-T2-I117S is Associated with a Narrow Optimal pH Profile

pH is known to play an important role in substrate binding and catalysis by influencing the ionization of the essential active site amino acid residues (Jeffery et al., 2000; Zhang et al., 2004). A cysteine, C191 substitution in a mutant *E. coli* aspartate aminotransferase changed the activity of the enzyme due to a resulting pH shift, which subsequently altered the catalytic efficiency of the enzyme on pH dependence (Jeffery et al., 2000). To investigate the effect of the introduced point mutation on the pH required for optimum L-asparaginase activity, the activity profile of each enzyme was investigated using identical buffers at a pH range between pH 5.5 and 10 (Figures 3.13 – 3.16).
Figure 3.12. Thermal unfolding of plant L-asparaginases.

The thermostability studies were monitored at 222 nm between the temperature range of 5 °C – 95 °C. Ellipticities were analyzed and converted to fraction (F) unfolded and curves generated using Boltzmann sigmoidal function of GraphPad Prism 5 software. The enzymes without K⁺ means potassium was completely excluded in the experiment while enzymes with K⁺ means potassium was present in the experiment, and F on y axis represent fraction unfolded.

Table 5. Unfolding temperature of *P. vulgaris* L-asparaginases in the presence or absence of potassium

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Unfolding Temperature Tₘ (°C)</th>
<th>With potassium</th>
<th>Without potassium</th>
</tr>
</thead>
<tbody>
<tr>
<td>PvAspG1</td>
<td>48.5</td>
<td>48.0</td>
<td></td>
</tr>
<tr>
<td>PvAspG1S118I</td>
<td>50.0</td>
<td>49.1</td>
<td></td>
</tr>
<tr>
<td>PvAspG-T2</td>
<td>46.9</td>
<td>46.3</td>
<td></td>
</tr>
<tr>
<td>PvAspG-T2I117S</td>
<td>50.0</td>
<td>47.0</td>
<td></td>
</tr>
</tbody>
</table>
PvAspG1 and PvAspG-T2-I117S were assayed in the presence of 50 mM KCl, since they are activated by potassium. All enzymes displayed an optimal pH value of 7.5. PvAspG-T2 and PvAspG1-S118I presented a broad pH profile, with activity progressively increasing from pH 5.5 to 7.5 followed by a progressive decrease to pH 10. By contrast, the potassium-sensitive variants PvAspG1 and PvAspG-T2-I117S exhibited a narrow pH profile, with a sharp peak in enzyme activity between pH 6.5-7 and pH 8.0. These data suggest that the serine residue and potassium influence the ionization of catalytic amino acid residues at the active site of the enzyme.
Figure 3.13. PvAspG1 activity across a pH spectrum.

Figure 3.14. PvAspG1-S118I activity across a pH spectrum.
Figure 3.15. Profile showing PvAspG-T2 activity across a pH spectrum.

Figure 3.16. PvAspG-T2-I117S activity across a pH spectrum.
Chapter 4: Discussion

4.1 Research Overview

A difference of single amino acid residue in the activation loop appears to exert considerable effects on the physicochemical properties of the *P. vulgaris* L-asparaginases. Within plants the K$^+$ dependent and K$^+$ independent L-asparaginase isoforms are known to share a high degree of sequence identity and structural similarity (Bruneau et al., 2006; Bejger et al., 2014). However, these enzymes perform different roles in plants. PvAspG1 hydrolyses asparagine, and PvAspG-T2, in addition to asparagine hydrolysis, also plays an important role in eliminating aberrant and toxic isoaspartyl-dipeptides formed during seed ageing in plants (Michalska and Jaskolski, 2006). The crystal of the K$^+$ independent L-asparaginase showed the presence of a sodium binding site (stabilization loop) which contributes to the stability of the enzyme’s active site (Michalska et al., 2006). However, in K$^+$ dependent L-asparaginase, in addition to the sodium binding site, there is another metal binding site called the activation loop. This loop controls the conformation of the active site residues for proper substrate recognition and docking when activated by potassium binding (Bejger et al., 2014).

This study investigated the mechanism of K$^+$ activation of L-asparaginase by replacing Ser-118, the residue suggested to be responsible for potassium coordination in the activation loop of PvAspG1 (Bejger et al., 2014), with isoleucine, and Ile-117 for serine in PvAspG-T2. The potassium dependence of plant L-asparaginase activation was subsequently studied. This study reports for the first time the kinetics of PvAspG-T2 in the presence and absence of potassium. The conformational studies of PvAspG1, PvAspG-T2 and their respective mutants provide information on the effect potassium and a point mutation on the secondary structures and stability of plant L-asparaginases. Also the binding of potassium in plant L-asparaginase was examined using isothermal titration calorimetry.
4.1.1 Structure – Function Architecture of Potassium Binding in Plant L-Asparaginase

According to a classification of enzymes activated by monovalent cations, the K\(^+\) dependent asparaginases belong to the Type II category of allosteric potassium-activated enzymes (Page and Di Cera, 2006; Vasak and Schnabl, 2016). These are the group of enzymes that are indirectly activated by the metal cation through a conformational change of the active site residues without a direct contact with the metal cation. The metal cation present in the activation loop of PvAspG1 is located approximately 18 Å away from the active site (Bejger et al., 2014). Therefore, it cannot directly influence substrate binding and catalysis. Rather, allosteric potassium activation is achieved through conformational changes that influence the position and/or conformation of amino acid residues involved in substrate binding and/or catalysis (Di Cera, 2006). In PvAspG1, metal cation binding in the activation loop determines a catalytic switch mechanism setting the active site in an ON or OFF state depending on the presence of potassium or sodium, respectively (Bejger et al., 2014), in a way reminiscent of dialkylglycine decarboxylase (Toney et al., 1993). In PvAspG1, there was a significant structural rearrangement of the activation loop when the coordinated potassium ion was replaced by sodium; this rearrangement resulted into changes in the conformation of the side chains of the catalytic switch residues His-117, Arg-224 and Glu-250, hence setting the active site to an OFF state. In an OFF state when sodium is coordinated, there is a reconfiguration of the side chains of the catalytic residues which affects the interaction of the side chain of Glu-250 with the side chains of His-117 and Arg-224 thereby preventing proper substrate anchoring for catalysis (Figure 1.4).

When sodium is replaced with potassium in the active site, one of the O atoms of the side chain of Glu-250 interacts with the main chain amide and side chain hydroxyl groups of Ser-118. This new interaction is compatible with the ON state of the catalytic switch. Because of its role in the activation loop, including H-bonded support of the water molecules completing the coordination sphere of the bound cation. The fact that Ser-118 mediates the conformational change of Glu-250 in the catalytic-switch triad, it was hypothesized to be the key important residue for the potassium dependence of
PvAspG1 (Bejger et al., 2014). Notably, this serine residue is absent in K⁺ independent asparaginases.

In the present study, the isolation of a cDNA coding for the K⁺ independent asparaginase from *P. vulgaris* confirmed the absence of the serine residue, which is replaced by isoleucine. In this study reciprocal site-directed mutagenesis of this residue followed by analysis of recombinant proteins was performed. The main finding from this study is that Ser-118 is necessary and sufficient for potassium activation of plant asparaginases. A unique site-directed replacement of Ile-117 by serine conferred the ability to bind potassium and activate PvAspG-T2. Conversely, the PvAspG1-S118I mutant lost potassium binding and activation. Potassium activation affected both the affinity for the substrate, as determined by the *Kₘ* values, and the rate of the reaction, the combined effect being manifested by an increased catalytic efficiency (*V_{max}/K_{m}*).

The serine residue is also required for potassium binding, as determined by ITC experiments. The stoichiometry of binding of ≈2 is consistent with the presence of two metal binding sites in the activation loops of a heterotetramer. The values determined for *K_d* of 2-5 mM are consistent with the fact that nearly maximal activation of plant asparaginases can be measured at a KCl concentration of 10 mM (Sodek et al., 1980). The reduced value of stoichiometry of binding and increased *K_d* of PvAspG-T2-I117S suggests a possible reduction in site occupancy and decreased affinity for potassium in the mutant enzyme. The CD results indicated that the presence of the serine residue is associated with similar secondary structural features. These include a higher percentage of α-helices in the absence of potassium in PvAspG1 and PvAspG-T2-I117S, which is reduced following the addition of potassium. The CD data demonstrated that no conformational changes were elicited by potassium in PvAspG-T2 and PvAspG1-S118I, as indicated by very similar percentages of α-helices.

The present results raise the question of how the binding of potassium elicits changes in conformation resulting in catalytic activation. However, the lack of potassium would almost certainly affect the conformation of the activation loop residues, His-117 and Ser-118. In turn, these may affect the other residues of the catalytic switch triad, Glu-
250 and Arg-224. Interestingly, in the absence of potassium, the presence of the Ser-118 residue was associated with a reduced affinity for L-asparagine in both PvAspG1 and PvAspG-T2-I117S as compared with PvAspG1-S118I and PvAspG-T2. The affinity could be restored by the addition of potassium. These observations are consistent with a role of Arg-224 in mediating the changes in the affinity for L-asparagine.

The conformational changes are also associated with an increased rate of catalysis. This may be explained by a more favorable position of the substrate determined by the interaction of imidazole ring of His-117 which is directed towards the surface of the protein core and with Arg-224 which is directed towards the active site holding the substrate in place with a form of salt-bridge in interaction with the nucleophilic residue, Thr-196. The differences in the profiles of activity versus pH associated with the presence of the serine residue also suggest possible effects on the ionization of catalytic residues, including the N-terminal nucleophile, Thr-196 hypothesized to play a key role in the catalysis (Michalska and Jaskolski, 2006). However, the remaining elements of the active site, Met-221, Glu-250, and oxyanion hole, have conserved conformation that is unresponsive to the type of ligand or cation bound in the activation loop.

Ser-118 is almost certainly also involved in determining the substrate preference of plant asparaginases. The main difference between the plant K⁺ dependent and K⁺ independent asparaginases is the high catalytic activity of the former with L-asparagine (Gabriel et al., 2012), while both subtypes exhibit a comparable level of catalytic activity with isoaspartyl dipeptides. Ser-118, by conferring potassium activation, contributes to this higher catalytic activity with L-asparagine. Another residue influencing substrate preference is Phe-162 in PvAspG1, replaced by isoleucine in PvAspG-T2. When the corresponding residues were exchanged in Arabidopsis thaliana K⁺ dependent and independent asparaginases, they specifically affected the reaction of the K⁺ dependent asparaginase with L-asparagine, and of the K⁺ independent enzyme with β-Asp-His, suggesting that the size of the side chain affected the binding in relation to the size of the substrate (Gabriel et al., 2012).
4.1.2 Mechanism of Potassium Selectivity of Plant L-Asparaginase and other Potassium Activated Enzymes.

Potassium has been identified as the most abundant alkali metal ion for optimal metabolic activity in the plant cell (Rodriguez-Navarro, 2000). Potassium plays an important physiological role in the transport and signaling of plant cell metabolites and enzyme activation. Several enzymes that require metal ion for optimal activity have been identified in almost all organisms, and the monovalent group 1 elements are mostly preferred by enzymes for optimal catalytic function (Evans and Sorger, 1966; Sueltzer, 1970). However, the mechanism behind selectivity of metal cations for enzyme optimum activation is poorly understood, and mostly depends on the structural arrangement of the catalytic apparatus of the particular enzyme under study.

In the potassium dependent plant asparaginase, previous studies showed that the presence of potassium caused appreciable increase in catalytic efficiency by decreasing the apparent \( K_m \) and increasing the \( V_{\text{max}} \) for substrate catalysis (Bruneau et al., 2006; Bejger et al., 2014). In the present study, the results showed that the kinetic parameters of the L-asparaginases are improved in the presence of potassium, this is in agreement with previously reported results; there is an 8-fold increase in \( V_{\text{max}} \) and 2-fold decrease in the apparent \( K_m \) in the presence of \( K^+ \). Also, there is a concomitant change in the conformation of plant L-asparaginases in the presence of potassium. This type of mechanism has been previously described as an allosteric type II mechanism (Monod et al., 1963), whereby the binding of a metal cation at one site causes increase in catalytic efficiency through a conformational change that is caused without a direct interaction with the substrate.

The crystal structure of PvAspG1 revealed that \( K^+ \) is centrally positioned in the activation loop formed by eight amino acid residues (Val-111 - Ser-118), with an octahedral coordination sphere created by four main-chain carbonyl groups and two water molecules positioned by the side chain of the last residue Ser-118 (Figure 4.1) (Bejger et al., 2014). The residues of the activation loop are directly involved in \( K^+ \) coordination through the main-chain carbonyl O atoms of Val-111, Met-112, Ser-115 and
His-117. The binding of potassium at this site controls indirectly L-aspartate/L-asparagine binding, enzyme activity and conformational changes. It is suggested that the selectivity and preference of K\(^+\) in PvAspG1 can be attributed to the structural fitness of K\(^+\), in terms of ionic size, to the constrained binding site.

This allosteric type mechanism of metal cation selectivity has been studied in several enzymes such as tryptophan synthase and tryptophanase (Rhee et al., 1996; Isupov et al., 1998). Similar to PvAspG1, the functional unit of tryptophan synthase is a heterotetramer of α\(_2\)β\(_2\) subunits arrangement. The crystal structure of tryptophan synthase revealed that K\(^+\) coordination is formed by 3 main-chain carbonyl groups of Gly-232, Phe-306, and Ser-308 which is also supplemented by at least one water molecule (Rhee et al., 1996), as observed in the crystal structure of PvAspG1 (Bejger et al., 2014). Likewise in tryptophanase, the enzyme that catalyzes the breakdown of L-tryptophan to indole, pyruvate and ammonia, the activity and conformation is influenced by the presence of K\(^+\) in the activation loop. The crystal structure revealed that the K\(^+\) coordination is formed by the three main-chain carbonyl atoms of Gly-53, Glu-70 and Asn-271 with three water molecules appropriately positioned by the side chain of Glu-70 (Isupov et al., 1998). It is observed that most of the K\(^+\) activated enzymes have almost similar arrangement of the catalytic apparatus responsible for K\(^+\) selectivity and activation.

In the present study, it is proposed that Ser-118 is essential in K\(^+\) selectivity for activation in PvAspG1. A substitution of the Ser-118 in the activation loop of PvAspG1 to isoleucine from PvAspG-T2 resulted in a loss of potassium dependency for optimum activation which resulted in decreased catalytic efficiency, and a conformational change. However, the introduction of serine to replace isoleucine in the position 117 of PvAspG-T2 conferred a K\(^+\) activation property as observed from the consequent increase in catalytic efficiency and conformational changes.
4.1.3 Potassium and Ser-Ile Substitution does not Necessarily Improve *P. vulgaris* L-asparaginase Stability

Thermal stability is one of the important characteristics of enzymes. This is the ability of enzymes to remain active at high temperature. Recent studies showed that bacterial type L-asparaginases unfold at higher temperatures in the range of 55 – 65 °C, compared to plant type L-asparaginases (Mahajan et al., 2014; Upadhyay et al., 2014; Yaacob et al., 2014; Kishore et al., 2015). Plant type asparaginases achieve optimum activity at about 37 °C (Karamitros et al., 2013; Kishore et al., 2015; Mohamed et al., 2015), and start to unfold at higher temperatures. In the present study, the *P. vulgaris* L-asparaginase maintained a thermal stability in the range of 47.0 – 50.0 °C in the presence and absence of potassium. At this temperature range, about half of the enzyme has completely unfolded as shown in Figure 3.12.

However, it is remarkable to note that there is no substantial difference in the thermal unfolding temperature (*T*_m) values of both the wild type (PvAspG1 and PvAspG-T2) and mutant enzymes (PvAspG1-S118I and PvAspG-T2-I117S) in the presence and absence of potassium. This means that neither the point mutation nor potassium have a compelling effect on the stability of the enzyme. There have been a few situations where a single amino acid residue substitution contributed significant effects on enzyme thermal stability (Imanaka et al., 1986; Imanaka et al., 1992). The thermal stability of penicillinase repressors was significantly enhanced from 30 °C to 48 °C, by simply substituting Leu-70, an amino acid residue uniquely located in the β – turn structure of the enzyme with a proline (Imanaka et al., 1992). Generally, the β – turn structures of enzymes are known to be mostly occupied by non-polar amino acid residues such as Pro, Asp, Asn, and Gly, whereas amino acid such as Leu, Ile, Thr and Val are rarely present in β – turn structures (Chou and Fasman, 1974; Farnsworth et al., 1997). Therefore the idea behind this involves the improvement of the internal hydrophobicity of the enzyme by appropriate introduction of any of the non-polar or neutral amino acid residue into the β – turn chain of the enzyme which may significantly contribute to the thermostability. Contrary to the present study, potassium was earlier reported to improve the stability of L-asparaginase partially purified from pea cotyledons (Sodek et al., 1980). The potassium
dependent asparaginase isolated from the testa of *Pisum sativum* was reported to be both less active and unstable in the absence of potassium, and potassium was required to maintain the stability of the enzyme (Sodek et al., 1980). Sodek et al., (1980) experiments however was performed using partially purified asparaginases and under different conditions. The thermostability results obtained from CD in the present study shows no substantial difference in the $T_m$ of the potassium dependent asparaginases, both in the presence and absence of potassium. This result is in agreement with the denaturation studies of recombinant L-asparaginase isolated from *Arabidopsis* that potassium ion does not influence the stability of plant L-asparaginases (Bruneau et al., 2006).

4.2 Plant L-asparaginase: A potential enzyme with anti-leukemic properties

Microbial sources of asparaginase have been extensively utilized for the treatment of childhood acute lymphoblastic leukemia (ALL) (Pieters et al., 2011; Vrooman et al., 2016). Leukemic cells are unable to synthesize asparagine like other normal cells and therefore depend on the plasma tissues for asparagine supply (Li et al., 2006). Asparaginase however catalyzes the hydrolysis of asparagine into aspartate and ammonia, hence causing depletion of the asparagine required for tumor cell growth. This consequently deprives leukemic cells of asparagine and hence causes cell death. However several side effects have been linked to the use of bacterial asparaginase for ALL treatment such as thrombosis, anaphylaxis, and several other allergic reactions in children (Couturier et al., 2015; Merlen et al., 2015). Therefore a different source of asparaginase, possibly a plant asparaginase, may serve as a therapeutic agent with little or no side effects for the treatment of ALL. However, plant L–asparaginases have low affinity for asparagine when compared to the bacterial asparaginases. In order to be able to use plant L–asparaginase as a chemotherapeutic agent for the treatment of ALL, more work has to be done to improve its substrate binding and affinity. This could be achieved by performing a series of site-directed mutagenesis of the active site residues that are involved in substrate binding and catalysis.
Figure 4.1 The activation loop of PvAspG1 binding to potassium.

The representation of the activation loop of PvAspG1 formed by 8 amino acid residues Val-111–Ser-118 showing the coordination of the centrally positioned potassium ion (green ball). Adapted from (Bejger et al., 2014).
Chapter 5: Conclusion

L-asparagine serves as an important compound for nitrogen transport and storage in plants; it plays an important role in protein biosynthesis and redistribution of nitrogen in the sink tissues of plants when broken down to aspartate and ammonia. L-asparaginase catalyzes the hydrolysis of L-asparagine. The plant L-asparaginases have been classified into potassium dependent and -independent subclasses, as a result of their potassium requirement for activation.

This study investigated the potassium dependency of the *P. vulgaris* L-asparaginase to understand the mechanism of activation. Ser-118 in the activation loop of the potassium dependent asparaginase was suggested to play an important role in coordinating potassium for activation. The Ser-118 is a characteristic feature of *PvAspG1*, which is replaced by isoleucine in the potassium independent asparaginase, *PvAspG-T2*. *PvAspG1* and *PvAspG-T2* mutants were synthesized by reciprocal substitution of serine and isoleucine at position 118 and 117 in *PvAspG1* and *PvAspG-T2* respectively, to synthesize *PvAspG1-S118I* and *PvAspG-T2-I117S*. Biochemical and biophysical studies provided information on the lack of potassium coordination in *PvASPG1-S118I* and *PvAspG-T2*. There was no difference in their kinetic parameters and conformation, as supported by the enzymatic and biophysical studies. Meanwhile, *PvAspG-T2-I117S* gained activation in the presence of potassium.

Although the substitution of serine and isoleucine in *PvAspG1* and *PvAspG-T2* did not necessarily affect the optimum pH of the enzymes, the pH profile showed that the potassium sensitive variants (*PvAspG1* and *PvAspG-T2-I117S*) have a narrower pH range when compared to the potassium insensitive enzymes (*pvAspG1-S118I* and *PvAspG-T2*). This suggests that the serine residue (Ser-118) while coordinating potassium also influences the ionization of catalytic amino acid residues at the active site of the enzyme. It is important to note that potassium only has an allosteric effect on the potassium sensitive asparaginases, and is not directly involved in substrate binding. Further
experiments should be performed to understand the mechanism involved in asparaginase-substrate binding, in interaction with potassium. Site directed mutagenesis of some of the active site residues that are involved in substrate docking and positioning should be attempted in order to understand and/or improve substrate affinity of the enzymes. His-117 and Arg-224 are more likely to be affected by potassium binding and contribute significantly to substrate binding for catalysis. Furthermore, while Ser-118 plays a critical role in coordinating the centrally positioned potassium ion for activation, Glu-250 forms hydrogen bond with the guanidinium group of Arg-224 and imidazole ring of His-117 in order to maintain the conformation necessary for catalysis.

Other residues in the catalytic pocket with conserved conformation, such as Thr-196, Met-221 and the oxyanion hole are not likely to influence asparagine affinity upon potassium binding due to their insensitivity to the presence or absence of metal cation at the active site. In the future, crystallographic studies of the mutant enzymes synthesized in this study will shed light on the conformational changes associated with the catalytic switch residues resulting from the Ser-Ile substitution and catalytic activation upon potassium binding.
References


Evans HJ, Sorger GJ (1966) Role of mineral elements with emphasis on the univalent cations. Annual review of plant physiology 17: 47-76


Kaplan L, Lynch TF, Smith C (1973) Early cultivated beans (Phaseolus vulgaris) from an intermontane Peruvian valley. Science 179: 76-77


Khan JA, Dunn BM, Tong L (2005) Crystal structure of human taspase1, a crucial protease regulating the function of MLL. Structure 13: 1443-1452


Rhee S, Parris KD, Ahmed SA, Miles EW, Davies DR (1996) Exchange of K⁺ or Cs⁺ for Na⁺ induces local and long-range changes in the three-dimensional structure of the tryptophan synthase α2β2 complex. Biochemistry 35: 4211-4221


Upadhyay AK, Singh A, Mukherjee K, Panda AK (2014) Refolding and purification of recombinant L-asparaginase from inclusion bodies of E. coli into active tetrameric protein. Front Microbiol 5: 486


Curriculum Vitae

Name: Ebenezer Ajewole

Post-secondary Education and Degrees:
- Obafemi Awolowo University, Ile-Ife, Osun state, Nigeria
- Degrees: 2006-2011 B.Agric. (Plant Science)
- 2014-2016 M.Sc. (Biology)

Honours and Awards:
- Western Graduate Research Scholarship (2014-2016)
- Meena and Amit Chakma Global opportunities Award (2016)
- Lester Libby Prize for Best Graduating Student in Plant Science (2012)
Related Work  Graduate Teaching and Research Assistant
Experience  The University of Western Ontario
2014-2016

Conferences:
independent asparaginase from common bean. Oral presentation. 54th Annual Conference
of the Phytochemical Society of North America. Urbana-Champaign, IL, USA. Aug 7-10.

Ajewole E, Pajak A, Jaskolski M, Marsolais F (2016). Mechanism of activation of L-
asparaginase from Phaseolus vulgaris. Oral presentation. Eastern Regional Meeting of
the Canadian Society of Plant Biologists. Toronto, ON, Canada, Nov. 20-21.