DRYING OF RED SPRING WHEAT SEEDLINGS (*TRITICUM AESTIVUM* L.) BY VARIOUS METHODS AND INVESTIGATION OF ITS PHENYLALANINE AMMONIALYASE STABILITY IN AN *IN VITRO* PROTEIN DIGESTION

by

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Abstract

Phenylketonuria and hyperphenylalanemia are autosomal recessive inborn errors of phenylalanine metabolism that are caused by mutations in the phenylalanine hydroxylase gene. Due to the stringency of the present dietary therapy, alternative treatments are being studied. Phenylalanine ammonia-lyase (PAL) is one of the potential dietary supplements for these patients. PAL is a well-studied plant enzyme which breaks down phenylalanine into *trans*-cinnamic acid and ammonia (Camm and Towers, 1973). It is found in the cytoplasm of the plant cells and is naturally encapsulated by plant cell walls which may protect it against the acidic pH environment in the gastrointestinal tract. It presumably degrades ingested Phe that circulates in the intestinal lumen. In this study, red spring wheat seedlings (*Triticum aestivum* L.) found to contain high PAL activity naturally were investigated as a potential alternative oral therapy. Specifically, the objectives were (1) to evaluate different drying methods on generating concentrated and dried preparation of wheat seedlings containing high levels of PAL activity; (2) to examine the retention of PAL activity over three months of storage under various storage conditions; (3) to determine the stability of PAL activity in simulated human digestion condition to establish if further study of using plant source enzyme *in vivo* is warranted.

Freeze-drying (FD) was found to retained the most activity (>90 % recovery dry wt basis) compared to air-drying (AD) and vacuum-microwave drying (VMD) for both leaf and residual seed/root samples. Pre-freezing of leaf tissues at -18 °C before FD significantly retained the highest PAL activity compared to pre-freezing at -25 °C, -35 °C, and -80 °C ($P<0.05$). Over three months of storage, 60-80 % of PAL activity was recovered in leaf and ~100 % was recovered in residual seed/root tissues after storage at -20 °C. After *in vitro* protein digestion, 36 % and 42 % of PAL activity was recovered in fresh leaf and root tissues respectively; however, FD tissues were found to be susceptible to proteases and acidic environment and no activity was

recovered after three hours of *in vitro* protein digestion. High performance liquid chromatography (HPLC) analysis of the residual Phe after *in vitro* protein digestion confirmed that fresh tissues had significantly higher conversion of Phe than that of FD tissues. Together, these results suggest that red spring wheat seedlings may have potential as a dietary supplement for phenylketonuric patients while further study to enhance PAL activity in plant preparations is required.

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AD	air-drying
a_w	water activity
BH_4	6(R)-L-erythro-5,6,7,8-tetrahydrobiopterin
BSA	bovine serum albumin
EVA	ethylene vinyl acetate
FD	freeze-drying
GLM	general linear model
HAL	histidine ammonia-lyase
HDPE	high density polyethylene
HPA	Hyperphenylalanemia
HPLC	High performance liquid chromatography
LDPE	low density polyethylene
MIO	3,5-dihydro-5-methylidene-4H-imidazol-4-one
NLAA	neutral large amino acids
PAH	Phenylalanine hydroxylase
PAL	Phenylalanine ammonia-lyase
Phe	phenylalanine
PKU	Phenylketonuria
PP	polypropylene
PS	polystyrene
PVC	polyvinyl chloride
PVPP	polyvinylpolypyrrolidone
TCA	trichloroacetic acid
TFA	trifluroacetic acid
VMD	vacuum-microwave drying

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In Memory of my uncle

I. Introduction

Phenylketonuria (PKU) and non-PKU-hyperphenylalaninemia (HPA) are autosomal recessive inborn errors of phenylalanine metabolism that are caused by mutations in the phenylalanine hydroxylase (PAH) gene. The complete or near-complete deficiency of the PAH activity results in elevation of blood level of phenylalanine and accumulation of phenylketones. If left untreated, patients develop neurological disorders, which is presumed to be a consequence of the toxic effects of the amino acid on brain development (Caballero and Wurtman, 1988). Dietary treatment was first introduced in 1953 and remains the main therapy for PKU/HPA patients. Patients must maintain a very strict diet and ingest a synthetic dietary supplement composed of a phenylalanine-free amino acid mixture, and a source of minerals and vitamins. Due to the stringency of the diet, many patients deviate from the therapy. The proportion of patients with Phe concentration above the recommended level was less than 30% for those younger than aged 10 years but almost 80% for those aged 15 years or older (Walter et al., 2002). Hence, alternative therapeutic methods for treating the condition are being investigated.

Oral enzyme therapy with phenylalanine ammonia-lyase (PAL) in combination with a control low protein diet has been suggested as a plausible alternative to the current therapy of PKU (Scriver et al., 1989). PAL catalyzes the non-oxidative deamination of L-Phe to yield *trans*-cinnamic acid and ammonia. *Trans*-cinnamic acid is further converted to benzoic acid and rapidly excreted in urine as hippurate (Hoskins et al., 1984). PAL is a well-studied enzyme that is widely distributed in plants, some fungi, yeasts and bacteria (Uniprot Knowledgebase). It requires no cofactors in the degradation of phenylalanine and is stable under a wide temperature range (Camm and Towers, 1973). Its product, *trans*-cinnamate is the precursor of numerous phenylpropanoid compounds generated by secondary metabolism in plants, including

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anthocyanins, flavonoids, lignins, furanocoumarin and isoflavonoid-phytoalexins (Liang et al., 1989; Howles et al., 1996; Levee and Seguin, 2001). As PAL is the major enzyme in the defence mechanism of plants, it can also be strongly induced by external physical, biological, and chemical stimulants (Shields et al., 1982; Solecka and Kacperska, 1995; Kim et al., 2001; Levee and Seguin, 2001; Khan et al., 2003; Shadle et al., 2003; Ritter and Schulz, 2004; D'Cunha, 2005). At different stage of plant development, the expression of PAL also varies between ages, tissues and organs (Bellini and Van Poucke, 1970; Camm and Towers, 1973; Blankenship and Unrath, 1988; Ishikawa et al., 2007)

Proof of concept of the efficacy of oral PAL therapy has been established using PKU mutant mouse model by Sarkissian et al. in 1999. Different methods of addressing this problem have been investigated such as PAL immobilization in semipermeable microcapsules or entrapped in silk fibroin (Bourget and Chang, 1985; Inoue et al., 1986), immobilization in expression cells (Sarkissian et al., 1999), or the use of recombinant PAL (Sarkissian et al., 1999; Liu et al., 2002). However, an immune response has been reported after repeated subcutaneous injection of PAL and the efficacy of PAL is limited by its susceptibility to intestinal proteases (Gilbert and Jack, 1981; Sarkissian et al., 1999). The cost of recombinant PAL as a daily supplement for patients have prevented any consideration of therapy as well. Hence, the current challenges of using PAL as a supplement include developing a method of protecting the enzyme from proteolytic degradation while still allowing the enzymes access to Phe. From previous work, among all the plant sources that were screened with PAL activity, red spring wheat seedling was found to be one of the best sources of the enzyme (Goldson et al., 2007). The use of seedlings provides a source of PAL that is renewable, readily available, and can be easily managed. Therefore, having a renewable source of seedlings at the optimal stages is a distinct

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advantage over other source of PAL. In this study, 7-day old red spring wheat seedlings (*Triticum aestivum* L.) with high PAL activity were investigated as the potential supplement for PKU/HPA patients. The retention of PAL after dehydration and over 3 months of storage were evaluated. In addition, simulated human protein digestion and various incubation conditions were performed to estimate the stability of PAL. The results from this work provide insights for future development and preparation of wheat seedlings as the dietary supplement for PKU/HPA patients to lift much of their dietary burdens.

II. Research Hypotheses and Objectives

In this study, the possibility of using red spring wheat (*Triticum aestivum* L.) as an alternative oral therapy for PKU and HPA patients was under investigation. The natural encapsulation of PAL within plant cells might be able to protect PAL within the gastrointestinal digestion, thus it could degrade Phe that circulates in the intestinal lumen. Hence, this approach might be utilized to increase dietary Phe and protein tolerance and could lift some of the burden of the PKU diet. The hypotheses of this study were:

1. PAL activity in red spring wheat seedlings can be retained after drying and over three months of storage;

2. PAL activity in red spring wheat seedlings is stable under acidic and basic environments, and proteolytic hydrolysis in an *in vitro* protein digestion.

Figure 1 and 2 provide flow-diagram overview of the schematic experimental designs. As such, the hypotheses were examined by the following objectives:

1. To generate concentrated and dried preparation of wheat seedlings containing high levels of PAL activity using different methods (vacuum-microwave-drying, freeze-drying and air-drying);

2. To examine the retention of PAL activity over three months of storage under various storage conditions;

3. To evaluate the stability of PAL activity under acidic and basic environments;

4. To simulate the conditions of human protein digestion and to determine the stability of PAL activity as well as the conversion of Phe during *in vitro* protein digestion.

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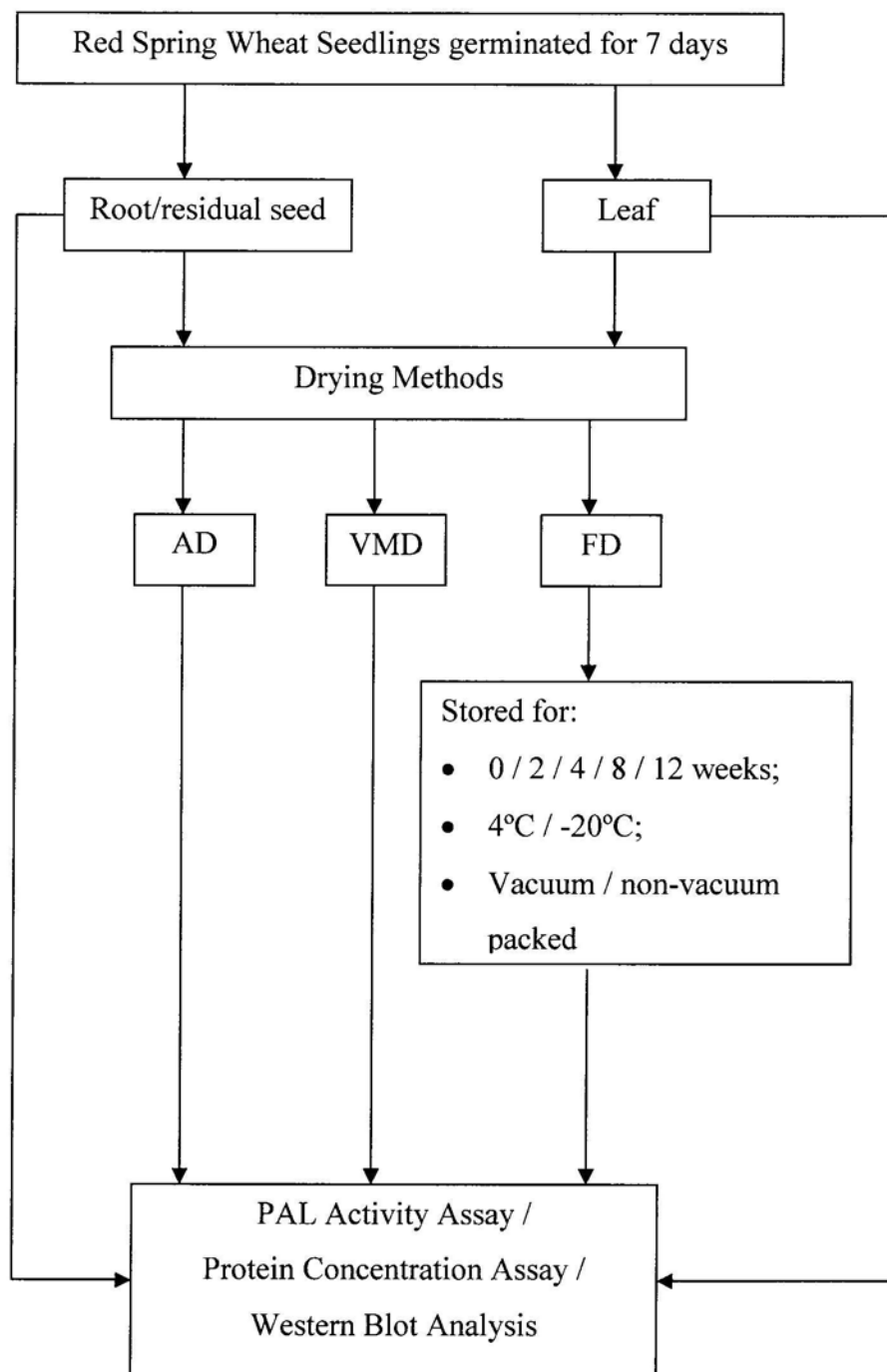
Figure 1. The Retention of Phenylalanine Ammonia-lyase Activity after Dehydration and Over Three Months of Storage
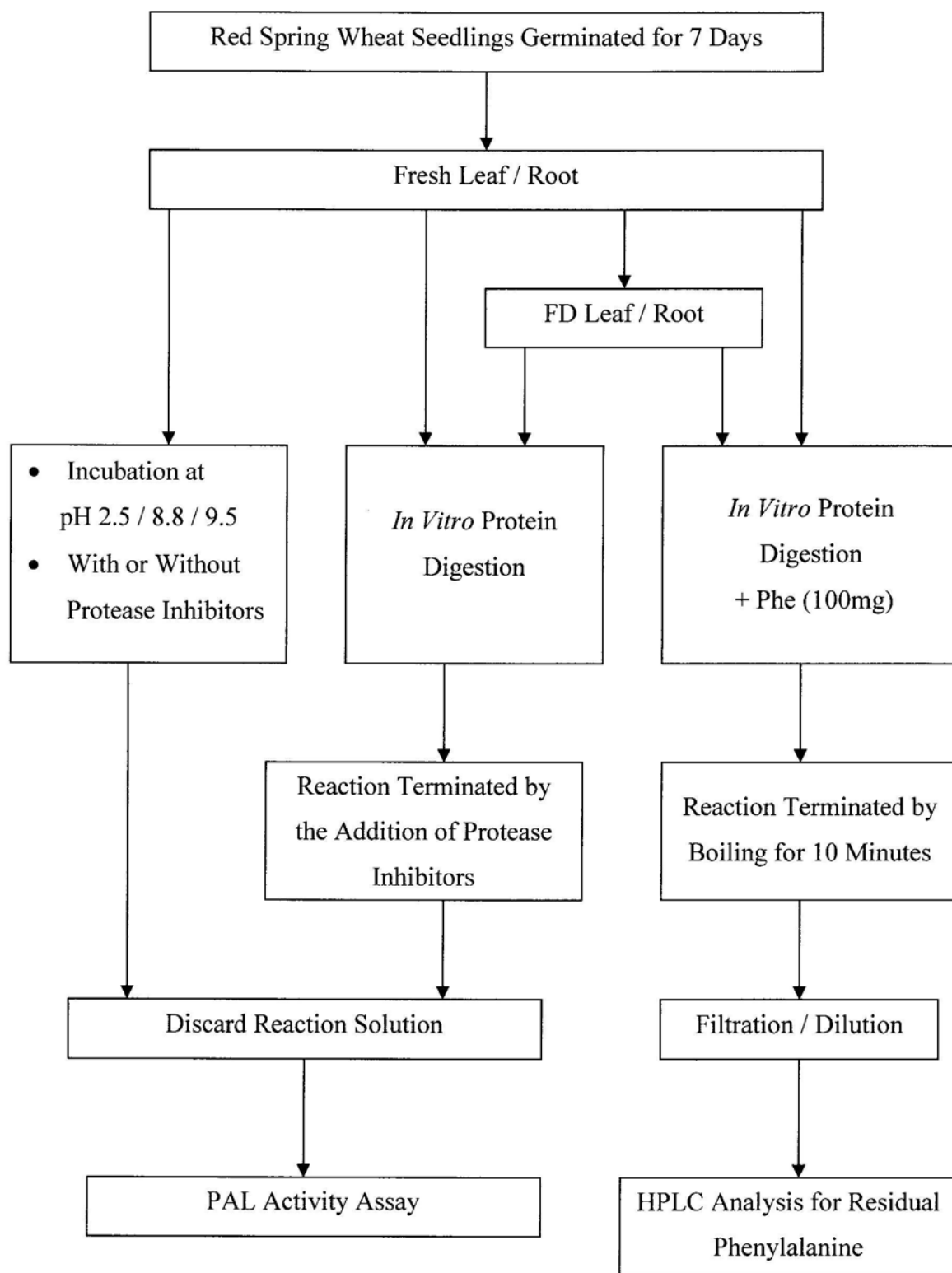
Figure 2. Phenylalanine Ammonia-lyase Stability and Activity under Various Conditions

III. Literature Review

III.A. Phenylketonuria

Classical phenylketonuria (PKU; MIM 261600) was first diagnosed by Dr. Asbjørn Følling in 1934 who detected the presence of phenylketones in the urine of patients (Centerwall and Centerwall, 1961). PKU and non-PKU-hyperphenylalanemia (HPA) are autosomal recessive inborn errors of amino acid metabolism that are caused by complete or near-complete deficiency in phenylalanine hydroxylase (PAH; EC 1.14.16.1) activity. If left untreated, elevated levels of Phe cause neurological disorders, which are presumed to be a consequence of the toxic effects of the amino acid on brain development (Caballero and Wurtman, 1988). The overall incidence of PKU and HPA varies between ethnic groups as shown in Table 1. Over the past 70 years, the disease has been widely studied on its gene and mutations, the enzyme structure and functions, as well as newborn screening and treatments.

III.A.1. Phenylalanine Hydroxylase

PAH functions in the liver and converts L-phenylalanine into L-tyrosine using co-factor tetrahydrobiopterin (BH_4). As of August 2007, 531 mutations of PAH had been identified (PAH Mutation Analysis Consortium Database, http://mcgill.ca/pahdb). The protein structure of PAH is approximately 100,000 Daltons in both rat and human liver composed of three domains with 4 monomeric subunits (Ledley and Woo, 1984; Kim et al., 2006). The human DNA fragment of PAH (chromosomal locus: 12q22-q24.2) contains about 2500 base pairs, with an open reading frame of 1,353 base pairs encoding 452 amino acids. The nucleotide sequence shows that the amino acid composition of the human PAH gene is 96 % conserved with that of the rat; therefore

mutant mouse strains with HPA is widely used in research studies (Güttler et al., 1984; Sarkissian et al., 1997).

PAH activity is used to classify the four types of PAH deficiency. The classification scheme was proposed by Guldberg et al. (1998). Normal blood level of Phe in adults is 58 ± 15 µmol/L (Cleary and Walter, 2001). Without a restricted diet, classical PKU patients have less than 1 % of PAH activity, and blood levels of Phe typically >20 mg/dL (>1200 µmol/L) with only 250-350 mg of dietary Phe tolerance per day; moderate PKU patients have 1-3 % of PAH activity with 350-400 mg of dietary Phe tolerance per day; mild PKU patients have over 3 % of PAH activity, and blood Phe of >10 mg/dL (>600 µmol/L) with 400-600 mg of dietary Phe tolerance per day (Trefz et al., 1984; Guldberg et al., 1998; Hanley, 2004). Mild HPA patients have blood Phe concentrations lower than 10 mg/dL (<600 µmol/L) on a normal diet and therefore studies have recommended that they do not need dietary treatment (Levy et al., 1971).

III.A.2. Dietary Therapy

Untreated PKU patients have demonstrated impaired brain development with symptoms include microcephaly, epilepsy, severe mental retardation, and behaviour problems (Williamson et al., 1984). Due to the efficiency of the newborn screening and treatment, these symptoms can be effectively prevented. Dietary treatment was first introduced by Bickel in 1953 and Woolf in 1954. Patients must maintain a highly restricted low protein diet and ingest a synthetic dietary supplement with Phe-free amino acid mixture, minerals and vitamins (Woolf et al., 1955; Levy, 1999). The supplement is offensive in odour and taste; and because of the stringency of the diet, emotional stress in these families is often high (Levy, 1999). It is clear that the dietary treatment should be initiated as soon as possible after birth; however, no consensus concerning the length of the diet should be maintained (Mitchell and Scriver, 2007). Studies have revealed that

discontinuation of the diet around 6 years of age does not allow optimal growth in mental abilities, and resulted in lower scores on measures of intelligence quotient, attention and reaction time (Williamson et al., 1984; Hanley, 2004). Some adults with PKU who discontinued the dietary treatment around 12 years of age have developed psychiatric difficulties including depression, anxiety, social withdrawal, neurotic behaviour, and other phobias (Williamson et al., 1984; Bowersox and Panel, 2001; Hanley, 2004). Also, other difficulties that affect normal living such as reduction in attention span, slow information-processing abilities, and slow motor reaction time are discovered (Mitchell and Scriver, 2007). However, different countries have various recommendations on the blood Phe concentrations to which adults should adhere (Table 2). In general, evidences show that the patient with PKU should maintain a lifelong restricted Phe diet in order to maintain the blood Phe concentration within the recommended normal value (Bowersox and Panel, 2001).

III.A.3. Alternative Therapies

Due to the difficulties in adhering to the diet treatment, many patients deviate from the dietary guidelines, which results in excess Phe concentration levels in blood. The proportion of patients with Phe concentrations above the recommended level was less than 30% for those younger than aged 10 years but almost 80% for those aged 15 years or older (Walter et al., 2002). Hence, a number of attempts are ongoing to find other treatments modalities for PKU. Somatic gene therapy uses a recombinant adeno-associated virus vector containing the murine Pah-cDNA, which is delivered into the liver of PKU mice via single intraportal or tail vein injections (Ding et al., 2004; Ding et al., 2006). Another therapy uses large neutral amino acids (LNAA), except Phe, as a dietary supplement to compete with Phe for the LNAA transporter at the blood-brain barrier (Kalkanoglu et al., 2005; Matalon et al., 2006; Matalon et al., 2007). The enzyme

replacement therapy using phenylalanine ammonia-lyase is another approach that is being widely studied. Table 3 summarizes the major studies of PAL in treatment of PKU. Due to its susceptibility to gastrointestinal proteases, purified PAL in free solution was rapidly broken down by gastrointestinal proteases (Fritz et al., 1976). Therefore, studies have been targeted on the use of different encapsulation methods such as nylon tubing, gelatine capsule, silk fibroin, artificial cell, and cellulose nitrate membrane to protect PAL against proteolytic attacks (Pedersen et al., 1978; Hoskins et al., 1980; Gilbert and Jack, 1981; Bourget and Chang, 1985; Inoue et al., 1986; Habibi-Moini and D'mello, 2001). Wieder et al. (1979) have reported that PAL of *Rhodotorula glutinis*, covalently attached to methoxypolyethylene glycol, was able to resist proteolytic digestion although it readily induced antibody formation. Later on, Sarkissian et al. (1999) demonstrated the use of recombinant *Rhodosporidium toruloides* PAL expressed in *E. coli* by oral administration and intraperitoneal injection. Other recombinant PALs from different sources and with modifications have also been evaluated (Liu et al., 2002; Gamez et al., 2005). However, low specific activity was commonly found in these encapsulated and recombinant PAL compared to native PAL (Kim et al., 2004). Further, the costs of recombinant PAL as a daily supplement for patients have prevented any consideration of therapy (Sarkissian et al., 1999). Overall, these approaches have shown some decrease of blood Phe in animal models with PKU but with certain limitations. These findings suggest that, with further research, PAL may lighten the burden of the highly restricted diet for PKU patients and their families, and also relieve the physiological and psychological difficulties that some patients experience.

III.B. Phenylalanine Ammonia-Lyase

III.B.1. Structures

Phenylalanine ammonia-lyase (PAL; EC4.3.1.5) was first discovered by Koukol and Conn (1961). It has been widely studied and isolated from over 60 plant sources, yeasts and fungi (UniProt Knowledgebase, http://www.pir.uniprot.org/) (Table 4). PAL has also been found in some bacteria which usually have both PAL and histidine ammonia-lyase (HAL; EC4.3.1.3) activities (Xiang and Moore, 2005; Moffitt et al., 2007). Conservation of PAL sequence is very high among species, with over 90 % conservation in protein sequence and about 700 amino acids in length (UniProt Knowledgebase). In plants and the yeast *Rhodosporidium toruloides*, the enzyme exists as a tetramer with subunits typically of 77-83 kDa and an alkaline pH optimum of 8.0 – 9.5 (Camm and Towers, 1973; Calabrese et al., 2004).

PAL catalyses the deamination of L-phenylalanine to yield *trans*-cinnamic acid and ammonia. The PAL crystal structure isolated from *Rhodosporium toruloides* has demonstrated similar structure and mechanism with HAL (Alunni et al., 2003; Calabrese et al., 2004). Both PAL and HAL do not require exogenous cofactors. They rely on the 3,5-dihydro-5-methylidene-4H-imidazol-4-one (MIO) cofactor which is formed autocatalytically from cyclization and dehydration of Ala-Ser-Gly (Schwede et al., 1999; Calabrese et al., 2004). In general, PAL differs from HAL in that it has an additional 215 residues located in both the N-terminal and C-terminal regions (MacDonald and D'Cunha, 2007).

III.B.2. Functions

PAL is the key enzyme that provides a bridge between primary metabolism and the phenylpropanoid pathways of secondary metabolism. Its product, *trans*-cinnamate is the

precursor of numerous compounds such as 4-coumarate, caffeate, ferulate and sinamate as shown in Figure 3 (Street and Cockburn, 1972; Ellis, 1997). These compounds are further synthesized and function as pigments (anthocyanins), UV protectants (flavonoids), structural polymers (lignins), antimicrobial compounds (furanocoumarin, isoflavonoid-phytoalexins and caffeic acid derivatives) (Liang et al., 1989; Howles et al., 1996; Levee and Seguin, 2001; Zabala et al., 2006). As PAL is the major enzyme in the defence mechanism of plants, it can be strongly induced by external stimulants like physical, biological, and chemical agents. Research studies have found that PAL is induced in response to pathogenic attack (Fungal *Cercospora nicotianae,* chitin, and chitosan) (Khan et al., 2003; Shadle et al., 2003), UV and certain visible light irradiation (Loschke et al., 1981; Levee and Seguin, 2001), tissue wounding (Shields et al., 1982), low temperature (Solecka and Kacperska, 1995), ultra-sonication, detergent (D'Cunha, 2005), amino acids (Kim et al., 2001), or low levels of nitrogen, phosphate, or iron (Ritter and Schulz, 2004). PAL reacts to these stimulants through increaseed expression and transcription of its gene (Liang et al., 1989). It has been reported that inhibition of PAL mRNA transcription by *trans*-cinnamic acid controls the down regulation of the phenylpropanoid pathway (Orr et al., 1993). PAL activity is inhibited by its end product, *trans*-cinnamic acid and its derivatives (*p*-hydroxycinnamate and *o*-chlorocinnamate), and related compounds (*p*-hydroxybenzoate and 2-naphthoate) (Orr et al., 1993). Phenol, 4-coumarate, *o*-cresol, and *p*-cresol have also been found to inhibit PAL (Orr et al., 1993; Alunni et al., 2003).

The level of PAL activity is controlled by the age and development, and is organ-tissue-specific in plants. Maximal PAL activity is found to differ with the age of different species. For example, PAL activity has appeared to be highest in 7-day old germinated wheat as compared to that of 5-day old radish (Bellini and Van Poucke, 1970; Camm and Towers, 1973). In the skin of grapes, PAL activity is high during early development, but rapidly decreases thereafter

(Blankenship and Unrath, 1988; Hiratsuka et al., 2001). In addition, different plant tissues exhibit huge variations in PAL activity. On a fresh weight basis, roots of radish show higher levels of PAL than hypocotyls but opposite results have been found in *Glehnia littoralis* (Bellini and Van Poucke, 1970; Ishikawa et al., 2007); in berries and apples, the majority of PAL is detected in the skins (Sapers et al., 1987; Blankenship and Unrath, 1988).

III.B.3. Applications

Due to the fact that no exogenous cofactor is required for enzymatic reaction, its importance in secondary metabolism, and its abundance in prokaryotes and eukaryotes, PAL is being used widely in industries and research studies. A major application of PAL in industry is to produce L-Phe as the precursor of aspartame (MacDonald and D'Cunha, 2007). Preparation of low Phe diets (PAL-treated casein hydrolysates) for PKU patients has been suggested as well (MacDonald and D'Cunha, 2007). In the present research area, PAL has been studied extensively for its potential to break down excess amounts of Phe in PKU patients, which has been discussed and summarized in section III.A.3. As proposed in this study, plants might be a good source of PAL for the treatment of PKU.

III.C. Nutritional Values of Red Spring Wheat Seedlings

As shown in Table 4 earlier, the PAL enzyme of red spring wheat is found in the cytoplasm and consists of 700 amino acids in length with molecular weight of 75,938 Da (Uniprot Knowledgebase). Two closely linked, highly homologous PAL genes have also been isolated from a wheat genomic library and southern blot analysis indicates the presence of a multiple gene family of PAL in wheat (Li and Liao, 2003). Besides the PAL enzyme, red spring wheat seedlings contain many other nutrients.

III.C.1. Health Benefit of Wheat Sprouts

Sprouts, in general, have a greater nutritive value in comparison to seeds due to a better quality of protein, a better bioavailability of essential minerals and trace elements, and a higher content of polyunsaturated fatty acids in dry weight basis (Harmuth-Hoene et al., 1987; Lintschinger et al., 1997). It has also been reported that the antioxidant activity of wheat grains strongly increases during the germination process (Calzuola et al., 2004). An increase of vitamin C content has been found during germination while no vitamin C was found in dry wheat grain (Yang et al., 2001). Vitamin E and β-carotene contents have been shown to gradually increase during germination and reach their peak on day 8 (Yang et al., 2001). Studies have established that the concentration of antioxidants, minerals, and sources of vitamins in wheat grains is increased by the germination process, and is dependent on the length of germination, reaching maximum values after about seven days (Yang et al., 2001; Calzuola et al., 2004).

Other than the health benefits mentioned above, wheat sprouts are a significant source of dietary fibre as well. Dietary fibre is the polysaccharides and lignin in the diet that are not digested by the endogenous secretions of the human digestive tract (Selvendran and Verne,

1988). Wheat seedling belongs to the group of graineceous monocots, which have a cell wall that is composed of about 50 – 70 % hemicellulose (xyloglucan, arabinoxylan and mixed-linkage β-D-glucan), 10 % pectin, 10 % protein (extensin and glycoprotein) and 15 % cellulose (Carpita, 1988). The cell walls provide mechanical support and protect the plant cells from organisms and abiotic stresses (Obel et al., 2002). Furthermore, as a dietary component, the insoluble cell walls improve health in the intestinal tract of humans such as relieving constipation, treating diverticular disease and preventing colon-rectal cancer, diabetes, gallstones, irritable bowel (Dreher, 1987). Hence, considering of all the dietary benefits of wheat sprouts with low intrinsic Phe level, as well as their high PAL activity protected within the dietary fibres, wheat seedlings may represent an excellent food source for PKU patients.

III.D. Drying Methods

Dehydration is the oldest method of food preservation used by mankind. It is a process in which water is removed to halt or slow down the growth of spoilage microorganisms, as well as the occurrence of chemical reactions. In turn, it extends the shelf life due to the lowered water activity (Figure 4). The drying process usually provokes a change of the physical, chemical, and biological properties, and modifies the characteristics of food products. In this study, various drying methods were used to dry wheat seedlings. The advantages and disadvantages of each method are discussed as follows.

III.D.1. Air Drying

Air drying (AD) is commonly used to dry herbs, spices, and some fruits. There are a number of different conventional hot air-driers such as cabinet drier, tunnel drier, conveyor drier, bin drier, fluidized bed drier, pneumatic drier, and rotary drier. These driers are built for different purposes such as various quantities and qualities of food, heat transfers, and air velocities.

In the dehydration process, it is by convection that the moisture of the materials is carried away from the drying surface to the air stream; while the heat current continuously transfers the moisture to the surface by conduction (Brennan, 2006). The water diffusivity, shrinkage properties and the presence of cell walls differed among foods, and these factors affect the movement of water within the solids (Karathanos et al., 1996; Brennan, 2006). As drying proceeds, the rate of water evaporation on the surface may become faster than the rate of diffusion to the surface and usually results in case hardening and collapse of the porous structure (Brennan, 2006). Collapse in a porous structure decreases the transport properties of inorganic

substances, gases and liquids, which affects the rehydration capacity and rehydration rate (Karathanos et al., 1996). Changes in porous structure also affect the texture of the product. Besides, AD is done under an atmosphere of oxygen levels which facilitates oxidation reaction such as the rate of browning. Browning is caused primarily by the oxidation of phenolics by enzymatic activity of polyphenol oxidase (Karabulut et al., 2007). Polyphenol oxidase is widely distributed in fruits and vegetables. In turn, AD products are often resulted in unfavourable colour changes (Krokida et al., 2001). Further, reduction in nutrients and volatile compounds inevitably occurs during AD. Volatile compounds are vaporized and nutrients which are sensitive to heat and oxygen are lost due to long exposure to temperatures and oxygen during AD process (Yousif et al., 2000; Cui et al., 2004). Overall, other than sun drying, AD is the most inexpensive process to operate but it affects the texture, colour, flavour, and nutritional value of the products extensively.

III.D.2. Freeze Drying

Freeze drying (FD) was first used in the 1950s and it is now wide spread in the chemistry, biotechnology, pharmaceutical, as well as food industries. It is considered the best dehydration method, producing end products of highest quality compared with other dehydration methods.

The drying involves a preliminary freezing of the products followed by drying under reduced pressure with a sufficient heat supply to sublimate ice. The initial dehydration occurs at low temperature, and without heating the oven shelves, the product temperature rises to room temperature; therefore heat damage to the products is minimal (Oetjen and Haseley, 2004). It provides dried products of porous structure and preserves most of the initial raw material's physical and chemical properties such as shape, appearance, taste, colour, flavour, and texture (Krokida et al., 2001; Shishehgarha et al., 2002; Oetjen and Haseley, 2004). Biological activity

and high rehydration capacity of products can be retained as well (Hellman et al., 1983; Oetjen and Haseley, 2004). With the addition of cryoprotectants, enzymes activity of pure phosphofructokinase, lactate dehydrogenase, L-asparaginase, and alkaline phosphatase are recovered after FD (Hellman et al., 1983; Carpenter et al., 1993; Ford and Dawson, 1993). Jiang and Nail (1998) also reported that pure catalase, β-galactosidase and lactate dehydrogenase activity was retained at higher concentrations after FD in the absence of protective agents.

However, there are some limitations that apply to the FD method. Because FD is generally conducted at absolute pressures of below 1 Torr, flavour components (mainly volatile compounds) are largely lost during the process (Flink, 1975). FD products are often sensitive to storage conditions such as temperature or oxygen atmosphere due to the open porous structures (Jiang and Nail, 1998; Oetjen and Haseley, 2004). Bulkiness of the dried products requires additional care during handling and storage. Although a lower freezing temperature is normally preferred as smaller ice crystals are formed which would have little physical damage to the cells, very high freezing rates may cause stresses that result in splitting or cracking of the tissues (Paine and Paine, 1992a; Fellows, 2000). Further, during rapid cooling, the formation of extracellular ice and the concentration of solutes occur more rapidly than the exosmosis of cell water, which therefore results in the cytoplasm becoming supercooled and damaged by the intracellular ice (Acker and McGann, 2002; Mcgann and Farrant, 1976). Nonetheless, it is complicated to determine the optimal pre-freezing temperature for each product. The operation of FD is much slower than AD and VMD which significantly increases the operating cost and the capital costs of the refrigerator and vacuum pump. Although it is beneficial to use FD method, the complexity of the process and the equipment requires trained technicians to operate; also the high operating and maintenance costs limit its applications to high added-value products.

III.D.3. Vacuum Microwave Drying

Microwave heating is now commonly used by households and industries. The mechanism of microwave heating is by the interaction between microwave energy and dielectric materials such as foods. The excitation of dielectric materials dissipates as heat by the application of electromagnetic microwave energy (Kaensup et al., 2002; Scaman and Durance, 2005). The energy penetrates into the interior of the food and provides a rapid heating process (Scaman and Durance, 2005). Vacuum drying is normally used for sensitive materials that can be damaged at high temperature; therefore drying takes place at reduced boiling points in a low-pressure chamber (King et al., 1989). In 1985, Decareau utilized the combination of the microwave heating and vacuum drying together and established the process of vacuum-microwave drying (VMD) (Decareau, 1985). VMD allows drying at low temperature and fast mass transfer conferred by vacuum; the reduced pressure may also inhibit oxidation of the product.

Due to its generally rapid process, VMD has been successfully employed in various applications such as food, agricultural, and pharmaceutical products (Yousif et al., 1999; Kaensup et al., 2002; Cui et al., 2003, 2004; Farrel et al., 2005). Rajasse et al. (2004) found that the enzymatic stability of lipase B under VMD was higher than that of under conventional thermal heating. The carotenoid retention of carrot slices and chlorophyll retention of chive leaves under VMD has also shown to be very close to those dried by FD and much better than those dried by AD (Cui et al., 2004). VMD allows solid food pieces to be dried more rapidly than other dehydration methods (Scaman and Durance, 2005). Generally, VMD is less economical than AD because it processes under reduced pressure; but it is considerably more economical than FD due to no pre-freezing required and shorter dehydration duration

(Owusuansah, 1991). To summarize, VMD may be beneficial to dry food products with desirable quality at a lower cost than FD.

III.E. Packaging and Shelf Stability

Packaging plays an important role in providing the optimum protective properties to keep the product in good condition for its anticipated shelf life. Nowadays, packaging serves as an essential marketing strategy. It is very complicated to choose the appropriate package with the optimal barrier properties for the particular product as different products have different physical natures, nutritive values, and sensitivity to oxygen, temperature and moisture (Paine and Paine, 1992c). To design the right packaging for the product to be shelf stable, various factors such as water activity and moisture content, storage temperature, package condition, and the packaged materials have to be considered. Each of these factors is discussed below.

III.E.1. Water Activity and Moisture Content

When considering the stability of dehydrated products, the water activity is more crucial than the total moisture content. Water activity measures the amount of moisture that is available to support microbial growth and chemical activity. As shown in Figure 4, these factors are strongly dependent on the water activity level. In general, the rate of lipid oxidation is minimum in the a_w range 0.20-0.40; the rate of non-enzymatic browning is highest in the a_w range 0.60-0.80. Bacteria, moulds and yeast will not grow below the a_w range 0.70-0.90 (Drapron, 1985).

On the other hand, enzymatic reactions are also an important cause of food deterioration. They often contribute to the undesirable browning and changes in texture and flavour. Nonetheless, water activity necessary for the initiation of enzymatic reactions is different among enzymes. Enzymatic activities of the common enzymes have been tested as a function of water activity as summarized in Table 5. In summary, water activity has to be relatively high so that enzymes and substrates have sufficient solvents for the occurrence of catalytic reactions.

However, for enzymes involve in lipid oxidation, because the substrates are hygroscopic, their mobility will not be affected by the amount of water; low a_w threshold is often found in these enzymes for the initiation of enzymatic reactions.

III.E.2. Storage Temperature

Low temperature storage is usually practiced to extend the shelf life of products by preserving texture, colour, flavour, and nutrient content. In the frozen state, the molecular movement, microbial growth, chemical and enzymatic reactions usually slow down or even stop (Pardo and Niranjan, 2006). In households, refrigerators and freezers are generally used to slow down the degradation of foods by biochemical spoilage and to reduce growth of bacteria and fungi (Grandison, 2006). Sensitive products after dehydration, kept in low temperature storage, can further preserve the qualities of volatile compounds and enzymatic activity. For example, when dried food products are properly packaged and stored, they have shelf lives of up to 2 years but this will be reduced to 3-6 months at 37 °C (Paine and Paine, 1992b). Studies on the effects of the storage temperature are crucial to derive the optimal quality of dried products.

III.E.3. Package Materials and Conditions

When a food product is dehydrated, moisture is removed, porous structures are formed, and biochemical components are more concentrated. As a result, the dehydrated product readily absorbs water from the surroundings and can be more susceptible to oxidative changes (Eichner, 1985). Minor changes in temperature, moisture, and oxygen level can largely affect the quality. Therefore, dehydrated products should be barrier packaged in a vacuum or inert gas to keep out moisture vapour, air and light (Paine and Paine, 1992c; Brennan and Day, 2006). There are lots

of materials that are currently used for packaging such as low density polyethylene (LDPE), high density polyethylene (HDPE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), ethylene vinyl acetate copolymers (EVA), nylons etc. Each of these has its own characteristics which offer both advantages and disadvantages. For example, LDPE can be extruded into different shapes with low permeability to water vapour but high permeability to organic vapours, oxygen and essential oils; HDPE is more rigid but is more costly than LDPE; PP has excellent grease resistance; PVC has good gas barrier properties and grease resistance and is a moderate barrier to moisture vapour; PS has high tensile strength but is a poor barrier to moisture vapour; EVA has high water vapour and gas resistance and stress cracking resistance, it also has a high impact strength at low temperatures; nylons has high tensile strength and is flexible over a wide temperature range (Eichner, 1985). Appropriate packaging materials can be selected to preserve quality if the specific sensitivities and tolerance values of low moisture foods to water vapour and oxygen uptake are known (Eichner, 1985). Temperature resistance is another factor that has to be considered because some packaging films that become brittle when exposed to low temperatures are not suitable for packaging frozen products. By combining all these factors, each product should have its own specifications of storage temperature, packaging methods and materials, and the optimal moisture content/water activity to extend its shelf life.

III.F. Human Protein Digestion

The human digestive system is made up of the oral cavity, the esophagus, the stomach, duodenum and the small intestine. Digestion of food is carried out by the endogenous secretion of enzymes and is controlled by nervous and hormonal excitatory and inhibitory mechanisms (Magee and Dalley, 1986). There are three phases of enzymatic secretion (cephalic phase, gastric phase, and intestinal phase) which fuse into a continuous process during digestion (Hawker, 1981; Bolt et al., 1983). Table 6 summarizes the major enzymes involved in digestion.

III.F.1. Cephalic Phase

Saliva is secreted to lubricate the food to make swallowing easy. It also contains amylase and a trace of lipase for digestion purposes (Magee and Dalley, 1986). During the cephalic phase, conditioned reflexes established by response to sight and smell in relation to eating induce secretion of gastric juice in preparation for the entry of food into the stomach (Bolt et al., 1983). Gastric juice is secreted for 30 minutes with the rate of approximately 500mL/hour (Bolt et al., 1983).

III.F.2. Gastric Phase

The gastric phase takes place in the stomach where saliva, food, and gastric juices are mixed to form a semi-solid chyme (Versantvoort et al., 2004). The mechanical pressure of the food against the mucosa causes the secretion of acid and pepsin. The gastric mucosa consists of different types of cells, each specialized to synthesize and secrete mucus, pepsinogen, HCl, intrinsic factor or the hormone gastrin ((Hawker, 1981; Magee and Dalley, 1986). Mucus is the

lubricant which helps to keep mucosa damp throughout the gastrointestinal tract while HCl helps to maintain the low pH environment in the stomach. During fasting period, the gastric pH is about 1.5 to 2. The gastric pH rises temporarily to pH 3-7 upon eating a meal. During gastric emptying, the gastric pH gradually declines until the fasted-state pH environment has been re-established (Versantvoort et al., 2004). Normal HCl secretion is 1-5 mmol/hour during resting and 5-40 mmol/hour during stimulation (Hawker, 1981). The acidity of the stomach instantaneously activates pepsinogen at pH 2; however, it is inactivated at pH values above 6 (Magee and Dalley, 1986). Pepsin is most efficient in cleaving bonds involving the aromatic amino acids: phenylalanine, tryptophan and tyrosine and contributes 10-20 % of total protein digestion (Hawker, 1981).

III.F.3. Intestinal Phase

Following the gastric phase, the chyme enters the duodenum and is further mixed, digested, and absorbed in the duodenum. The entry of chyme into the duodenum initiates hormonal responses and nervous reflexes which affect gastric secretion and gastric motility (Hawker, 1981). Pancreatic juice, which contains bicarbonate for neutralizing the acid from stomach, electrolytes, and enzymes for digesting all three major types of macronutrients such as protein, fat, and carbohydrates, is secreted into the duodenum (Versantvoort et al., 2004). The composition of pancreatic juice includes water, bicarbonate, chloride, sodium, potassium, calcium and magnesium, proteases (trypsinogen, chymotrypsinogen, procarboxypeptidase A and B, leucine aminopeptidase, elastase, dipeptidase, oligopeptidase), lipases, amylase, dextrinase, glucoamylase, lactase, sucrase, nucleases (ribonuclease, deoxyribonuclease, alkaline phosphatase), phospholipase A and B, and cholesterol esterase (Hawker, 1981; Bolt et al., 1983). Bile is also secreted in the duodenum from the gallbladder when there is fat in the meal. Sodium

and potassium salts of bile acids are conjugated with taurine and glycine to form taurocholic and glycocholic acid in the ratio of 1:3 (Versantvoort et al., 2004). Bile acid helps to emulsify the large fat particles of the food into small particles so as to be further digested by the lipases; it further aids in the transport and absorption of the digested fat to and through the intestinal mucosal membrane. In the proximal small intestine, fasting bile concentrations are in the range of 1.5-5 mM (1-3 g/L chyme) and increased to 7-15 mM (5-10 g/L chyme) after eating (Versantvoort et al., 2004).

In general, protein digestion is extremely efficient; up to 99 % of ingested protein is absorbed as amino acids (Magee and Dalley, 1986). Enterokinase activates trypsinogen by cleaving at the lysine-isoleucine linkage close to the N terminal end of the trypsin molecule (Hawker, 1981). The activated trypsin thus further activates other trypsinogen moleculesand proteases such as chymotrypsin, elastase and carboxypeptidase. Trypsin cleaves peptide bonds preferentially at amino acids that have positively charged R groups, such as lysine and arginine; chymotrypsin cleaves peptide bonds preferentially at aromatic amino acids such as tyrosine, tryptophan, and phenylalanine while carboxypeptidase cleaves amino acids at the carbonyl end. Final digestion is completed by enzymes located in the brush border of the small intestine, including aminopeptidase and dipeptidase which split the remaining peptides into single or pairs of amino acids. Amino acids, small amount of dipeptides and tripeptides are absorbed in the intestinal mucosal cells by specific membrane transport systems (Hawker, 1981).

III.F.4. *In Vitro* Digestion

In vitro digestion model is a commonly used method to gather information on bioavailability of nutrients (Garrett et al., 2000), allergens (Astwood et al., 1996; Fu et al., 2002), bioactive peptides (Lo and Li-Chan, 2005), as well as contaminants (Oomen et al., 2003;

Versantvoort et al., 2005). Compared to animal models, it is much more simple, rapid, safe and inexpensive to carry out. However, it does not represent the complicated digestion *in vivo*. Most *in vitro* digestion methods involve two phases: gastric digestion phase to simulate the stomach and a subsequent intestinal digestion phase. Several *in vitro* digestion models, briefly outlined in Table 7, have been published. In general, pepsin and hydrochloric acid are used to mimic the gastric phase; and pancreatin, bile salts, together with sodium hydroxide and sodium bicarbonate are used to mimic the intestinal digestion phase with incubation at 37 °C with shaking. The results obtained from *in vitro* digestion model can provide information to better understanding the bioavailability of the compounds of interest and for planning *in vivo* animal studies in the future.

Table 1. Prevalence of Phenylalanine Hydroxylase Deficiency in Live Births by Ethnic Group

Ethnic Group	PAH deficiency in live births	Citation
Turks	1/2600	(Ozalp et al., 1986)
Irish	1/3,500	(Hanley, 2004)
Caucasian	1/10,000	(Scriver and Kaufman, 2001)
East Asian	1/10,000	(Scriver and Kaufman, 2001)
Japanese	1/143,000	(Aoki and Wada, 1988)
Finnish	1/150,000	(Hanley, 2004)

Table 2. Recommendation on Blood Phenylalanine Level for Adults Phenylketonuric Patients

Country	Concentration of Blood Phenylalanine in mg/dL (μmol/L)
Canada	2-10 (120-600)
United States	<10 (600)
United Kingdom	11.5 (700)
Germany	<20 (1200)
France	<21.5 (1300)

Summarized from Hanley (2004).

Table 3. Summary of the Mode of Phenylalanine Ammonia-Lyase Administration from Different Sources for Treatment of Phenylketonuria

Source of PAL	Mode of Administration	Reference
Rhodotorula glutinis	Absorbed in the walls of hollow fibres and covalently bound to the innerwall of small bore nylon tubing	(Pedersen et al., 1978)
Rhodotorula glutinis	Purified PAL freeze-dried and sterilized powder packed into hard gelatine capsules	(Hoskins et al., 1980)
Rhodosporidium toruloides	PAL contained within permeabilized cells	(Gilbert and Jack, 1981)
From microbial source	Semipermeable microcapsules	(Bourget and Chang, 1985)
Rhodotorula glutinis	Entrapped in silk fibroin	(Inoue et al., 1986)
Recombinant *Rhodosporidium toruloides* PAL expressed in *E. coli*	Oral administration and intraperitoneal injection	(Sarkissian et al., 1999)
Rhodotorula glutinis	Microencapsulated in cellulose nitrate membrane	(Habibi-Moini and D'mello, 2001)
Recombinant PAL *Petroselinum crispum* expressed in *L. lactis* cells	Liquid preparation for oral administration	(Liu et al., 2002)
Recombinant *Rhodosporidium toruloides* Polyethelene glycol-PAL expressed in *E. coli*	Intraperitoneal injection	(Gamez et al., 2007)

Table 4. Species and Length of Phenylalanine Ammonia-lyase Sequence (Uniprot Knowledgebase)

Species	Length of PAL Sequence
Yeasts:	
Saccharomyces cerevisiae (Baker's yeast)	635
Rhodosporidium toruloides (Yeast)	714,716
Rhodotorula rubra (Yeast)	713
Fungi:	
Ustilago maydis (Smut fungus)	724
Aspergillus fumigatus (Sartorya fumigata)	727
Aspergillus niger	703
Neurospora crassa	762
Chaetomium globosum (Soil fungus)	706
Plant Sources:	
Arabidopsis thaliana (Mouse-ear cress)	725,717,694,707
Beta vulgaris (Sugar beet)	719
Bromheadia finlaysoniana (Orchid)	703
Camellia sinensis (Tea)	714
Cicer arietinum (Chickpea) (Garbanzo)	718
Citrus limon (Lemon)	722
Cucumis melo (Muskmelon)	619
Daucus carota (Carrot)	708
Dianthus caryophyllus (Carnation) (Clove pink)	618
Digitalis lanata (Foxglove)	713
Glycine max (Soybean)	713
Helianthus annuus (Common sunflower)	667
Hordeum vulgare (Barley)	549,475,497
Ipomoea batatas (Sweet potato)	707,708
Lithospermum erythrorhizon	710,705
Medicago sativa (Alfalfa)	725
Nicotiana tabacum (Common tobacco)	712,715
Oryza sativa subsp. indica (Rice)	713
Oryza sativa subsp. japonica (Rice)	701,713,671
Persea americana (Avocado)	620
Petroselinum crispum (Parsley) (Petroselinum hortense)	716-718
Phaseolus vulgaris (Kidney bean) (French bean)	712, 710
Pinus taeda (Loblolly pine)	754
Pisum sativum (Garden pea)	723,724
Populus balsamifera subsp. Trichocarpa)	715
Populus jackii (Balm of Gilead)	711
Populus kitakamiensis (Aspen)	682-710
Populus sieboldii x Populus grandidentata)	715
Populus tremuloides (Quaking aspen)	711,714
Prunus avium (Cherry)	717
Rubus idaeus (Raspberry)	730
Solanum lycopersicum (Tomato)	704,721
Solanum tuberosum (Potato)	590,720
Stylosanthes humilis (Townsville stylo)	715
Trifolium subterraneum (Subterranean clover)	725
Triticum aestivum (Wheat)	700
Zea mays (maize)	703

Table 5. Enzymatic Reactions Observed in Food/Model Synthetic Media as a Function of Water Activity

Products / substrates	Enzymatic activities observed	Temperature (°C)	A_w threshold or percent moisture content to initiate enzymatic activity
Grains	Various glycoside-hydrolases	30	13% moisture
	Amylases	30	27% moisture
	Lipases	30	16-18% moisture
	Phytases	30	16-18% moisture
Wheat flour dough	Proteases	35	0.96
Rye flour	Amylases	30	0.75
	Proteases	30	0.75
Starch	Various amylases	35	≥ 0.75 in the presence of salts
Casein	Trypsin	30	0.50
L-alanine $4NO_2$ anilide, HCl	Aminopeptidase M	25	0.50
Galactose	Galactosidase	30	$0.40 - 0.60$
Bread	Amylases	30	0.36
	Proteases	30	0.36
Wheat germ	Various glycoside-hydrolases	20	> 0.20
Indoxylacetate	Esterase	20	0.10
Olive oil	Lipase	$20 - 40$	0.025

Adapted from Drapron (1985).

Table 6. Enzymes Involved in Digestive System

Enzyme	pH	Function(s)
Mouth:		
α-amylase	6.5 – 7.0	Starch → oligosaccharides
Stomach:		
Pepsins	1.5 –	Protein → polypeptides
Lipase	3.5	Triglycerides → diglycerides and fatty acids (emulsification of fat)
Small intestine:		
α-amylase	7.0 –	Starch → oligosaccharides
α-dextrinase	8.5	Oligosaccharides → glucose
glucoamylase		Maltotriose/maltose/isomaltose → glucose
Lactase		Lactose → glucose + galactose
Sucrase		Sucrose → fructose + glucose
Lipase A & B		Triglycerides → monoglycerides/diglycerides/free fatty acids/glycerol
Phospholipase		Phospholipids → fatty acids
Ribonuclease		RNA → oligoribonucleotides & ribonucleotides
Deoxyribonuclease		DNA → oligodeoxyribonucleotides & deoxyribonucleotides
Alkaline phosphatase		Removal of phosphate group in proteins/nucleotides/alkaloids
Cholesterol esterase		Sterol ester → sterol + fatty acid
Enterokinase		Trypsinogen → Trypsin
Trypsin		Oligopeptides/protein → large peptides
Chymotrypsin		Oligopeptides/protein → large peptides
Elastase		Oligopeptides/protein → large peptides
Carboxypeptidase		Oligopeptides/large peptides → small peptides & amino acids
Oligopeptidase		Small peptides/tripeptides/dipeptides → amino acids
Dipeptidase		Small peptides → dipeptides
Aminopeptidase		Oligopeptides/small peptides → amino acids

Summarized from Hawker (1981) and Bolt et al.,(1983)

Table 7. *In Vitro* Digestion Methods of Human Digestive System

Testing Compound	Enzyme/chemicals	pH	Mixing Speed (rpm)	Time
Soil contaminant (lead)[a]	Saliva:			
	α-amylase, uric acid, mucin, KCl, NaCl, NaOH	6.5	55	5 minutes
	Gastric Phase:			
	Pepsin, mucin, HCl, NaCl, KCl, NaH_2PO_4	1.07	55	2 hours
	Intestinal Phase:			
	$CaCl_2 . 2H_2O$, pancreatin, lipase, NaCl, HCl, $NaHCO_3$	7.8	55	2 hours
Carotenoid[b]	Gastric Phase:			
	HCl, porcine pepsin	2.0	95	1 hour
	Intestinal Phase:			
	$NaHCO_3$, NaOH, bile extract, pancreatin	7.5	95	2 hours
Food allergens[c]	Gastric Phase:			
	Pepsin, NaCl	1.2	N/A	0-120 mins
	Intestinal Phase:			
	KH_2PO_4, pancreatin	7.5	N/A	0-120 mins

(Oomen et al., 2003[a]; Garrett et al., 2002[b]; Astwood et al., 1996[c])
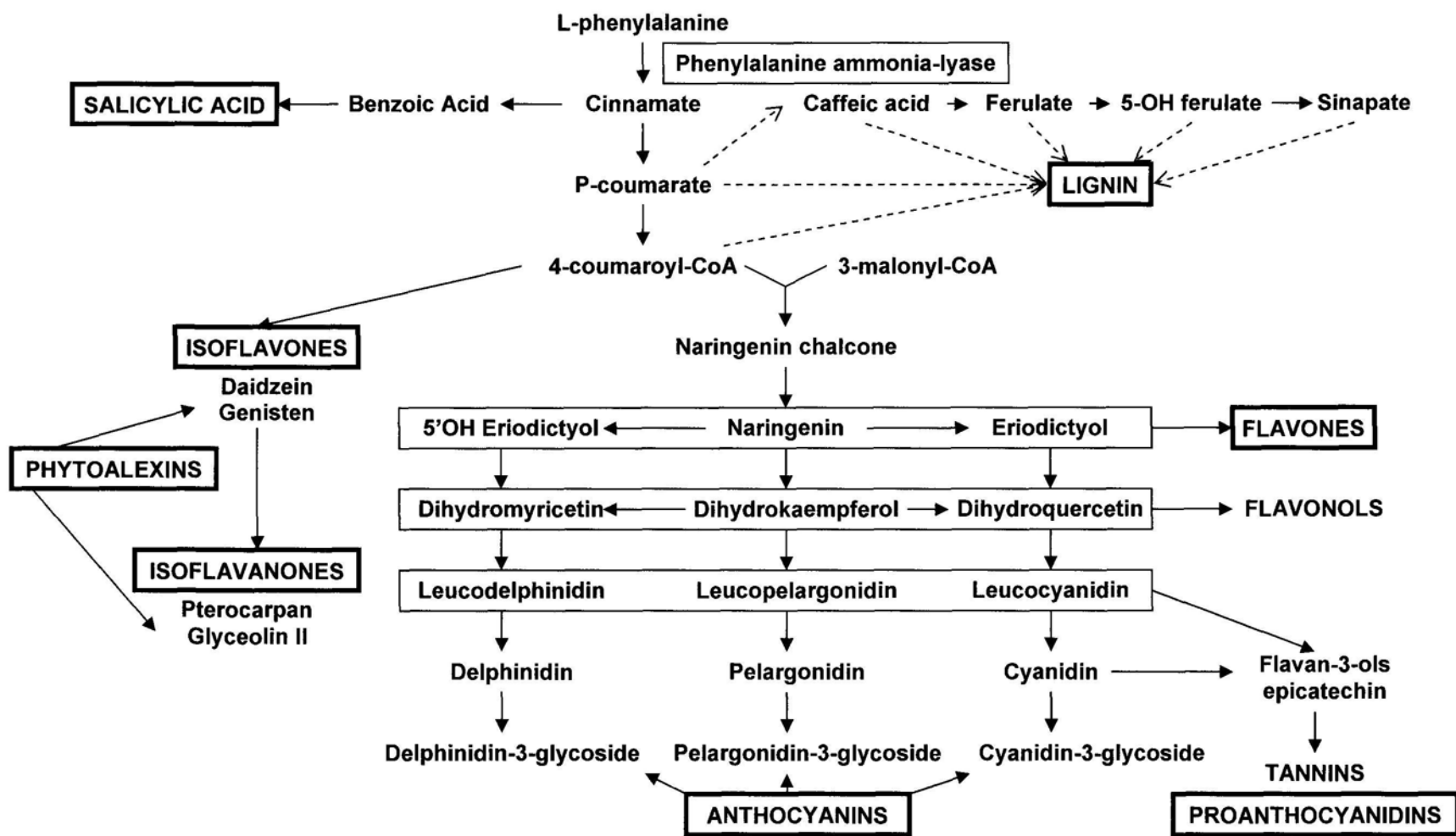
Figure 3. Plant Phenylpropanoid Metabolic Pathway Initiates by Phenylalanine Ammonia-lyase. Adapted from Zabala et al. (2006).
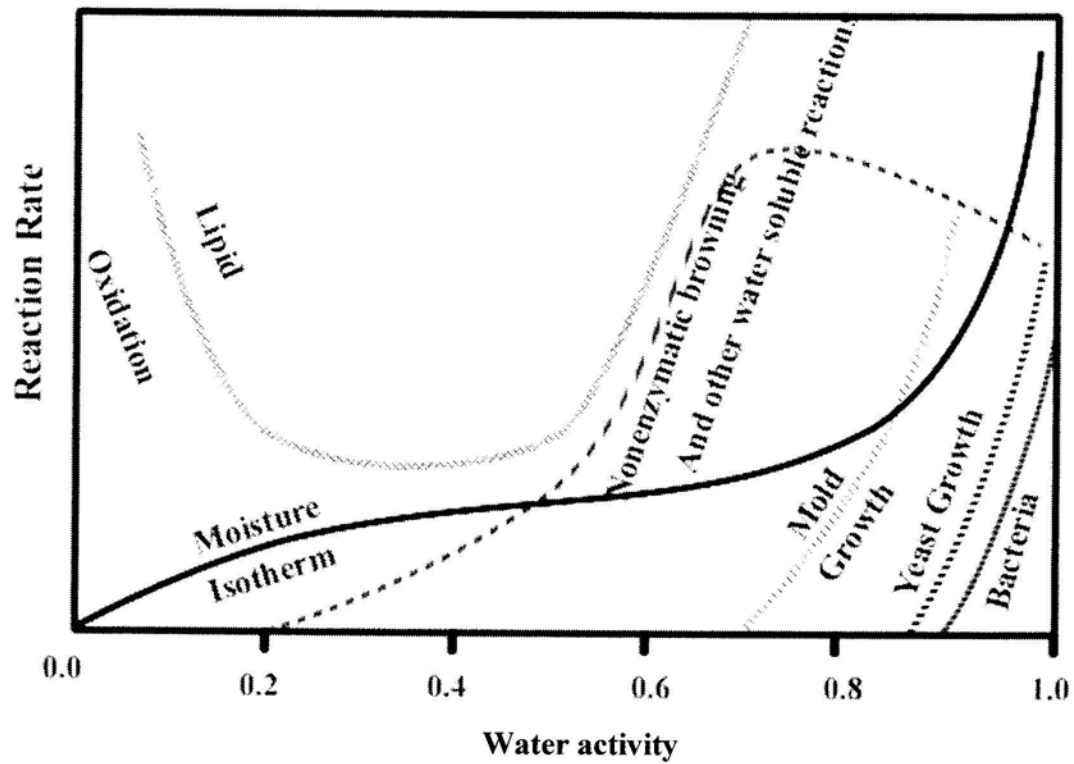
Figure 4. Influence of Water Activity on the Stability of Foods; adapted from Brennan and Day (2006).

IV. Materials and Methods

IV.A. Chemicals

Enzymes, antibodies, and substrates were purchased from Sigma Chemical Co. unless otherwise specified. All chemicals were of reagent grade.

IV.B. Sample Preparation

IV.B.1. Germination of Red Spring Wheat Seedlings

Three bags of red spring wheat seeds, purchased from two different suppliers (WestCoast Seeds; B.C., Canada and Quality Seeds West; B.C., Canada) over 3 years were evaluated. Seeds were soaked in 2.625 % sodium hypochlorite (50% (v/v) chlorine bleach) solution for 15 minutes, rinsed with sterile deionized water, and germinated at room temperature (~25 °C) for 7 days. Seedlings were germinated in sterilized paper towels (Section V.A), and in Freshlife Automatic Sprouters (Tribest Corp., model 2000; California, USA) which provided intermittent hydration with distilled water every 15 minutes (Section V.B). For Section V.A.1, seeds were germinated in the dark with and without regular hydration. After 7 days of germination, leaf tissues were separated from residual seed/root tissues for further analyses. Residual seed is defined as the starchy endosperm, aleurone, pericarp, and seed coats. Only leaf and root tissues were used for analysis in Section V.B.

IV.B.2. Preparation of Dried Samples

Four g of plant tissue were used for each drying process. For freeze drying (FD), wheat seedlings were kept at -80 °C, -35 °C, -25 °C or -18 °C overnight and were dried under reduced pressure (0.027 kPa) for 24 hours. Vacuum-microwave drying (VMD) was performed with a dryer (EnWave Inc.,Whisper model VMD 900 W; B.C., Canada), at 250 W and 4 kPa, for 36 minutes for leaf samples and 40 minutes for residual seed/root samples. Air drying (AD) was carried out at 25 °C using a 240 V, 50 Hz Laboratory Fluid Bed Dryer (Sherwood Scientific Ltd.; Cambridge, United Kingdom) with blower at level 8 (3.10 metres/second) for 20 hours. After drying, plant material was stored in desiccators and assayed for PAL activity within 3 hours. Percent recovery of PAL activity in dried samples was calculated with respect to the fresh samples from the same batch.

IV.B.3. Preparation of Freeze-Dried Samples for Shelf Stability Determination

Freeze-dried leaf and residual seed/root tissues were pre-frozen at -18 °C overnight and freeze-dried for 24 hours under reduced pressure (0.027 kPa). After drying, samples were stored in desiccators and assayed for PAL activity within 3 hours or packed under vacuum and/or non-vacuum in the Vak 3.0R pouch (0.80 mil Nylon / 2.2 mil EVA; West Coast FoodPak System Ltd.; B.C., Canada). Packed samples were stored at 4 °C and/or -20 °C and assayed for PAL activity after storing for 2, 4, 8, and 12 weeks. Freeze-dried samples assayed within 3 hours after hydration were then used as the control to calculate the percent recovery of PAL activity.

IV.C. Analyses

IV.C.1. Water Activity (a_w) and Moisture Content Determinations

The water activity (a_w) of all fresh and dried samples was determined using the Aqualab (Myer Service & Supply Ltd. Model #539; Ontario, Canada). Moisture content of samples was determined using a vacuum air oven at 70 °C and 14 kPa. All water activity readings and moisture content analysis were done in duplicate for each sample.

IV.C.2. Extraction of Phenylalanine-Ammonia-lyase in Red Spring Wheat Seedlings

Fresh wheat seedlings were assayed on the day of harvest, after drying by various methods, and after incubating at various conditions. The relative distribution of PAL activity in the leaves, residual seed/root, and roots of the wheat seedling was determined on day 7 following transfer of seeds to germination conditions.

Plant samples (fresh and dried samples) were homogenized in extraction buffer (50 mM Tris-HCl, pH 8.8, 1 mM EDTA, 10 mM 2-mercaptoethanol,) and 2.5 % (w/v) polyvinylpoly-pyrrolidone (PVPP) in a 1:2 ratio (sample fresh weight : buffer) by grinding with mortar and pestle. After homogenization, samples were kept at 4 °C for 1 hour, and then centrifuged at 23,500 x g (Sorvall RC 5B Plus, Mandel Scientific Co. Ltd.; Ontario, Canada) for 15 minutes at 4 °C. Aliquots of supernatant were desalted using a PD-10 column (GE Healthcare; Quebec, Canada). After filtered through the PD-10 column, 200 µl of the sample filtrate (or 200 µl deionized water used as a blank) was added to 400 µl of reaction buffer (100 mM Tris-HCl, pH 8.8) and 200 µl substrate (40 mM L-phenylalanine, 100 mM Tris-HCl, pH 8.8) and incubated at 37 °C for 15 minutes. An aliquot of 200 µl 25 % trichloroacetic acid (TCA; w/v) was added after incubation to terminate the reaction, and samples were centrifuged at 13,000 x g for 15

minutes (Mikro 20, Hettich Zentrifugen; Germany). Absorbance at 290 nm was used to determine the amount of *trans*-cinnamic acid produced, calculated using a standard curve (Unicam UV/Vis spectrometer; Cambridge, United Kingdom).

For Section V.B.1, extraction buffer was adjusted to pH 2.5 and 9.5, and homogenized with plant samples as mentioned above. Plant samples were cut into ~1-2 cm lengths and submerged in extraction buffer adjusted to pH 2.5 with PVPP in a 1:2 ratio. After 1 hour incubation, samples were all adjusted to pH 8.8 then ground with a mortar and pestle. Percent recovery of PAL activity with pH adjustment was calculated with respect to samples without pH adjustment.

IV.C.3. Protein Concentration Assay

Protein concentration of samples was determined using the Bradford method. A standard curve was constructed by preparing bovine serum albumin (BSA) at different dilutions ranging from 0.0 – 0.9 mg/ml. Bio-Rad protein assay dye reagent concentrate (Sigma; 500-0006) was diluted with deionized water in 1:4 ratios. An aliquot of 2.5 ml diluted dye reagent was mixed with 50μl of sample filtrate or BSA protein standards. Absorbance of all standards and samples were measured at 595 nm using a spectrophotometer (Unicam UV/Vis Spectrometer; Cambridge, United Kingdom).

IV.C.4. Gel Electrophoresis and Western Blot Analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed according to Laemmli (1970) using 10 % acrylamide resolving gels and PageRuler™ prestained protein ladders (#SM0671, Fermentas). Lanes were loaded with 100 μg protein. Proteins were stained with Coomassie Brilliant Blue R-250. Western blots were carried out using a nitrocellulose

membrane, pore size 0.45 μm, using a semi-dry electroblotter. The blot was blocked with 10 ml

5 % non-fat milk powder in phosphate buffered saline Tween-20 (PBST) for 1 hour on a

gyratory shaker. It was then probed with rabbit anti-poplar PAL polyclonal antibodies (1:1000)

in 1.5 ml 3 % non-fat milk powder in PBST buffer for 3 hours. The blot was washed using 3 x

10 ml PBST buffer washes of 20 minutes each, and then incubated for 1 hour (1:2000 dilution in

PBST) with goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma; A 3687). Excess

secondary antibody was removed with 2 x 10 ml of PBST for 20 minutes followed by a 10

minute wash with phosphate buffered saline (PBS) buffer. Alkaline phosphatase activity was

detected on the blot using nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl

phosphate in alkaline phosphate buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5).

IV.C.5. High Performance Liquid Chromatography Analysis of Residual Phe after *in vitro* Protein Digestion

The amount of Phe remaining after incubation was determined by reverse phase high

performance liquid chromatography (RP-HPLC) using a modified method of Qi et al. (2001). A

Hewlett Packard HPLC system with an auto sampler and a diode array UV/vis detector was used

with a reverse-phase Zorbax SR-C18 column (4.6 mm x 150 mm; 5 μm particle size). Flow rate

was 1.0 ml/minute with column temperature at 40 °C. The UV detector was set to monitor the

eluent at 250 nm wavelength. An aliquot of 20 μl of the diluted samples was injected at room

temperature. Table 1 shows the elution method. A standard curve was constructed and the

amount of Phe in the samples was calculated accordingly.

IV.D. Incubation of Red Spring Wheat Seedlings at Various Conditions

IV.D.1. Incubation at pH 8.8 for Determining Phenylalanine Ammonia-lyase Activity

Four g of fresh leaf and root/residual seed samples were cut into ~1-2 cm lengths separately and incubated with 25 ml water adjusted to pH 8.8 using 1 N NaOH for 3 hours at 37 °C incubator with shaking at 100 rpm (Innova 4000, New Brunswick Scientific; New Jersey, USA). In some samples, protease inhibitors, PMSF and EDTA, were added to achieve final concentrations of 0.5 mM and 5 mM respectively. Wheat seedling tissues were removed from the incubation solution and assayed for PAL activity. Percent recovery of PAL activity after incubation was calculated with respect to fresh samples from the same batch without incubation.

IV.D.2. *in vitro* Stability of Phenylalanine Ammonia-lyase to Digestion

Four g of fresh leaf/root and FD leaf/root samples (compensated for moisture loss in weight) were cut into ~1-2 cm lengths and incubated separately with 25 ml of 4 % pepsin (w/w, enzyme/fresh weight basis; 5,120 – 16,000 units/mL) (Sigma; P-7000; 800 – 2,500 units/mg protein) adjusted to pH 2.5 with 1 N HCl for 1 hour at 37 °C with shaking at 100 rpm. The pH was then raised to 5.3 with 1 M $NaHCO_3$ and further raised to pH 7.5 with 1 N NaOH. Pancreatin (4 % w/w, enzyme/fresh weight basis) (Sigma; P-8096; 1x U.S. Pharmacopeia specifications) was added and incubated for 2 hours. At each half-hour interval, samples were removed and reaction was terminated by raising the pH to 7.5 in the first 1 hour (gastric phase) or by addition of 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF, Sigma; A-8456) and EDTA to achieve final concentrations of 0.1 mM and 1 mM, respectively. Wheat seedling tissues were

removed from the reaction solution and subjected to PAL activity assay. Percent recovery of PAL activity after this *in vitro* protein digestion was calculated with respect to fresh samples without digestion from the same batch.

IV.D.3. Phenylalanine Conversion during Incubation at pH 8.8

Four g of fresh leaf and root samples were cut into ~1-2 cm lengths or ground with a mortar and pestle to homogenize with 25 ml of water adjusted to pH 8.8 using 1 N NaOH for 2 hours at 37 °C incubator with shaking at 100 rpm. At each half-hour interval, samples were removed and reaction was terminated by boiling for 10 minutes. Samples were then centrifuged at 13,000 x g for 10 minutes (Mikro 20, Hettich Zentrifugen; Germany). Supernatants of samples were diluted for 40 times and filtered through 0.22 μm filter units. A blank control was performed without the addition of wheat samples and a sample control was performed without incubated for 2 hours. The amount of Phe left after *in vitro* protein digestion was measured by HPLC analysis. The amount of Phe being digested by wheat seedlings was calculated by difference with respect to the sample control.

IV.D.4. Phenylalanine Conversion during *in vitro* Protein Digestion

Four g of fresh or FD leaf and root samples, ground with a mortar and pestle or intact, were subject to *in vitro* protein digestion. On the other hand, four g of fresh leaf and root samples were ground with extraction buffer in a 1:2 ratio in a mortar and pestle. The cellular fluids of these samples were collected, adjusted to 25 ml with distilled deionized water and to pH 2.5 with 1 N HCl, and subjected to *in vitro* protein digestion. The *in vitro* protein digestion is described in section IV.D.2 with slight modifications. One hundred mg of L-phenylalanine

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(equivalent to 24.2mM) was added to *in vitro* protein digestion. At each half-hour interval, samples were removed and subjected to boiling for 10 minutes to denature all the proteins and enzymes. Samples were then centrifuged at 13,000 x g for 10 minutes (Mikro 20, Hettich Zentrifugen; Germany). Supernatants of samples were diluted 40 times and filtered through 0.22 micron filter units. A blank control was performed without the addition of wheat samples and a sample control was performed without incubated for 3 hours.

The residual Phe after the *in vitro* protein digestion was measured by HPLC analysis. The amount of Phe converted by wheat seedlings was calculated by difference in the Phe with respect to the sample control.

IV.E. Statistical Analysis

For spectrophotometric analysis of PAL activity, all experiments were done at least twice with duplicate samples, and triplicate readings obtained from each sample. For HPLC analysis, all experiments were done at least twice with duplicate samples unless otherwise stated.

Minitab statistical software (Version 12.0) was used for statistical analysis. One-way analysis of variance was used to compare means. Tukey's significant difference test was used for pairwise comparison of means. Linear regression model was used to establish correlations between different variables. Significance of difference was defined at $p < 0.05$.

Table 8. Solvents and Gradients of HPLC Analysis

Time (minute)	Solvents/Gradients	
	Solvent A: Methanol	Solvent B: 0.2% TFA
0	10.0%	90.0%
0.1	10.0%	90.0%
9.0	35.0%	65.0%
9.1	50.0%	50.0%
14.0	50.0%	50.0%
18.0	75.0%	25.0%
21.0	75.0%	25.0%
30.0	100.0%	0.0%
35.0	100.0%	0.0%

V. Results and Discussion

V.A. Dehydration and Shelf Stability of Phenylalanine Ammonia-lyase Activity in Red Spring Wheat Seedlings

V.A.1. Effects of Growth Conditions on Phenylalanine Ammonia-lyase Activity on Fresh Tissues

Three bags of red spring wheat seeds purchased from two different suppliers over 3 years were used in this study. No significant difference (p< 0.05) in the PAL activity was observed between these samples. Similarly, work from McCallum and Walker (1990) has also reported that no difference was found in PAL activity of different cultivars in red spring wheat. On a dry weight basis, PAL activity of leaf and root/residual seed samples germinated with regular hydration in the light had 11.90 ± 2.64 µmol/h/g and 6.48 ± 1.59 µmol/h/g, respectively. It has been suggested that both leaf and root/residual seed produce coumarins, lignans and lignin, which are derived from the activity of PAL, whereas flavonoid production is leaf-specific (Olenichenko and Zagoskina, 2005). Hence the difference in PAL activity between leaves and root/residual seed samples might correspond to the production of flavonoid in leaf samples.

Variation in expression of PAL activity of wheat seedlings was observed with different germination conditions. Sterilized wheat seeds were germinated in the dark or exposed to natural light for 7 days with or without hydration. The effects of light exposure and hydration on PAL expression were found as shown in Table 9. The major challenge for germinating in the dark without hydration was the presence of mold and non-germinated seeds. With the exposure of sunlight, longer green leaves and more roots were developed in comparison to those grown in the dark. PAL activity was found to be higher in leaf samples with light exposure. PAL activity was shown to be stimulated and induced by sunlight in maize vegetative tissues and lettuce seeds as

well (Daines and Minocha, 1983; Singh et al., 1999). In addition, the availability of water was found to be a critical factor to affect PAL activity. Wheat seedlings grown on paper towels in the dark without watering were significantly lower in PAL activity and moisture content. On a dry weight basis, leaf tissue was highest in PAL activity followed by root and root/residual seed. Based on these results, wheat seedlings for further experiments were germinated with light exposure with regular hydration and used for drying of samples using various methods; for *in vitro* protein digestion and shelf-stability determination of wheat seedlings, automatic sprouters which provided intermittent hydration with distilled water every 15 minutes were used to maintain high PAL expression and mold-free wheat seedlings.

V.A.2. Dehydration of Wheat Seedlings

Various dehydration methods of wheat seedlings were evaluated to concentrate plant-encapsulated PAL activity and to produce a stable product. Two common methods of dehydration, FD and AD, and the relatively novel technique of VMD were performed. Table 10 shows the moisture content and water activity of both fresh and dried seedling samples. The initial moisture content of the leaves and root/residual seed tissues of 7-day-old wheat seedlings was 88.6 % (wb) and 74.1 % (wb), respectively. Although the moisture content of fresh leaf tissues was higher than that of the root/residual seed tissues by ~14 % (wb), the water activity for the root/residual seed tissue was slightly higher, suggesting that more water-binding compounds were present in the leaves. After dehydration by various methods, the water activity of all samples decreased to shelf-stable levels ($a_w = 0.3 - 0.4$) (Rockland and Beuchat, 1986). The moisture content of all root samples was similar, however, the moisture content of leaf samples that were subject to VMD was significantly higher than the FD or AD leaf samples.

V.A.3. Effects of Different Drying Methods on Phenylalanine Ammonia-lyase Activity

The method of dehydration had a significant effect on retention of PAL activity (Figure 5). FD was optimal for retaining PAL activity in all seedling tissues, with 108 ± 6 % and 90 ± 13 % of PAL activity recovered in leaf and root/residual seed tissues respectively. PAL activity was significantly decreased following AD of leaf tissue; root/residual seed tissue retained a similar proportion of PAL activity following AD and VMD treatments. Acid proteinase activity has been reported to be highest in the leaves and lowest in the roots of wheat seedlings with maximum proteolytic activity observed at high temperature (52 °C) (Frith et al., 1975). Therefore, it is possible that PAL in leaf tissue suffered greater proteolysis during the extended air-drying time, compared to that in the roots. AD was carried out at room temperature, because higher temperatures (35-75 °C) used in preliminary work resulted in very low recovery of activity (data not shown).

Other studies have typically shown that the retention of enzymes or other bioactive components of plant tissues subjected to VMD is intermediate between that found with AD and FD treatments (Lin et al., 1998; Cui et al., 2003). Several factors can contribute to this. The temperature of the samples during the majority of the VMD process is low, as they are maintained at boiling point of water at the reduced pressure of the chamber (~30 °C at 4 kPa). In this work, the temperature of leaf samples subjected to VMD was maintained at approximately 27 °C while the temperature of root/residual seed samples was approximately 25 °C throughout the dehydration. Also, VMD is a rapid process, and in this case, dehydration was completed within 36 - 40 minutes, compared to 20 hours required for air drying. As well, the process occurs under very low oxygen pressure, inhibiting degradative oxidation reactions.

V.A.4. Abundance of Phenylalanine Ammonia-lyase-Related Polypeptides

In red spring wheat seedlings, three PAL-related polypeptides (74, 83 and 103 kDa) were detected by Western blot analysis (Figure 6). Separate analysis of the leaf and the residual seed/root of seedlings revealed that the leaves contained all three polypeptides while the root/residual seed tissue contained only the 74 kDa polypeptide. Nari et al. (1972) has reported the 75 kDa and 85 kDa polypeptides separated by electrophoresis of highly purified wheat PAL. These two bands may correspond to the smaller bands revealed in this work. As mentioned previously, PAL genes often exhibited differential patterns of accumulation in response to environmental stimuli and at different stage of development (Bolwell et al., 1985; Gowri et al., 1991; Ishikawa et al., 2007). It has also been reported that subunits of PAL can be subjected to proteolytic processing (Bolwell et al., 1985; Jorrin and Dixon, 1990). Therefore, the different polypeptides identified by Western blot analysis may arise from different protein processing such as N-linked glycosylation, proteolytic processing, or phosphorylation.

All three PAL-related polypeptides remained after FD and VMD but 103 kDa polypeptide was found to be relatively weak in VMD. In AD leaf sample, only the 83 kDa polypeptide was detectable. This suggests that there was less degradation of PAL during FD, and the polypeptide abundance correlated well with the higher retention of activity following FD. In root/residual seed samples, there were no differences in the abundance of the 74 kDa polypeptide with the different drying methods.

V.A.5. Effects of Freezing Temperatures on Phenylalanine Ammonia-lyase Activity of Freeze-Dried Tissues

The FD process was further investigated by subjecting samples to various pre-freezing temperatures of -18 °C, -25 °C, -35 °C and -80 °C (Table 11). After dehydration, the moisture content and water activity were not significantly different among the samples. PAL activity in leaf samples was more sensitive to pre-freezing temperatures than root/residual seed samples. Pre-freezing of leaves at -18 °C resulted in the best retention of the PAL activity; but no significant difference was observed with the different temperatures for the root/residual seed samples. The proportion of enzyme activity retained as a result of a given pre-temperature treatment may be related to the size of ice crystals formed during freezing (Fellows, 2000). Little physical damage should occur to cells subjected to lower freezing temperatures, as smaller ice crystals form quickly within both cells and intercellular spaces; however, very high freezing rates may cause dimensional stresses leading to the disintegration of sensitive products (Paine and Paine, 1992a; Fellows, 2000). During rapid cooling, the formation of extracellular ice and the concentration of solutes occur more rapidly than the exosmosis of cell water, which therefore results in the cytoplasm becoming supercooled and damaged by intracellular ice (Acker and McGann, 2002; Mcgann and Farrant, 1976). The filamentous fungus (*Laccaria fraternal*) has also been found to be sensitive to pre-freezing temperature (Sundari and Adholeya, 1999). Maximum rehydration property was obtained with a low freezing rate (approximate 1 °C/minute) (Sundari and Adholeya, 1999). Leaf samples might be more sensitive to the pre-freezing temperature; therefore a slower freezing rate at -18 °C may have prevented the cells from freeze damage. As a result, pre-freezing at -18 °C resulted in the highest recovery of PAL activity in leaf samples.

V.A.6. Shelf stability of Phenylalanine Ammonia-lyase activity of freeze-dried tissues under various storage conditions

Work from Rees and Jones (1996) has established that loss of PAL activity in concentrated PAL powder is rapid when exposed to the water activity of above 0.5 due to covalent and non-covalent aggregation of enzyme. In addition, wheat tissues might have to be prepared and consumed by the patients on a daily basis; therefore, the shelf stability of plant preparations must be taken into consideration. As discussed in V.A.4, FD was found to be the best method to retain PAL activity in both leaf and root/residual seed tissues. FD samples were then chosen for further analysis of the shelf stability of PAL under various storage conditions. Three factors were evaluated here: storage temperature (4 °C and -20 °C), package condition (vacuum packed and non-vacuum packed), and storage duration (2-week, 4-week, 8-week and 12-week). The percent recovery of PAL activity at different weeks was calculated with respect to FD samples assayed within 3 hours (Time 0). PAL activity of leaf and root/residual seed samples was found to follow different trends with respect to these factors. Figure 7 demonstrated the effects of each factor on PAL activity of leaf tissue. Significant differences were found between storage duration, batches, storage temperature, and package condition with no interaction among factors. Since batch 1 and batch 2 leaf samples were significantly different, all factors were analyzed separately and summarized in Table 12. PAL activity of batch 2 was significantly higher than that of batch 1 which might be due to variation in wheat sprouts. Although all leaf tissues were germinated in the same condition, differences may still be found within the same batch due to variations in each independent wheat seedling. Over 2-week, 4-week, 8-week and 12-week, PAL activity in leaf tissues was observed to retain better under -20 °C storage than 4 °C. In the frozen state, the molecular movement, and chemical and enzymatic reactions slow down (Pardo and Niranjan, 2006). The mobility of endoproteases was limited; therefore PAL activity was retained better during storage at –20°C than storage at 4 °C.

Vacuum-packed versus non-vacuum-packed did not show any significant impact on activity retention, suggesting that the activity of PAL is less affected by the oxidation of amino acids than temperature. The PAL activity of the FD leaf tissues assayed within 3 hours after dehydration was 7.63 ± 0.52 μmol/h/g dry wt in batch 1 and 8.85 ± 0.49 μmol/h/g dry wt in batch 2. Figure 8 showed the percent recovery of PAL activity in leaf samples in week 12 with respect to FD samples at time 0. In both batches, leaf tissues stored at -20 °C under vacuum-packed best preserved PAL activity followed by -20 °C under non-vacuum-packed; while significantly lower PAL activity was retained at 4 °C with or without vacuum-packed. Hence, PAL in leaf tissues was more sensitive to storage temperature than package condition.

Unlike leaf samples, FD root/residual seed samples were significantly different between storage temperature, storage duration, and package condition but not between batches. General main effect plots of each factor were displayed in figure 9. Similar to leaf samples, PAL activity in root/residual seed samples was more sensitive to storage temperature than the package condition. Also root/residual seed samples did not show interactions among factors. To examine each factor independently, Tukey's test was performed on 2-week, 4-week, 8-week and 12-week samples at each combination of the package condition and storage temperature factors (Table 13). In all four selected storage durations, PAL activity was best retained under -20 °C and vacuum-packed treatment followed by -20 °C and non-vacuum-packed treatment. Storage of root/residual seed sample at 4 °C significantly decreased its PAL activity as early as week 2. Although PAL activity seemed to have an increase over three months of storage, no significant difference was found between storage duration in all four groups (at each combination of storage temperature and package condition).

The PAL activity at time 0 of FD root/residual seed tissues was 7.09 ± 2.37 μmol/h/g dry wt. Figure 10 shows the percent recovery of PAL activity in root/residual seed samples in week 12 with respect to FD samples at time 0. The results have demonstrated that PAL activity in both

leaf and root/residual seed tissues were affected by storage temperature but less dependent on package condition. Root/residual seed tissues exhibited stable PAL activity over time while leaf tissue PAL activity was found to decrease over 12 weeks of storage. In summary, considering all the factors above, PAL activity was best retained at -20 °C over three months in both leaf and root/residual seed tissues.

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V.B. Stability and Activity of Phenylalanine Ammonia-lyase in Red Spring Wheat Seedlings under Various Incubation Conditions

In this work, both HPLC analysis and spectrophotometric method were used to determine the stability and activity of PAL in red spring wheat seedlings under various incubation conditions. The HPLC analysis approach was different from the spectrophotometric analysis of PAL activity. HPLC analysis measured the amount of residual Phe after digestion; while the spectrophotometric analysis of PAL activity measured the amount of *trans*-cinnamic acid produced. The amount of *trans*-cinnamic acid was not measured using HPLC method because during the three hour incubation in the solution, other enzymes might convert *trans*-cinnamic acid into other phenylpropanoid compounds. The spectrophotometric analysis uses PAL in the form of a crude extract to assay the amount of *trans*-cinnamic acid produced in 15 minutes of reaction time at 37 °C. Therefore, to summarize the two methods, spectrophotometric analysis of PAL activity measured the activity of PAL retained after digestion, while the HPLC analysis measured the conversion of Phe by PAL during the incubation. Due to different time factor in each analytical method, it is not suitable to compare the results from these two methods. The results obtained from HPLC analysis showed that while less Phe was converted during *in vitro* digestion, less enzymatic activity was retained after *in vitro* protein digestion using spectrophotometric analysis. This suggested that the retention of the enzyme after digestion corresponded with the conversion of Phe during digestion. Hence, the trend of the results from the two analytical methods agreed with each other.

V.B.1. Stability of Phenylalanine Ammonia-lyase Activity During Incubation at Various pH Conditions

In the human digestive system, the gastric phase functions at pH 1.8 – 3.5 and intestinal phase functions at pH 6.7 – 8.5 (Berne and Levy, 1996). This wide pH range may be harsh on the conformation and activity of PAL. Before evaluating the effect of proteases on PAL activity, the first step was to examine the stability of PAL activity under acidic and basic pH conditions. PAL activity assay was slightly modified to determine the effect of pH. Controls were set up by incubating ground samples with extraction buffer/PVPP at pH 8.8 in a 1:2 ratio for one hour. Samples tested at pH 9.5 and 2.5 were done by incubating ground samples with extraction buffer/PVPP at respective pH for one hour. An examination of the pH-dependence of PAL activity of wheat by McCallum and Walker (1990) has suggested that PAL has an optimum around pH 8.8 – 9.0. In turn, acidic environment might have a big impact on its activity. Therefore, during the pH 2.5 incubation, some samples were kept un-ground to monitor the protection of cell walls on PAL in acidic condition. The pH of all samples were changed to 8.8 then ground after one hour incubation and subjected to the normal assay at pH 8.8.

Incubation at different pH affected PAL activity of both leaf and residual seed/root samples (Table 14). Residual seed/root samples were more sensitive to pH changes. Ground root/residual seed samples incubated at pH 2.5 and pH 9.5 were significantly different from the control. However, no difference among control, ground samples incubated at pH 9.5, and non-ground samples at pH 2.5 was observed in leaf samples. For both leaf and residual seed/root samples, the percent recovery of ground samples incubated at pH 2.5 were significantly lower than that of non-ground samples. This suggested that cell walls of wheat seedlings can protect PAL from an acidic environment.

V.B.2. Stability of Phenylalanine Ammonia-lyase Activity in Wheat Seedlings during Incubation at Optimal pH

The results discussed in section V.B.1 demonstrated that PAL activity in non-ground wheat seedlings is stable after incubation at pH 2.5 and pH 9.5; therefore further experiments were investigated to evaluate incubation at optimal pH over the duration of digestion on PAL at 37 °C. It was reported that wheat seedlings contain proteases which are expressed during the early stage of grain development (Frith et al., 1975; Dominguez and Cejudo, 1996). Also, the endoproteolytic activity in crude wheat leaf extracts is very stable over a wide temperature range (Frith et al., 1975). Therefore, it was essential to evaluate the effect of endoproteases at body temperature over three hours of incubation on PAL. Both leaf and root samples were incubated at pH 8.8 at 37 °C for three hours with or without the addition of serine-protease inhibitor (PMSF) and metalloprotease-inhibitor (EDTA). Solutions were removed from the leaf and root tissues after incubation; and samples were then subjected to the normal PAL activity assay. PAL activity of samples incubated for three hours was compared with that of the fresh samples without incubation.

The results revealed that three hours of incubation at 37 °C of wheat seedlings have a significant impact on PAL activity (Table 15). Although the incubation was held at the optimal pH of PAL, the activity in both fresh leaf and root tissues decreased by 31% and 68 % respectively. Root tissues appeared to be excessively sensitive to the incubation, which might be due to the fact that root tissues have the highest specific protease activity on a fresh weight basis (Frith et al., 1975). Dominguez and Cejudo (1996) have reported that endoproteases in wheat extracted in 70 % ethanol are successfully inhibited by PMSF, EDTA and pepstatin A and pHMB. They also demonstrated that serine proteases are predominant in the early stage of grain development. However, in this study, the addition of protease inhibitors did not improve the retention of PAL but rather further reduced its activity in leaf samples. Therefore, there might be

limited accessibility of PMSF and EDTA to the seedlings due to the protection of the cell walls which blocked their inhibitory effects.

V.B.3. Retention of Phenylalanine Ammonia-lyase Activity after *in vitro* Protein Digestion

It is important to further study the stability of PAL in red spring wheat seedlings after *in vitro* protein digestion such that its stability to the proteolytic attacks in the gastric phase and intestinal phase could be observed. To investigate the PAL stability in both leaf and root tissues during *in vitro* protein digestion, leaf and root tissues were incubated with pepsin at pH 2.5 for one hour and pancreatin at pH 7.5 for two hours. At half-hour interval, samples were removed and reaction was terminated by raising the pH to 7.5 for the gastric phase or by adding protease inhibitors such as 4-(2-aminoethyl)benzensulfonyl fluoride (AEBSF) and EDTA for the intestinal phase.

As shown in figure 11 and 12, fresh leaf and root samples exhibited similar patterns. The percent recovery of PAL activity in fresh leaf and root samples decreased over three hours of incubation. A significant decrease in percent recovery of PAL activity was seen in the first 1.5 hour of incubation. Only 44.3 ± 8.2 % in leaf tissues and 52.9 ± 26.1 % in root tissues were recovered after 1.5 hour incubation. For both tissues, the decrease of PAL activity stabilized after half hour incubation in the pancreatic phase. In total, there was a 35.4 % and 49.5 % drop in PAL activity over the first hour of digestion in gastric phase and 28.4 % and 9.7 % drop over the 2 hour of digestion in pancreatic phase in fresh leaf and root tissues respectively. This could be explained by the fact that the optimal pH of PAL is in the range of pH 8.0 – 9.5 (Camm and Towers, 1973); therefore, the acidic environment might not be favourable for PAL as denaturation might have occurred. Root tissues were revealed to be more susceptible to gastric

digestion than pancreatic digestion. This result agreed with section V.B.1 that root samples were more sensitive to low pH than leaf samples.

At the end of three hours *in vitro* protein digestion, 36.2 % and 41.8 % of PAL activity was recovered in leaf and root tissues respectively, which is equivalent to 4.3 μmol/h/g dry wt and 3.7 μmol/h/g dry wt. As found in section V.B.2, 68.7 % and 31.5 % of PAL activity were recovered after three hours incubation at optimal pH in leaf and root tissues respectively. PAL activity in fresh wheat seedlings was able to protect against the proteolytic attacks from *in vitro* protein digestion solutions but it was more susceptible to endoproteolytic attacks.

In part A of this study, FD was found to be the best method to retain PAL activity; however, FD leaf tissues appeared to be very sensitive to the gastric digestion. Over 50 % of PAL activity decreased in the first half hour of simulated gastric digestion and almost no activity was recovered after 3 hours of digestion. FD root tissues did not show any recovery after half hour of simulated gastric digestion (data not shown). The differences in PAL recovery between fresh and FD samples might be explained by the following reasons. Freeze-drying usually forms a porous structure in the samples (Fellows, 2000) which allowed the PAL enzyme to be more susceptible to the digestive enzymes, or more easily lost to the solution. In addition, nuclear magnetic resonance studies of freeze-dried parenchyma apple tissue revealed that freeze-drying method has destroyed membrane integrity and caused cell wall collapse (Hills and Remigereau, 1997).

Gilbert and Jack (1981) have reported that PAL in yeast *Rhodotorula glutinis* was completely inactivated by duodenal juice after 30 minutes due to its susceptibility to chymotrypsin, subtilisin and trypsin. Considering that fresh leaf and root samples in this study have retained approximately 36 % and 42 % of PAL activity respectively after three hours of digestion, the rigidity of cell walls in fresh tissues must have an effect on encapsulating PAL and

protecting it against the acidity and proteases whereas FD samples failed to retain the activity due to loss of cell wall integrity during dehydration process.

V.B.4. Amount of Phenylalanine Digested during *in vitro* Protein Digestion Quantified by High Performance Liquid Chromatography (HPLC) analysis

It is crucial to evaluate the PAL activity of wheat seedlings during *in vitro* digestion; however, it is not possible to verify the production of *trans*-cinnamic acid by spectrophotometric analysis due to the limitations of the spectrophotometric method. Instead, reverse phase HPLC was used for analysis. *Trans*-cinnamate is the precursor of numerous compounds such as 4-coumarate, caffeate, ferulate and sinamate (Street and Cockburn, 1972; Ellis, 1997). Evaluating the amount of *trans*-cinnamic acid produced during *in vitro* digestion would not be the best method because it may be further broken down into other products and result in an underestimation of PAL activity. The residual Phe after digestion was quantified instead. Four grams of fresh or FD wheat seedlings (calculated on a fresh weight basis) were incubated with pepsin at pH 2.5 for one hour and pancreatin at pH 7.5 for two hours together with the addition of 100 mg Phe. The *in vitro* protein digestion reaction was terminated at half-hour intervals by boiling for 10 minutes to denature all the proteins and enzymes. The residual Phe after each half-hour interval was determined. Based on a Phe standard curve (Appendix E), the conversion of Phe by wheat seedlings was calculated by difference.

Ground and non-ground leaf and root tissues were first subjected to two hours incubation at pH 8.8 at 37 °C to determine the conversion of Phe by PAL at optimal pH. Significant differences were found among treatments but no difference was found among time intervals (Table 16). The conversion of Phe in ground tissues was significantly less than non-ground tissues. Similar results were obtained from section V.B.1, ground samples incubated at pH 2.5

had significantly lower PAL activity recovery than that of the non-ground samples. During grinding, endoproteases and PAL within the cytoplasm might both be released into the solutions. Consequently, PAL would be more susceptible to endoproteases and being denatured.

The ground and non-ground fresh and freeze-dried leaf and root tissues were further investigated in the *in vitro* protein digestion. Conversion of Phe during digestion by fresh/FD, leaf/root tissues, with/without grinding were shown in Table 17 and 18. For all samples, no difference in the conversion of Phe was found among different time intervals, suggesting that the enzymatic reaction was mainly active in the first half hour of the incubation. Interestingly, ground and non-ground samples in both leaf and root tissues did not show any significant difference. The reason for this remains unknown. One may explain that the grinding process released proteins from the plant cells into the reaction solution; the gastrointestinal proteases and endoproteases preferentially hydrolysed these proteins that were more abundant than PAL, and therefore provided a protecting effect for PAL. Cellular fluid released from grinding leaf and root tissues in extraction buffer were also subjected to digestion. After three hours of digestion, only 6.3 mg and 6.1 mg of Phe were converted by leaf and root tissues, respectively. Compared to ground leaf and root tissues (18.3 and 13.5 mg of Phe conversion respectively), extracts had relatively low Phe conversion. Juice extracts of PAL had excluded the cell walls and some other proteins present in plant cells. Consequently, PAL enzyme was more susceptible to proteases without the cell walls and proteins that could be used as the protecting effect. Further study must be carried out before conclusion could be drawn.

Similar to the results from section V.B.3, conversion of Phe by FD samples was significantly less than fresh samples. The results further confirmed that FD samples were more sensitive to the digestive environments. This may be due to the lack of protection from the rigid cell walls resulting in higher rates of enzyme inactivation.

Both HPLC and spectrophotometric analyses demonstrated that there was significantly lower Phe conversion and *trans*-cinnamic acid production in FD samples, followed by fresh root tissues and fresh leaf tissues accordingly. In addition, the reactions were observed mainly at the beginning of the digestion. The major loss of PAL activity was also found in the gastric phase by spectrophotometric analysis. The amount of Phe was shown to be taken up in the first half hour of digestion using HPLC analysis. Both of the results suggested that the conversion of Phe took place at the early stage of the digestive process.

Table 9. Phenylalanine Ammonia-lyase Activity calculated on a Dry Weight Basis in Fresh Plant Tissues under Different Growth Conditions.

Growth condition(s)	$A_w{}^a$	Moisture content (% wb)[a]	PAL activity (μmol/hr/g dry wt)[a]
Root and residual seed: dark /no watering	0.976 ± 0.008	51.0 ± 14.8	3.38 ± 1.24^x
Root and residual seed: dark / with watering	0.993 ± 0.001	82.8 ± 2.0	10.96 ± 4.10^y
Root and residual seed: light / with watering	0.994 ± 0.030	74.7 ± 8.3	6.48 ± 1.59^y
Leaf: dark / with watering	0.989 ± 0.009	89.4 ± 1.2	8.88 ± 1.28
Leaf: light / with watering	0.973 ± 0.069	88.6 ± 2.2	11.90 ± 2.64
Root: light / with watering	0.976 ± 0.003	91.3 ± 0.99	8.16 ± 3.11

xy Within the same tissue, treatments denoted by different letters are significantly different ($p < 0.05$).

[a]Values represent the mean of $4 - 21$ determinations $\pm$ standard deviation.

Table 10. Moisture Content and Water Activity of Fresh and Dried Wheat Seedling Tissues

Sample & dehydration method	Water activity $(a_w)^b$	Moisture content $(\%wb)^b$
Fresh leaf	0.973 ± 0.069	88.6 ± 2.2
AD leaf	0.369 ± 0.007	4.7 ± 3.5^x
FD leaf	0.404 ± 0.064	5.0 ± 0.9^x
VMD leaf	0.376 ± 0.029	12.6 ± 2.6^y
Fresh root/residual seed	0.994 ± 0.030	74.7 ± 8.3
AD root/residual seed	0.352 ± 0.026	8.4 ± 0.3
FD root/residual seed	0.410 ± 0.050	11.6 ± 3.1
VMD root/residual seed	0.348 ± 0.074	10.5 ± 1.6

[b]Values for samples are the mean of 3-5 determinations $\pm$ standard deviation.
[xy] Within the same tissue, treatments denoted by different letters are significantly different ($p < 0.05$).
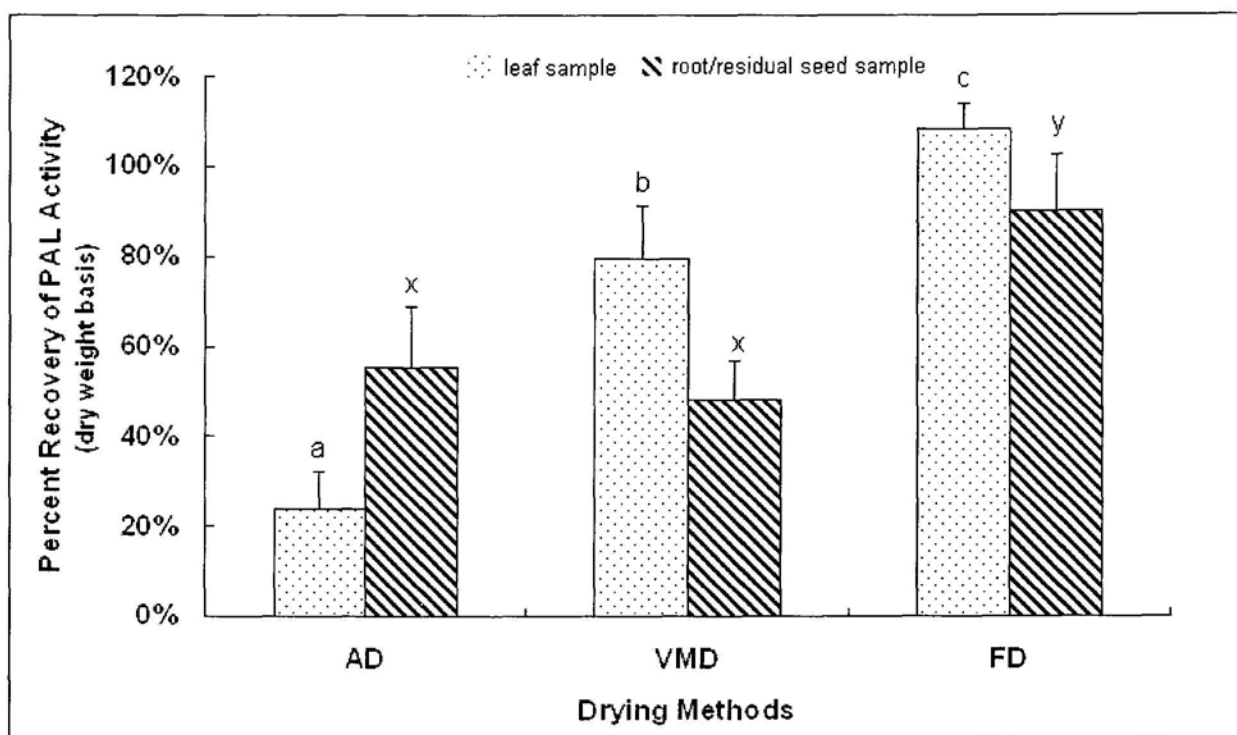
Figure 5. Percent Recovery of Phenylalanine Ammonia-lyase Activity in Dehydrated Wheat Seedling Tissues (Leaf and Root/Residual seed). Leaf and root/residual seed samples were subjected to air- (AD), vacuum microwave- (VMD), and freeze-drying (FD) with pre-freezing at -18 °C. Percent recovery was calculated on a dry weight basis relative to fresh samples. Within the same tissue sample, treatments denoted by different letters are significantly different ($p <$ 0.05).
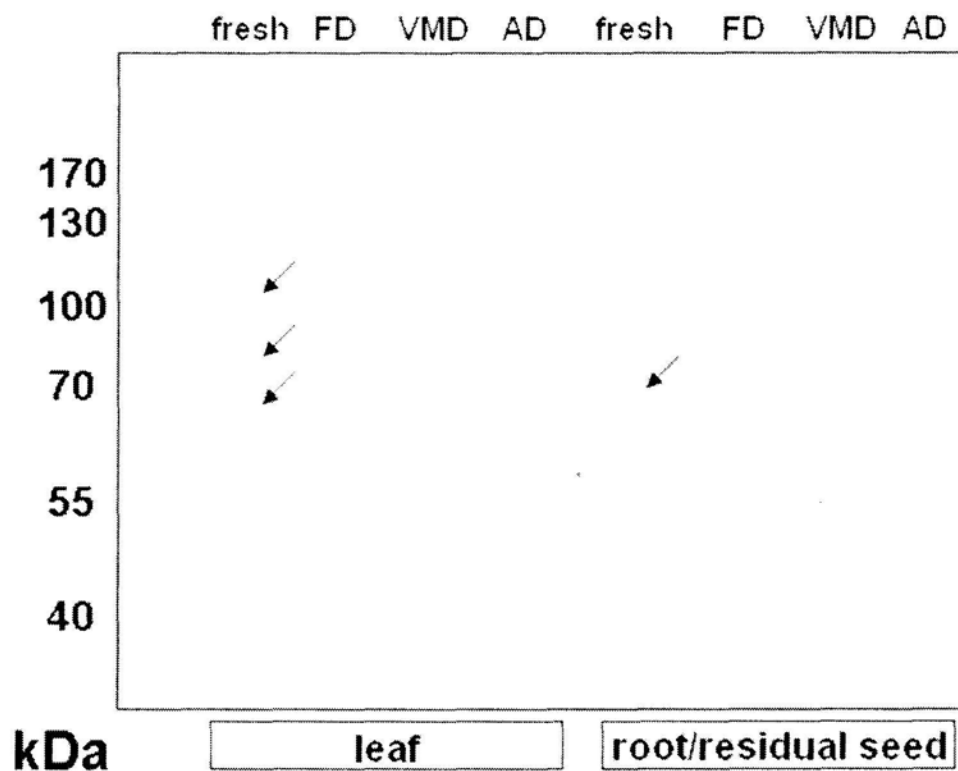
Figure 6. Western Blot Analysis of Proteins Extracted from Dehydrated Wheat Seedling Tissues. Arrows point to PAL-related polypeptides. Three PAL-related polypeptides were detected in leaf tissues (103, 83, 74 kDa); the 74 kDa polypeptide was detected in the residual seed/root tissues. Fresh: non-dried; AD: air-dried; FD: freeze-dried; VMD: vacuum-microwave dried.

Table 11. Moisture Content, Water Activity and Percent Recovery of Phenylalanine Ammonia-lyase Activity in Freeze-Dried Seedling Tissue Subjected to Various Pre-Freezing Temperatures

Sample	Pre-freezing temp (°C)	Water activity $(a_w)^b$	Moisture content (% wb)^b	PAL activity (% fresh sample) [abc]
Leaf	-18	0.404 ± 0.063	5.0 ± 0.9	108 ± 6^x
	-25	0.450 ± 0.055	6.0 ± 1.1	69 ± 12^y
	-35	0.502 ± 0.060	7.5 ± 1.4	89 ± 7^z
	-80	0.449 ± 0.061	6.4 ± 1.4	75 ± 11^{yz}
Root/residual seed	-18	0.410 ± 0.050	11.6 ± 3.1	90 ± 13
	-25	0.468 ± 0.043	6.3 ± 1.8	88 ± 12
	-35	0.464 ± 0.037	8.2 ± 5.2	95 ± 9
	-80	0.451 ± 0.052	6.0 ± 2.8	88 ± 8

[a]Percent recovery of PAL activity was calculated with respect to activity in fresh samples (dry weight basis).
[b]Values represent the mean of 3 to 10 determinations ± standard deviation.
[c]Within the same sample, treatments denoted by different letters are significantly different ($p < 0.05$).

**Figure 7.** Main Effect Plot of Phenylalanine Ammonia-lyase Activity in Freeze-dried Leaf Samples with Respect to Storage Duration (weeks), Storage Temperature (°C), Package Condition, Replicate Readings of Samples and Batches.

Table 12. The Effects of Storage Duration, Storage Temperature and Package Condition on Phenylalanine Ammonia-lyase Activity in Two Different Batches of Freeze-dried Leaf Samples

Package condition		PAL activity (μmol/h/g dry wt)[yx]			
		Vacuum-packed		Non-vacuum-packed	
Batch # Storage temperature Storage duration		4 °C	-20 °C	4 °C	-20 °C
Batch 1	Week 2	3.36 ± 0.74^b	3.90 ± 0.54^b	2.53 ± 0.56^c	5.84 ± 0.17^a
	Week 4	1.17 ± 1.60^{ab}	0.57 ± 0.72^b	0.19 ± 0.61^b	2.56 ± 0.95^a
	Week 8	0.58 ± 0.21^c	2.53 ± 0.56^b	0.81 ± 0.28^c	3.22 ± 0.19^a
	Week 12	0.31 ± 0.12^c	5.05 ± 0.52^b	1.76 ± 0.31^d	4.44 ± 0.24^a
Batch 2	Week 2	12.78 ± 0.37	11.89 ± 1.56	11.28 ± 1.93	10.89 ± 1.07
	Week 4	5.60 ± 0.70^{ab}	7.84 ± 0.56^b	2.16 ± 1.38^b	5.77 ± 1.30^a
	Week 8	7.66 ± 0.99^b	13.19 ± 2.55^{ab}	4.47 ± 0.40^b	11.53 ± 1.88^a
	Week 12	3.87 ± 0.65^b	8.06 ± 0.40^a	2.96 ± 0.48^b	7.80 ± 0.96^a

[x]Values represent the mean of 2 determinations $\pm$ standard deviation.
[y]For the sample within the same week, treatments denoted by different letters are significantly different ($p < 0.05$).

Figure 8. Percent Recovery of Phenylalanine Ammonia-lyase Activity in Freeze-dried Leaf Samples after 12 Weeks of Storage at Various Storage Temperatures and Package Conditions. Leaf samples of batch 1 and batch 2 were subjected to 12 weeks of storage at 4 °C and -20 °C, in vacuum pack and non-vacuum pack. Percent recovery was calculated on a dry weight basis relative to freeze-dried leaf samples assayed within 3 hours. Within the same batch, treatments denoted by different letters are significantly different ($p < 0.05$).
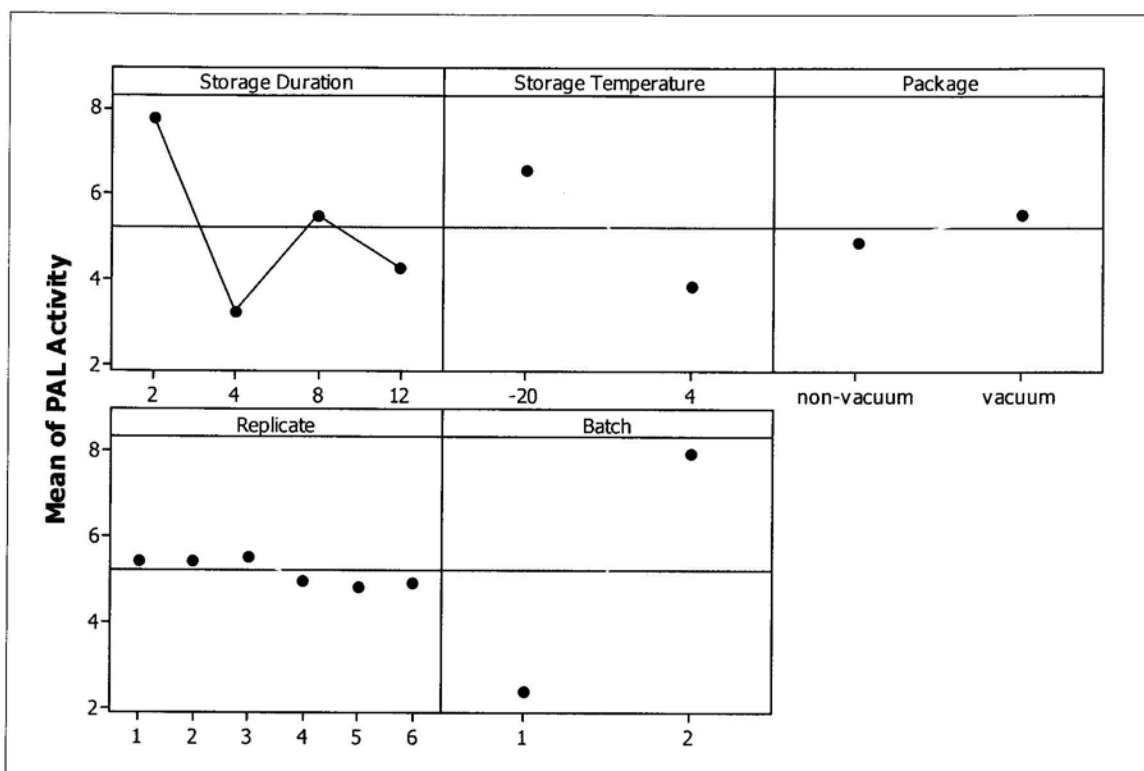
Figure 9. Main Effect Plot of Phenylalanine Ammonia-lyase Activity in Freeze-dried Root/Residual seed Samples with Respect to Storage Duration (weeks), Storage Temperature (°C), Package Condition, Replicate Readings of Samples and Batches.

Table 13. The Effects of Storage Duration, Storage Temperature and Package Condition on Phenylalanine Ammonia-lyase Activity of Freeze-dried Root/Residual Seed Samples

	PAL activity (μmol/h/g dry wt)[yx]			
Package condition	Vacuum-packed		Non-vacuum-packed	
Storage temperature Storage duration	4 °C	-20 °C	4 °C	-20 °C
Week 2	1.29 ± 0.85^{c}	4.53 ± 0.66^{b}	0.71 ± 0.20^{c}	3.64 ± 0.74^{a}
Week 4	0.74 ± 0.31^{c}	3.66 ± 0.88^{b}	2.54 ± 1.10^{a}	2.41 ± 0.80^{a}
Week 8	1.65 ± 1.32^{c}	5.86 ± 0.78^{b}	1.33 ± 1.09^{c}	4.04 ± 0.59^{a}
Week 12	2.91 ± 0.67^{b}	6.00 ± 1.94^{a}	1.63 ± 0.44^{c}	6.59 ± 0.98^{a}

[x] Values represent the mean of 4 determinations ± standard deviation.

[y] For the sample within the same week, treatments denoted by different letters are significantly different ($p < 0.05$).
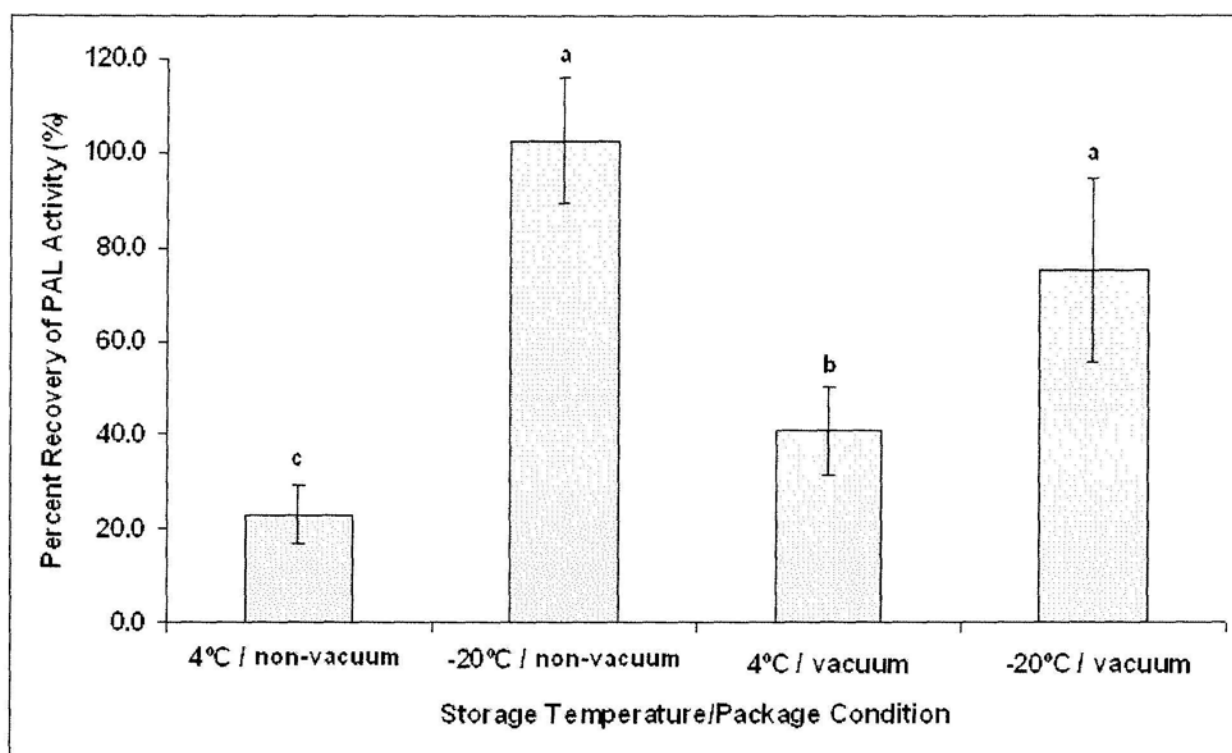
Figure 10. Percent Recovery of Phenylalanine Ammonia-lyase Activity in Freeze-dried Root/Residual seed Samples after 12 Weeks of Storage at Various Storage Temperatures and Package Conditions. Root/residual seed samples were subjected to 12 weeks of storage at 4 °C and -20 °C, under vacuum-packed and non-vacuum-packed. Percent recovery was calculated on a dry weight basis relative to freeze-dried root/residual seed samples assayed within 3 hours. Treatments denoted by different letters are significantly different ($p < 0.05$).

Table 14. Percent Recovery of Phenylalanine Ammonia-lyase Activity in Fresh Leaf and Root/Residual seed Samples Incubated at Various pH

Treatment(s)	PAL activity (% control fresh sample)[yxz] Leaf	Root
Control	100.6 ± 5.4^a	99.8 ± 10.0^e
Incubation: ground, pH 2.5	52.6 ± 6.4^b	48.4 ± 26.1^g
Incubation: non-ground, pH 2.5	91.4 ± 17.7^a	82.9 ± 18.6^{ef}
Incubation: ground, pH 9.5	100.1 ± 3.6^a	61.6 ± 19.0^{fg}

[x]Percent recovery of PAL activity was calculated with respect to activity in fresh samples incubated at pH 8.8 (dry weight basis).
[y]Values represent the mean of 4 to 8 determinations ± standard deviation.
[z]Within the same tissue, treatments denoted by different letters are significantly different ($p < 0.05$).

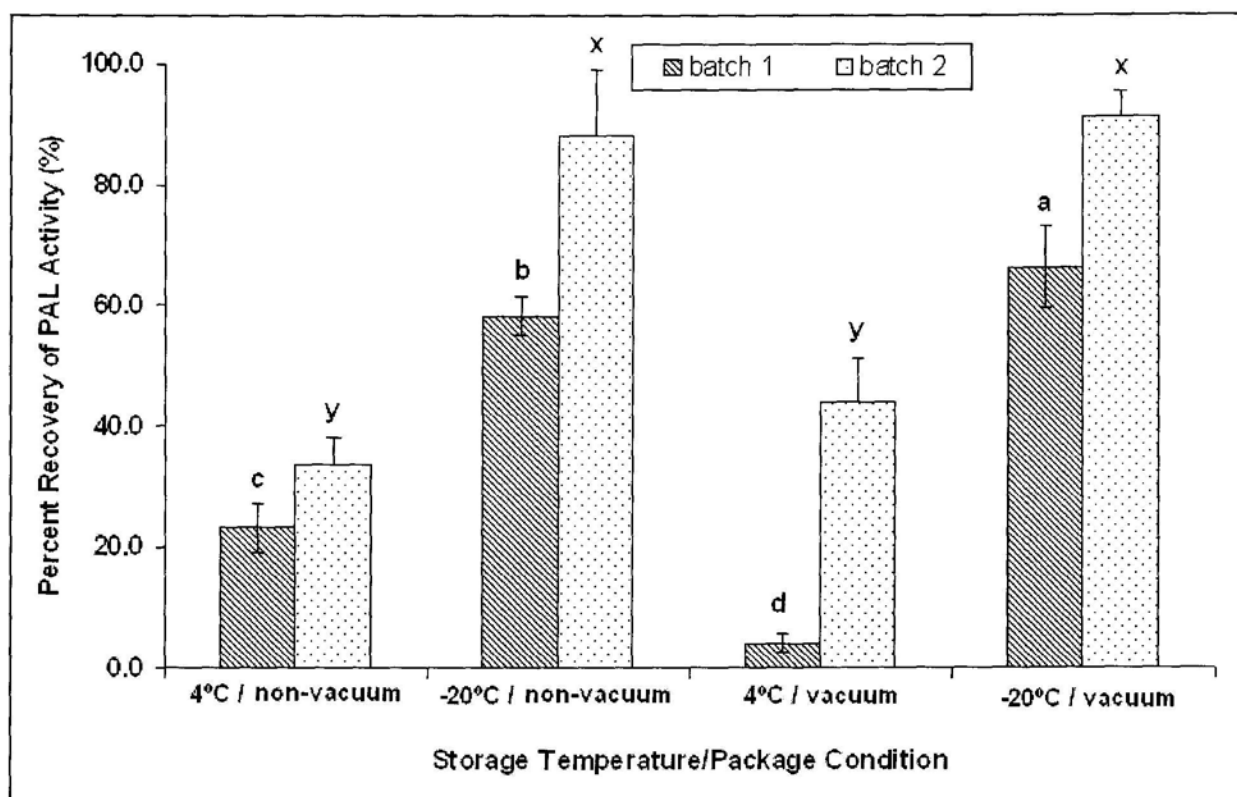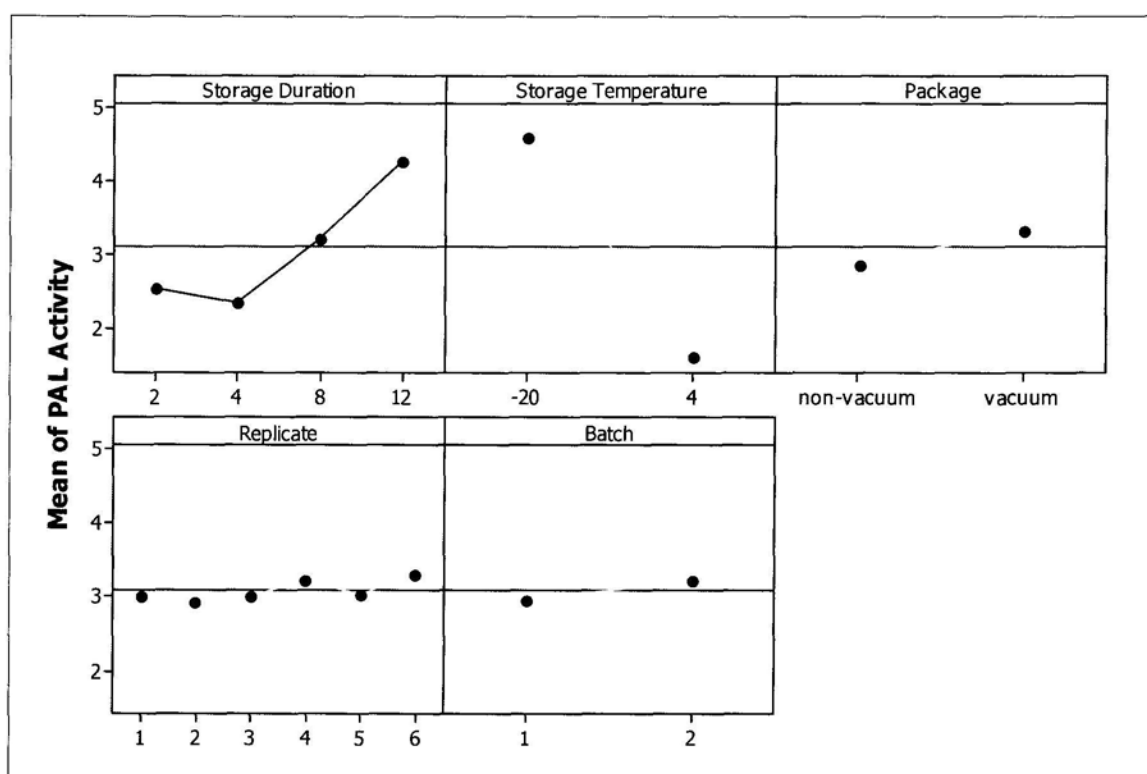**Table 15.** Phenylalanine Ammonia-lyase Activity in Fresh Leaf and Root Tissues after Three Hours Incubation at pH 8.8 at 37 °C with or without the Addition of Protease Inhibitor

Treatment(s)	PAL activity (% fresh sample)[yxz]	
	Leaf	Root
Control[p]	100.0 ± 4.3^a	100.0 ± 13.2^e
Incubation: 3 hrs, pH 8.8 and 37 °C	68.7 ± 2.5^b	31.5 ± 2.3^f
Incubation: 3 hrs, pH 8.8 and 37 °C with protease inhibitors[q]	42.8 ± 9.8^c	31.8 ± 3.0^f

[x]Percent recovery of PAL activity was calculated with respect to activity in fresh samples (dry weight basis).
[y]Values represent the mean of 6 determinations ± standard deviation.
[z]Within the same tissue, treatments denoted by different letters are significantly different ($p < 0.05$).
[p]For control, fresh leaf and root samples were assayed using the PAL activity assay method.
[q]Final concentrations of 0.5mM PMSF and 5mM of EDTA were added together with the samples before incubation.
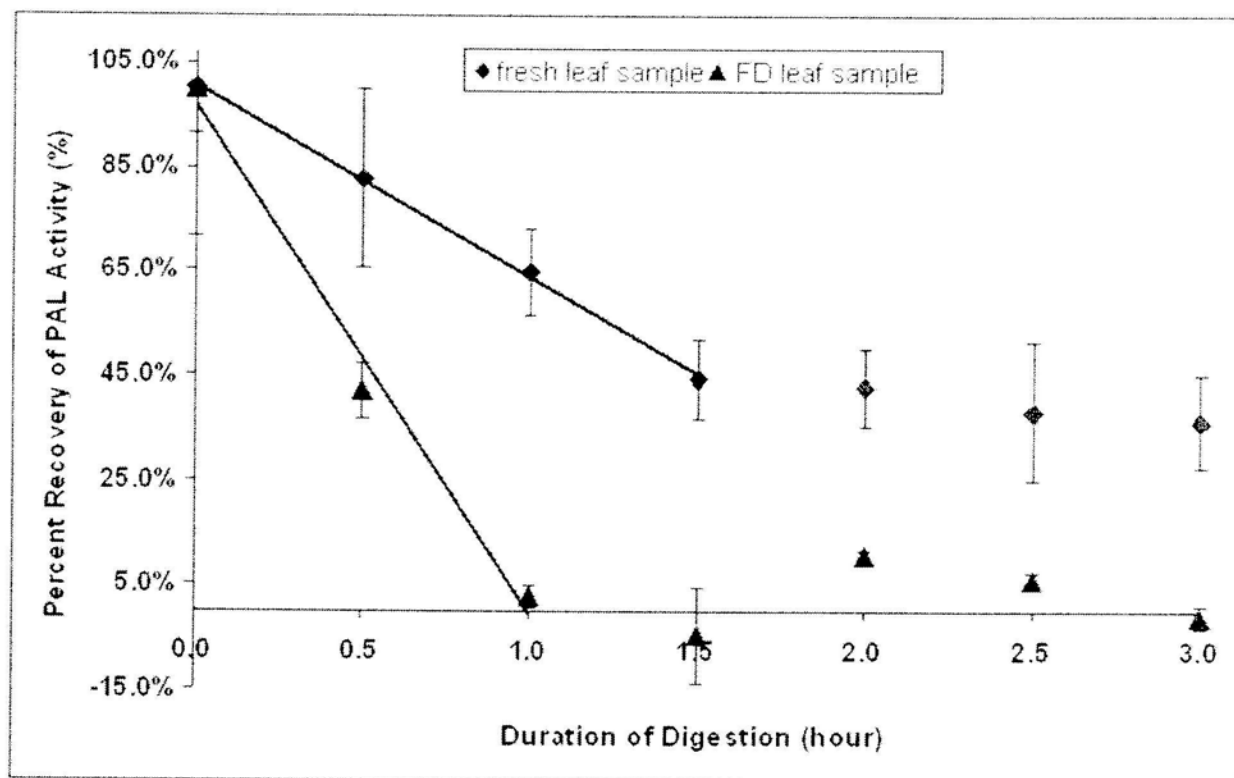
Figure 11. Percent Recovery of Phenylalanine Ammonia-lyase Activity in Fresh and Freeze-dried Leaf Tissues over 3 Hours of *in vitro* Digestion. Fresh and FD leaf tissues were subjected to 1 hour peptic digestion and 2 hours pancreatic digestion (4% of fresh samples by weight). Values represent the mean of 6-8 determinations ± standard deviation. Linear regressions were extrapolated on the first 4 digestion intervals in fresh leaf samples (percent recovery of PAL activity = -0.37(duration of digestion) + 1.01; R^2 = 1.00) and on the first 3 digestion intervals in FD leaf samples (percent recovery of PAL activity = -0.97(duration of digestion) + 0.97; R^2 = 0.99).
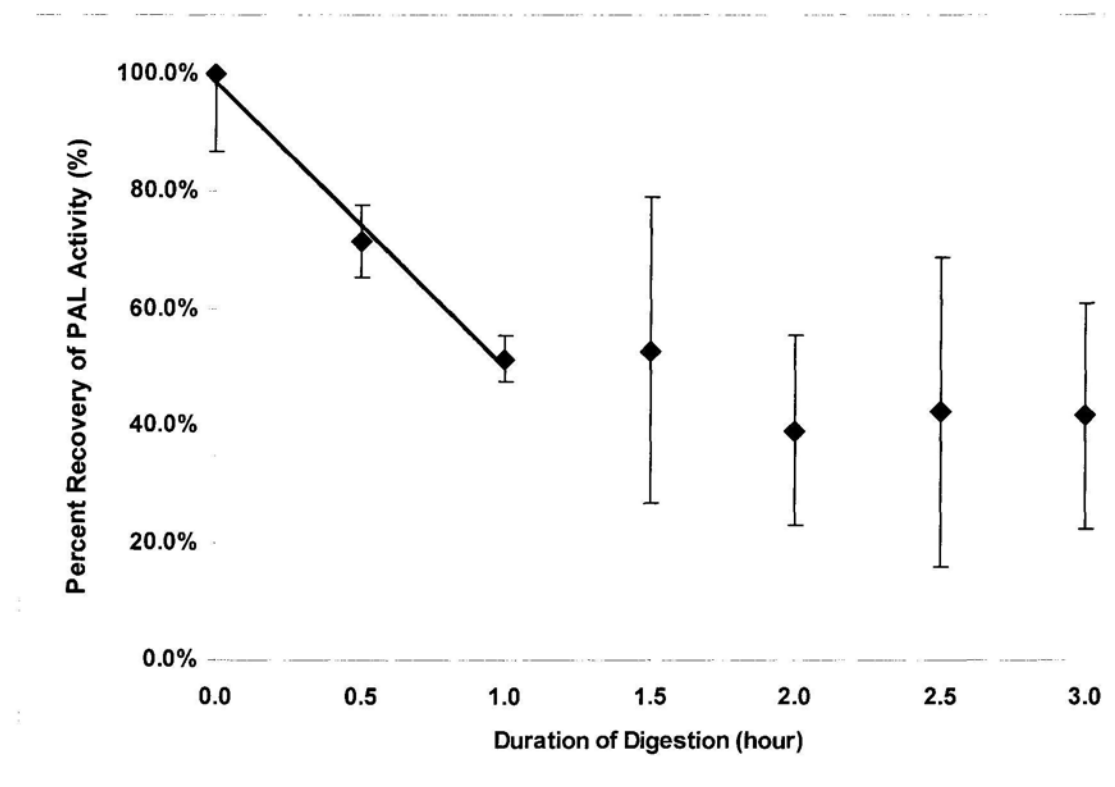
Figure 12. Percent Recovery of Phenylalanine Ammonia-lyase Activity in Fresh Root Tissues over 3 Hours of *in vitro* Digestion. Fresh root tissues were subjected to 1 hour peptic digestion and 2 hours pancreatic digestion (4% of fresh samples by weight). Values represent the mean of 6-8 determinations ± standard deviation. Linear regression was extrapolated on the first 3 digestion intervals in fresh root samples (percent recovery of PAL activity = -0.49(duration of digestion) + 0.99; R^2 = 0.99).

Table 16. Conversion of Phenylalanine after Two Hours Incubation with Fresh Leaf and Root Tissues at pH 8.8 at 37 °C

Duration of incubation (hr)	Conversion of phenylalanine (mg)[x]			
	Leaf		Root	
	Ground[a]	Non-ground[b]	Ground[c]	Non-ground[d]
0.5	4.0 (1.2)	11.4	6.7	17.7 (1.0)
1.0	6.0 (2.0)	13.0	10.4	16.1 (3.4)
1.5	5.8 (1.8)	15.8 (0.3)	11.2	17.6(0.8)
2.0	7.3 (1.4)	20.1	10.1 (0.1)	12.1(1.9)

[x]Values represent the mean (range) of determinations. Values without range were single determinations. Conversion of phenylalanine by four grams of tissues calculated in fresh weight basis.

[abcd]Different letters denoted difference between ground and non-ground samples in each tissue.

Table 17. Conversion of Phenylalanine after Three Hours *in vitro* Protein Digestion with Ground and Non-ground Fresh and Freeze-dried Leaf Tissues

Duration of digestion (hr)	Conversion of phenylalanine (mg)$^\beta$			
	Fresh leaf^x		Freeze-dried leaf^y	
	Ground^a	Non-ground^a	Ground^b	Non-ground^b
0.5	10.1 ± 3.5	14.7 ± 1.4	4.7	4.1 (1.1)
1.0	12.1 ± 7.3	13.6 ± 4.0	5.3	3.4 (1.3)
1.5	11.0 ± 0.8	10.9 ± 3.7	7.9	3.5 (1.8)
2.0	15.2 ± 3.4	12.6 ± 2.7	8.3	2.5
2.5	18.1 ± 2.7	12.2 ± 5.5	6.4	1.2
3.0	18.3 ± 4.0	14.4 ± 4.2	8.6	6.7 (3.7)

$^\beta$Conversion of phenylalanine by four grams of tissues calculated in fresh weight basis.
^{x}Values represent the mean of 3 determinations ± standard deviation.
^{y}Values represent the mean (range) determinations. Values without range were single determinations.
^{ab}Different letters denoted difference between different treatments.

Table 18. Conversion of Phenylalanine after Three Hours *in vitro* Protein Digestion with Ground and Non-ground Fresh and Freeze-dried Root Tissues

Duration of digestion (hr)	Conversion of phenylalanine (mg)$^\beta$			
	Fresh root^x		Freeze-dried root	
	Ground^a	Non-ground^a	Ground^{by}	Non-ground^{bx}
0.5	9.7 ± 4.4	5.1 ± 4.7	0.8	2.2 ± 0.8
1.0	8.6 ± 5.7	5.6 ± 2.3	0.3	2.4 ± 0.3
1.5	6.8 ± 2.5	9.9 ± 2.4	2.9	1.7 ± 0.7
2.0	15.8 ± 8.0	5.6 ± 0.8	4.5	2.5 ± 0.9
2.5	10.7 ± 2.2	11.8 ± 0.7	5.2	2.4 ± 2.0
3.0	13.5 ± 8.2	10.3 ± 3.7	5.1	1.5 ± 0.6

$^\beta$Conversion of phenylalanine by four grams of tissues calculated in fresh weight basis.
^{x}Values represent the mean of 3 determinations ± standard deviation.
^{y}Values represent single determination.
^{ab}Different letters denoted difference between different treatments.

VI. Conclusion

VI.A. Study Findings

This study has shown that red spring wheat (*Triticum aestivum* L.) seedlings have the potential to be used as an alternative oral therapy for PKU and HPA patients. The best condition to obtain high PAL expression in red spring wheat seeds was germination in the light with regular hydration for 7 days. Three PAL-related polypeptides (74, 83 and 103 kDa) in the seedlings were detected by Western blot analysis. Separate analysis of the leaf and the residual seed/root of seedlings revealed that the leaves contained all three polypeptides while the root/residual seed tissues contained only the 74 kDa polypeptide. The different polypeptides may arise from different protein processing such as N-linked glycosylation, proteolytic processing or phosphorylation. Higher PAL activity in leaf tissue might correspond to the two extra polypeptides not found in root/residual seed tissue. Concentrated and dried preparation of wheat seedlings containing high levels of PAL activity was successfully generated using different methods such as AD, VMD and FD. Among the three drying methods, FD was optimal for retaining PAL activity, with 108% and 90% of PAL activity recovered in leaf and root/residual seed tissues respectively. Leaf tissues might be sensitive to the freezing rate and the size of ice crystals formation during freezing. Therefore, the pre-freezing temperature prior to FD treatment showed a significant effect on leaf tissues. Pre-freezing of leaves at -18 °C resulted in the best retention of the PAL activity. PAL activity in FD wheat seedlings was stable over three months of storage at -20 °C. Further, the stability of PAL activity was investigated under various incubation conditions such as acidic and alkaline pH environments and *in vitro* digestion. Although PAL activity of FD samples was successfully retained after dehydration and over 3 months of storage, it was not well retained through the *in vitro* protein digestion. Over 50

% of PAL activity decreased in the first half hour of simulated gastric digestion and no activity were recovered after 3 hours of digestion of both FD leaf and root tissues. When comparing fresh and FD seedlings, the HPLC analysis of residual Phe after *in vitro* digestion demonstrated that <50 % and <35 % of Phe conversion found in FD leaf and root tissues than fresh tissues, respectively. In contrast, PAL activity in fresh red spring wheat seedlings was stable under various incubation conditions. It was retained after incubation at acidic and alkaline environments for two hours. After three hours of *in vitro* protein digestion, 36% and 42% of PAL activity was recovered in fresh leaf and root tissues respectively. As reported by Gilbert and Jack (1981), PAL in yeast *Rhodotorula glutinis* was completely inactivated by duodenal juice after 30 minutes due to its susceptibility to proteases. Compared to yeast, PAL in fresh seedlings has some protection against acidic environments and proteolytic attacks, possibly by the complex structures of the rigid cell walls. Overall, this study has established the success of dehydrating crude red spring wheat seedlings to retain PAL activity; however, PAL activity in FD seedlings does not sustain the acidic environment and under proteolytic hydrolysis. Nonetheless, fresh red spring wheat seedlings demonstrated that PAL was possibly protected by the cell walls in an *in vitro* protein digestion. This work has provided the basis for future investigation of using red spring wheat seedlings as the dietary supplement for PKU and HPA patients.

VI.B. Future Studies

In this work, the endoproteolytic activity in fresh red spring wheat seedlings contributed the major loss of PAL activity. Although PAL activity in fresh seedlings is resistant to digestive proteolysis as compared with that of the yeast, the endoproteolytic attacks degrade PAL within the plant tissues. Therefore, one major obstacle to overcome in the future is to suppress the endoproteolytic activity in the seedlings. Endoproteases in the grains hydrolyze the storage materials during germination to provide the initial nutrients for seedlings growth (Dominguez and Cejudo, 1996). They are more abundant in the germinative phase and decreased during the developmental phase (Dominguez and Cejudo, 1996). From previous work, PAL activity was found to decrease after 10 days of post-germinative growth of red spring wheat seedlings (Goldson et al., 2007). However, PAL activity during the developmental phase of seedlings was not determined. Hence, it is important to examine the PAL activity during developmental phase of seedlings which may have lower endoproteolytic activity. On the other hand, isolation and investigation of the activity of each polypeptide of PAL in red spring wheat seedlings would better help in understanding the characteristic of each polypeptide and its stability under different conditions. Further, PAL can be induced by external stimulants like physical, biological, and chemical agents. Different stimulations should be tested to further enhance PAL activity of red spring wheat seedlings or other plant sources.

In order to fully understand the PAL activity of crude red spring wheat seedlings, radio-labelled Phe should be incorporated in future study. Phe labelled with isotopic hydrogen at C-3 has been used to study the mechanism of PAL activity (Rao et al., 1967). It is useful to monitor Phe during germination of seedlings such that the changes of PAL activity can be closely investigated during the growth. Besides, by using radio-labelled Phe, the conversion of Phe by PAL at the beginning of the digestion can be monitored precisely both *in vivo* and *in vitro*.

Although the *in vitro* conditions used in this study do not represent the real digestion of human gastrointestinal system, it provides the basis for future *in vivo* testing such as animal studies and clinical trials which require more time, money and personnel expertise. If PAL activity in fresh red spring wheat seedlings can be enhanced by external stimulations which further increase the conversion of Phe in an *in vitro* protein digestion, *in vivo* animal study using mutant PKU mouse model should be carried out to examine the efficacy of using wheat seedlings as the dietary supplements for patients. As discussed earlier, PAH gene in human is 96 % conserved with that of the rat (Güttler et al., 1984). The mutant mouse strains with HPA are widely used in finding different alternative therapies in treatments of PKU/HPA (Sarkissian et al., 1999; Ding et al., 2006). Fresh red spring wheat seedlings can be used as the supplementary source in the diet for the mutant mouse to evaluate the efficiency of wheat sprouts in lowering blood Phe levels.

In summary, the results of this thesis study provide the first foundation on which to base further investigation of the administration of PAL naturally encapsulated in red spring wheat seedlings as an alternative oral therapy for PKU and HPA patients. Hence, the success of this approach could be utilized to increase dietary protein tolerance and lift some of the burdens of the PKU diet.

VII. References

Acker JP, McGann LE (2002) Protective effect of intracellular ice during freezing? Cryobiology. **46:** 197-202

Alunni S, Cipiciani A, Fioroni G, Ottavi L (2003) Mechanisms of inhibition of phenylalanine ammonia-lyase by phenol inhibitors and phenol/glycine synergistic inhibitors. Archives of Biochemistry and Biophysics **412:** 170-175

Aoki K, Wada, Y (1988) Outcome of the patients detected by newborn screening in Japan. Acta Paediatrica Japonica **30:** 429-434

Astwood JD, Leach JN, Fuchs RL (1996) Stability of food allergens to digestion *in vitro*. Nature Biotechnology **14:** 1269-1273

Bellini E, Van Poucke M (1970) Distribution of phenylalanine ammonia-lyase in etiolated and far-red irradiated radish seedlings. Planta **93:** 60-70

Berne RM, Levy, MN (1996) Gastrointestinal secretions. *In* Principles of Physiology, Ed 2nd ed. Mosby-Year Book Inc., St. Louis, pp 459 - 480

Blankenship SM, Unrath CR (1988) Pal and ethylene content during maturation of red and golden delicious apples. Phytochemistry **27:** 1001-1003

Bolt R, Ruebner BH, Palmer PES, Watson DW (1983) The Digestive System, Ed 1st. John Wiley & Sons, New York

Bolwell GP, Bell JN, Cramer CL, Schuch W, Lamb CJ, Dixon RA (1985) L-phenylalanine ammonia-lyase from *Phaseolus-Vulgaris* - characterization and differential induction of multiple forms from elicitor-treated cell-suspension cultures. European Journal of Biochemistry **149:** 411-419

Bourget L, Chang TMS (1985) Phenylalanine ammonia-lyase immobilized in semipermeable microcapsules for enzyme replacement in phenylketonuria. Febs Letters **180:** 5-8

Bowersox J, Panel NCD (2001) National institutes of health consensus development conference statement: phenylketonuria: screening and management, October 16-18, 2000. Pediatrics **108:** 972-982

Brennan J (2006) Evaporation and dehydration. *In* Brennan J, ed, Food Processing Handbook. Wiley-vch, Weinheim, pp 85-124

Brennan J, Day BPF (2006) Packaging. *In* Brennan J, ed, Food Processing Handbook. Wiley-vch, Weinheim, pp 291-350

Caballero B, Wurtman, RJ (1988) Control of plasma phenylalanine levels. *In* Wurtman RJ, Ritter-Walker E, ed, Dietary Phenylalanine and Brain Function. Birkhäuser, Boston, pp 3-12

Calabrese JC, Jordan DB, Boodhoo A, Sariaslani S, Vannelli T (2004) Crystal structure of phenylalanine ammonia lyase: multiple helix dipoles implicated in catalysis. Biochemistry **43**: 11403-11416

Calzuola I, Marsili V, Gianfranceschi GL (2004) Synthesis of antioxidants in wheat sprouts. Journal of Agricultural and Food Chemistry **52**: 5201-5206

Camm E, Towers G (1973) Phenylalanine ammonia-lyase. Phytochemistry **12**: 961-973

Carpenter JF, Prestrelski SJ, Arakawa T (1993) Separation of freezing-induced and drying-induced denaturation of lyophilized proteins using stress-specific stabilization .1. enzyme-activity and calorimetric studies. Archives of Biochemistry and Biophysics **303**: 456-464

Carpita N (1988) The chemical structure of the cell walls of higher plants. *In* Kritchevsky D, Bonfield C, Anderson JW, ed, Dietary Fiber Chemistry Physiology, and Health Effects. Plenum Press, New York, pp 15-30

Centerwall WR, Centerwall, SA (1961) Phenylketonuria (folling's disease) the story of its discovery. Journal of the History of Medicine and Allied Sciences **XVI**: 292-296

Cleary M, Walter JH (2001) Assessment of adult phenylketonuria. Annals of Clinical Biochemistry **38**: 450-458

Cui Z, Xu SY, Sun DW (2003) Dehydration of garlic slices by combined microwave-vacuum and air drying. Drying Technology **21**: 1173-1184

Cui Z, Xu SY, Sun DW (2004) Effect of microwave-vacuum drying on the carotenoids retention of carrot slices and chlorophyll retention of chinese chive leaves. Drying Technology **22**: 563-575

Daines RJ, Minocha SC (1983) Regulation of phenylalanine ammonia-lyase in germinating lettuce seeds (*Lactuca-Sativa* L Cv grand rapids) - effects of abscisic-acid and water-stress. Zeitschrift Fur Pflanzenphysiologie **110**: 69-76

D'Cunha GB (2005) Enrichment of phenylalanine ammonia lyase activity of *Rhodotorula* yeast. Enzyme and Microbial Technology **36**: 498-502

Decareau RV (1985) Microwaves in the food processing industry, Ed 1st. Academic Press, Orlando

Ding Z, Georgiev P, Thony B (2006) Administration-route and gender-independent long-term therapeutic correction of phenylketonuria (PKU) in a mouse model by recombinant adeno-associated virus 8 pseudotyped vector-mediated gene transfer. Gene Therapy **13**: 587-593

Ding ZB, Harding CO, Thony B (2004) State-of-the-art 2003 on PKU gene therapy. Molecular Genetics and Metabolism **81**: 3-8

Dominguez F, Cejudo FJ (1996) Characterization of the endoproteases appearing during wheat grain development. Plant Physiology **112**: 1211-1217

Drapron R (1985) Modalities of enzyme activities in low moisture media. *In* Mathlouthi M, ed, Food Packaging and Preservation Theory and Practice, Ed 1st. Elsevier Applied Science Publishers, England, pp 181-198

Dreher M (1987) The concept of dietary fiber. *In* Handbook of Dietary Fiber An Applied Approach. Marcel Dekker, New York, pp 1-17

Eichner K (1985) The influence of water content and water activity on chemical changes in foods of low moisture content under packaging aspects. *In* Mathlouthi M, ed, Food Packaging and Preservation Theory and Practice. Elsevier Applied Science Publishers, England, pp 67-92

Ellis BE (1997) Metabolism of defence and communication. *In* Dennis DT, Turpin DH, Lefebvre DD, Layzell DB, ed, Plant Metabolism, Ed 2nd. Longman Ltd., England, pp 148-160

Farrel G, McMinn WAM, Magee TRA (2005) Microwave-vacuum drying kinetics of pharmaceutical powders. Drying Technology **23**: 2131-2146

Fellows P (2000) Food Processing Technology: Principles and Practice. Woodhead Pub., Cambridge, England

Flink JM (1975) Process Conditions for Improved Flavor Quality of Freeze-Dried Foods. Journal of Agricultural and Food Chemistry **23:** 1019-1026

Ford AW, Dawson PJ (1993) The effect of carbohydrate additives in the freeze-drying of alkaline-phosphatase. Journal of Pharmacy and Pharmacology **45:** 86-93

Frith GJT, Bruce DG, Dalling MJ (1975) Distribution of acid proteinase activity in wheat seedlings. Plant and Cell Physiology **16:** 1085-1091

Fritz RR, Hodgins DS, Abell CW (1976) Phenylalanine ammonia-lyase - induction and purification from yeast and clearance in mammals. Journal of Biological Chemistry **251:** 4646-4650

Fu TT, Abbott UR, Hatzos C (2002) Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid - a comparative study. Journal of Agricultural and Food Chemistry **50:** 7154-7160

Gamez A, Sarkissian CN, Wang L, Kim W, Straub M, Patch MG, Chen L, Striepeke S, Fitzpatrick P, Lemontt JF, O'Neill C, Scriver CR, Stevens RC (2005) Development of pegylated forms of recombinant *Rhodosporidium toruloides* phenylalanine ammonia-lyase for the treatment of classical phenylketonuria. Molecular Therapy **11:** 986-989

Gamez A, Wang L, Sarkissian CN, Wendt D, Fitzpatrick P, Lemontt JF, Scriver CR, Stevens RC (2007) Structure-based epitope and PEGylation sites mapping of phenylalanine ammonia-lyase for enzyme substitution treatment of phenylketonuria. Molecular Genetics and Metabolism **91:** 325-334

Garrett DA, Failla ML, Sarama RJ (2000) Estimation of carotenoid bioavailability from fresh stir-fried vegetables using an in vitro digestion/caco-2 cell culture model. Journal of Nutritional Biochemistry **11:** 574-580

Gilbert HJ, Jack GW (1981) The effect of proteinases on phenylalanine ammonia-lyase from the yeast *Rhodotorula-Glutinis*. Biochemical Journal **199:** 715-723

Goldson A, Lam M, Scaman CH, Clemens S, Kermode A (2007) Screening of phenylalanine ammonia lyase in plant tissues, and retention of activity during dehydration. Journal of the Science of Food and Agriculture

Gowri G, Paiva NL, Dixon RA (1991) Stress responses in alfalfa (*Medicago-Sativa* L) .12. sequence-analysis of phenylalanine ammonia-lyase (Pal) cDNA clones and appearance of Pal transcripts in elicitor-treated cell-cultures and developing plants. Plant Molecular Biology **17**: 415-429

Grandison A (2006) Postharvest handling and preparation of foods for processing. *In* Brennan J, ed, Food Processing Handbook, Ed 1st. Wiley-vch, Weinheim, pp 1-32

Guldberg P, Rey F, Zschocke J, Romano V, Francois B, Michiels L, Ullrich K, Hoffmann GF, Burgard P, Schmidt H, Meli C, Riva E, Dianzani I, Ponzone A, Rey J, Guttler F (1998) A European multicenter study of phenylalanine hydroxylase deficiency: Classification of 105 mutations and a general system for genotype-based prediction of metabolic phenotype. American Journal of Human Genetics **63**: 71-79

Güttler F, Lidsky, AS, Woo, SLC (1984) Molecular genetics of PKU: prenatal diagnosis and carrier detection by gene analysis. *In* Bickel H, Wachtel U, ed, Inherited Diseases of Amino Acid Metabolism. Georg Thieme Verlag, New York, pp 18-34

Habibi-Moini S, D'mello AP (2001) Evaluation of possible reasons for the low phenylalanine ammonia lyase activity in cellulose nitrate membrane microcapsules. International Journal of Pharmaceutics **215**: 185-196

Hanley WB (2004) Adult phenylketonuria. American Journal of Medicine **117**: 590-595

Harmuth-Hoene A, Bognar AE, Kornemann U, Diehl JF (1987) The influence of germination on the nutritional value of wheat, mung beans and chickpeas. Z Lebensm Unters Forsch **185**: 386-393

Hawker R (1981) Notebook of Medical Physiology: Gastrointestinal with aspects of total parenteral nutrition, Ed 1st. Churchill Livingstone, New York

Hellman K, Miller DS, Cammack KA (1983) The effect of freeze-drying on the quaternary structure of L-asparaginase from Erwinia-Carotovora. Biochimica Et Biophysica Acta **749**: 133-142

Hills BP, Remigereau B (1997) NMR studies of changes in subcellular water compartmentation in parenchyma apple tissue during drying and freezing. International Journal of Food Science and Technology **32**: 51-61

Hiratsuka S, Onodera H, Kawai Y, Kubo T, Itoh H, Wada R (2001) Enzyme activity changes during anthocyanin synthesis in 'Olympia' grape berries. Scientia Horticulturae **90**: 255-264

Hoskins J, Jack G, Wade HE, Peiris RJ, Wright EC, Starr DJ, Stern J (1980) Enzymatic control of phenylalanine intake in phenylketonuria. Lancet **1**: 392-394

Hoskins JA, Holliday SB, Greenway AM (1984) The metabolism of cinnamic acid by healthy and phenylketonuric adults - a kinetic-study. Biomedical Mass Spectrometry **11**: 296-300

Howles PA, Sewalt VJH, Paiva NL, Elkind Y, Bate NJ, Lamb C, Dixon RA (1996) Overexpression of L-phenylalanine ammonia-lyase in transgenic tobacco plants reveals control points for flux into phenylpropanoid biosynthesis. Plant Physiology **112**: 1617-1624

Inoue S, Matsunaga Y, Iwane H, Sotomura M, Nose T (1986) Entrapment of phenylalanine ammonia-lyase in silk fibroin for protection from proteolytic attack. Biochemical and Biophysical Research Communications **141**: 165-170

Ishikawa A, Kitamura Y, Ozeki Y, Watanabe M (2007) Different responses of shoot and root cultures of *Glehnia littoralis* to yeast extract. Journal of Natural Medicines **61**: 30-37

Jiang S, Nail SL (1998) Effect of process conditions on recovery of protein activity after freezing and freeze-drying. European Journal of Pharmaceutics and Biopharmaceutics **45**: 249-257

Jorrin J, Dixon RA (1990) Stress responses in alfalfa (*Medicago-Sativa* L) .2. purification, characterization, and induction of phenylalanine ammonia-lyase isoforms from eicitor-treated cell-suspension cultures. Plant Physiology **92**: 447-455

Kaensup W, Chutima S, Wongwises S (2002) Experimental study on drying of chilli in a combined microwave-vacuum-rotary drum dryer. Drying Technology **20**: 2067-2079

Kalkanoglu HS, Ahring KK, Sertkaya D, Moller LB, Romstad A, Mikkelsen I, Guldberg P, Lou HC, Guttler F (2005) Behavioural effects of phenylalanine-free amino acid tablet supplementation in intellectually disabled adults with untreated phenylketonuria. Acta Paediatrica **94**: 1218-1222

Karabulut I, Topcu A, Duran A, Turan S, Ozturk B (2007) Effect of hot air drying and sun drying on color values and beta-carotene content of apricot (*Prunus armenica* L.). Lwt-Food Science and Technology **40**: 753-758

Karathanos VT, Kanellopoulos NK, Belessiotis VG (1996) Development of porous structure during air drying of agricultural plant products. Journal of Food Engineering **29**: 167-183

Khan W, Prithiviraj B, Smith DL (2003) Chitosan and chitin oligomers increase phenylalanine ammonia-lyase and tyrosine ammonia-lyase activities in soybean leaves. Journal of Plant Physiology **160**: 859-863

Kim SH, Kronstad JW, Ellis BE (2001) Induction of phenylalanine ammonia-lyase activity by tryptophan in *Ustilago maydis*. Phytochemistry **58**: 849-857

Kim SW, Jung JS, Oh HJ, Kim J, Lee KS, Lee DH, Park C, Kimm K, Koo SK, Jung SC (2006) Structural and functional analyses of mutations of the human phenylalanine hydroxylase gene. Clinica Chimica Acta **365**: 279-287

Kim W, Erlandsen H, Surendran S, Stevens RC, Gamez A, Michols-Matalon K, Tyring SK, Matalon R (2004) Trends in enzyme therapy for phenylketonuria. Molecular Therapy **10**: 220-224

King VA, Zall RR, Ludington DC (1989) Controlled low-temperature vacuum dehydration - a new approach for low-temperature and low-pressure food drying. Journal of Food Science **54**: 1573-1593

Koukol J, Conn, EE (1961) Metabolism of aromatic compounds in higher plants. IV. Purification and properties of phenylalanine deaminase of *Horden vulgare*. Journal of Biological Chemistry **236**: 2692-2698

Krokida MK, Maroulis ZB, Saravacos GD (2001) The effect of the method of drying on the colour of dehydrated products. International Journal of Food Science and Technology **36**: 53-59

Laemmli UK (1970) Cleavage of structural proteins during assembly of head of bacteriophage-T4. Nature **227**: 680-685

Ledley FD, Woo SLC (1984) Cloning and expression of the human phenylalanine hydroxylase gene. *In* Bickel H, Wachtel U, ed, Inherited Diseases of Amino Acid Metabolism. Georg Thieme Verlag, New York, pp 37-50

Levee V, Seguin A (2001) Inducible expression of the heterologous PAL2 promoter from bean in white pine (*Pinus strobus*) transgenic cells. Tree Physiology **21**: 665-672

Levy H, Shih VE, Karolkew V, French WA, Carr JR, Cass V, Kennedy JL, Maccread RA (1971) Persistent mild hyperphenylalaninemia in untreated state - prospective study. New England Journal of Medicine **285**: 424-429

Levy HL (1999) Phenylketonuria: old disease, new approach to treatment. Proceedings of the National Academy of Sciences of the United States of America **96:** 1811-1813

Li H, Liao YC (2003) Isolation and characterization of two closely linked phenylalanine ammonia-lyase genes from wheat. Yi Chuan Xue Bao **30:** 907-912

Liang XW, Dron M, Cramer CL, Dixon RA, Lamb CJ (1989) Differential regulation of phenylalanine ammonia-lyase genes during plant development and by environmental cues. Journal of Biological Chemistry **264:** 14486-14492

Lin TM, Durance TD, Scaman CH (1998) Characterization of vacuum microwave, air and freeze dried carrot slices. Food Research International **31:** 111-117

Lintschinger J, Fuchs N, Moser H, Jager R, Hlebeina T, Markolin G, Gossler W (1997) Uptake of various trace elements during germination of wheat, buckwheat and quinoa. Plant Foods for Human Nutrition **50:** 223-237

Liu JZ, Jia XY, Zhang J, Xiang H, Hu W, Zhou Y (2002) Study on a novel strategy to treatment of phenylketonuria. Artificial Cells Blood Substitutes and Immobilization Biotechnology **30:** 243-257

Lo WMY, Li-Chan ECY (2005) Angiotensin I converting enzyme inhibitory peptides from in vitro pepsin-pancreatin digestion of soy protein. Journal of Agricultural and Food Chemistry **53:** 3369-3376

Loschke DC, Hadwiger LA, Schroder J, Hahlbrock K (1981) Effects of light and of *fusarium-solani* on synthesis and activity of phenylalanine ammonia-lyase in peas. Plant Physiology **68:** 680-685

MacDonald MJ, D'Cunha GB (2007) A modern view of phenylalanine ammonia lyase. Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire **85:** 273-282

Magee D, Dalley AF (1986) Digestion and the Structure and Function of the Gut, Ed. Karger, Omaha

Matalon R, Michals-Matalon K, Bhatia G, Burlina AB, Burlina AP, Braga C, Fiori L, Giovannini M, Grechanina E, Novikov P, Grady J, Tyring SK, Guttler E (2007) Double blind placebo control trial of large neutral amino acids in treatment of PKU: Effect on blood phenylalanine. Journal of Inherited Metabolic Disease **30:** 153-158

Matalon R, Michals-Matalon K, Bhatia G, Grechanina E, Novikov P, McDonald JD, Grady J, Tyring SK, Guttler F (2006) Large neutral amino acids in the treatment of phenylketonuria (PKU). Journal of Inherited Metabolic Disease **29**: 732-738

Mccallum JA, Walker JRL (1990) Phenolic biosynthesis during grain development in wheat - changes in phenylalanine ammonia-lyase activity and soluble phenolic content. Journal of Cereal Science **11**: 35-49

Mcgann LE, Farrant J (1976) Survival of tissue-culture cells frozen by a 2-step procedure to - 196 degrees C .2. warming rate and concentration of dimethyl-sulfoxide. Cryobiology **13**: 269-273

Mitchell J, Scriver, CR (2007) Phenylalanine hydroxylase deficiency. *In* GeneReivews, Vol 2000, Seattle

Moffitt MC, Louie GV, Bowman ME, Pence J, Noel JP, Moore BS (2007) Discovery of two cyanobacterial phenylalanine ammonia lyases: kinetic and structural characterization. Biochemistry **46**: 1004-1012

Nari J, Mouttet C, Pinna MH, Ricard J (1972) Some physico-chemical properties of L-phenylalanine ammonia-lyase of wheat seedlings. Febs Letters **23**: 220-224

Obel N, Porchia AC, Scheller HV (2002) Dynamic changes in cell wall polysaccharides during wheat seedling development. Phytochemistry **60**: 603-610

Oetjen G, Haseley P (2004) Freeze-drying, Ed 2nd. Wiley-vch, Lübeck

Olenichenko NA, Zagoskina NV (2005) Response of winter wheat to cold: production of phenolic compounds and L-phenylalanine ammonia lyase activity. Applied Biochemistry and Microbiology **41**: 600-603

Oomen AG, Rompelberg CJM, Bruil MA, Dobbe CJG, Pereboom DPKH, Sips AJAM (2003) Development of an in vitro digestion model for estimating the bioaccessibility of soil contaminants. Archives of Environmental Contamination and Toxicology **44**: 281-287

Orr JD, Edwards R, Dixon RA (1993) Stress responses in alfalfa (*Medicago-Sativa* L) .14. changes in the levels of phenylpropanoid pathway intermediates in relation to regulation of L-phenylalanine ammonia-lyase in elicitor-treated cell-suspension cultures. Plant Physiology **101**: 847-856

Owusuansah YJ (1991) Advances in microwave drying of foods and food ingredients. Canadian Institute of Food Science and Technology Journal-Journal De L Institut Canadien De Science Et Technologie Alimentaires **24:** 102-107

Ozalp I, Coskun T, Ceyhan M, Tokol S, Oran O, Erdem G, Tekinalp G, Durmus Z, Tarikahya Y (1986) Incidence of phenylketonuria and hyperphenylalaninemia in a sample of the turkish newborn population. Journal of Inherited Metabolic Disease **9:** 237-239

Paine F, Paine HY (1992a) Dried and moisture sensitive foods. *In* A Handbook of Food Packaging, Ed 2nd. Blackie Academic & Professional, Glasgow, pp 296-314

Paine F, Paine HY (1992b) Frozen foods. *In* A Handbook of Food Packaging, Ed 2nd. Blackie Academic & Professional, Glasgow, pp 248-264

Paine F, Paine HY (1992c) Introduction to packaging. *In* A Handbook of Food Packaging, Ed 2nd. Blackie Academic & Professional, Glasgow, pp 1-32

Pardo J, Niranjan K (2006) Freezing. *In* Brennan J, ed, Food Processing Handbook. Wiley-vch, Weinheim, pp 125-145

Pedersen H, Horvath C, Ambrus CM (1978) Preparation of immobilized L-phenylalanine ammonia-lyase in tubular form for depletion of L-phenylalanine. Research Communications in Chemical Pathology and Pharmacology **20:** 559-569

Qi WW, Sariaslani FS, Tang XS (2001) Methods for the production of tyrosine, cinnamic acid and para-hydroxycinnamic acid. *In* World Patent, Vol 02/090523

Rao PVS, Moore K, Towers GHN (1967) Degradation of aromatic amino acids by fungi .2. purification and properties of phenylalanine ammonia-lyase from *Ustilago hordei.* Canadian Journal of Biochemistry **45:** 1863-1872

Rees DG, Jones DH (1996) Stability of L-phenylalanine ammonia-lyase in aqueous solution and as the solid state in air and organic solvents. Enzyme and Microbial Technology **19:** 282-288

Rejasse B, Lamare S, Legoy MD, Besson T (2004) Stability improvement of immobilized Candida antarctica lipase B in an organic medium under microwave radiation. Organic and Biomolecular Chemistry **2:** 1086-1089

Ritter H, Schulz GE (2004) Structural basis for the entrance into the phenylpropanoid metabolism catalyzed by phenylalanine ammonia-lyase. Plant Cell **16**: 3426-3436

Rockland L, Beuchat LR (1986) Mechanics and influence of water binding on water activity. *In* Water Activity: Theory and Applications to Food, New York and Basel, pp 1 - 26

Sapers GM, Matulaitis RM, Beck JA (1987) Detection of phenylalanine ammonia-lyase in the skin of blueberry and cranberry fruits. Journal of Food Science **52**: 155-158

Sarkissian CN, Shao Z, Blain F, Peevers R, Su H, Fuller MA, Scriver CR (1997) Effect of oral phenylalanine ammonia lyase (PAL) on plasma phenylalanine levels in a genetic mouse orthologue of PKU. American Journal of Human Genetics **61**: A35-A35

Sarkissian CN, Shao ZQ, Blain F, Peevers R, Su HS, Heft R, Chang TMS, Scriver CR (1999) A different approach to treatment of phenylketonuria: Phenylalanine degradation with recombinant phenylalanine ammonia lyase. Proceedings of the National Academy of Sciences of the United States of America **96**: 2339-2344

Scaman C, Durance TD (2005) Combined Microwave Vacuum Drying. *In* Sun DW, ed, Emerging Technologies in Food Processing. Academic Press, Elsevier, London

Schwede TF, Retey J, Schulz GE (1999) Crystal structure of histidine ammonia-lyase revealing a novel polypeptide modification as the catalytic electrophile. Biochemistry **38**: 5355-5361

Scriver C, Kaufman S, Woo SLC (1989) The Metabolic Basis of Inherited Disease. McGraw-Hill, New York

Scriver CR, Kaufman S (2001) The hyperphenylalaninemias. *In* Scriver CR, Beaudet AL, Sly SW, Valle D, Childs B, Kinzler KW, Vogelstein B, ed, The Metabolic and Molecular Bases of Inherited Disease, Ed 8. McGraw-Hill, New York, p Ch. 77

Selvendran R, Verne AVFV (1988) The chemistry and properties of plant cell walls and dietary fiber. *In* Kritchevsky D, Bonfield C, Anderson JW, ed, Dietary Fiber Chemistry, Physiology, and Health Effects. Plenum Press, New York, pp 1-15

Shadle GL, Wesley SV, Korth KL, Chen F, Lamb C, Dixon RA (2003) Phenylpropanoid compounds and disease resistance in transgenic tobacco with altered expression of L-phenylalanine ammonia-lyase. Phytochemistry **64**: 153-161

Shields SE, Wingate VP, Lamb CJ (1982) Dual control of phenylalanine ammonia-lyase production and removal by its product cinnamic acid. European Journal of Biochemistry **123:** 389-395

Shishehgarha F, Makhlouf J, Ratti C (2002) Freeze-drying characteristics of strawberries. Drying Technology **20:** 131-145

Singh A, Selvi MT, Sharma R (1999) Sunlight-induced anthocyanin pigmentation in maize vegetative tissues. Journal of Experimental Botany **50:** 1619-1625

Solecka D, Kacperska A (1995) Phenylalanine ammonia-lyase activity in leaves of winter oilseed rape plants as affected by acclimation of plants to low-temperature. Plant Physiology and Biochemistry **33:** 585-591

Street HE, Cockburn W (1972) Secondary plant products. *In* Plant Metabolism, Ed 2nd. Pergamon Press Ltd., New York, pp 186-211

Sundari SK, Adholeya A (1999) Freeze-drying vegetative mycelium of *Laccaria fraterna* and its subsequent regeneration. Biotechnology Techniques **13:** 491-495

Trefz F, Schmidt H, Bartholomé K, Mahle M, Matthis P, Pecht G (1984) Differential diagnosis and significance of various hyperphenylalaninemia. *In* Bickel H, Wachtel U, ed, Inherited Diseases of Amino Acid Metabolism. Georg Thieme Verlag, New York, pp 86-100

Versantvoort C, van de Kamp E, Rompelberg CJM (2004) Development and applicability of an in vitro digestion model in assessing the bioaccessibility of contaminants from food, Vol 320102002. RIVM Report, Bilthoven

Versantvoort CHM, Oomen AG, Van de Kamp E, Rompelberg CJM, Sips AJAM (2005) Applicability of an *in vitro* digestion model in assessing the bioaccessibility of mycotoxins from food. Food and Chemical Toxicology **43:** 31-40

Walter JH, White FJ, Hall SK, MacDonald A, Rylance G, Boneh A, Francis DE, Shortland GJ, Schmidt M, Vail A (2002) How practical are recommendations for dietary control in phenylketonuria? Lancet **360:** 55-57

Wieder KJ, Palczuk NC, Vanes T, Davis FF (1979) Some properties of polyethylene glycol-phenylalanine ammonia-lyase adducts. Journal of Biological Chemistry **254:** 2579-2587

Williamson M, Koch R, Friedman EG (1984) Diet termination: matched pair control between PKU children and their non-PKU siblings. *In* Bickel H, Wachtel U, ed, Inherited Diseases of Amino Acid Metabolism. Georg Thieme Verlag, New York, pp 151-162

Woolf LI, Griffiths R, Moncrieff A (1955) Treatment of phenylketonuria with a diet low in phenylalanine. British Medical Journal **1:** 57-64

Xiang LK, Moore BS (2005) Biochemical characterization of a prokaryotic phenylalanine ammonia lyase. Journal of Bacteriology **187:** 4286-4289

Yang F, Basu TK, Ooraikul B (2001) Studies on germination conditions and antioxidant contents of wheat grain. International Journal of Food Sciences and Nutrition **52:** 319-330

Yousif AN, Durance TD, Scaman CH, Girard B (2000) Headspace volatiles and physical characteristics of vacuum-microwave, air, and freeze-dried oregano (*Lippia berlandieri Schauer*). Journal of Food Science **65:** 926-930

Yousif AN, Scaman CH, Durance TD, Girard B (1999) Flavor volatiles and physical properties of vacuum-microwave- and air-dried sweet basil (*Ocimum basilicum* L.). Journal of Agricultural and Food Chemistry **47:** 4777-4781

Zabala G, Zou JJ, Tuteja J, Gonzalez DO, Clough SJ, Vodkin LO (2006) Transcriptome changes in the phenylpropanoid pathway of glycine max in response to *Pseudomonas syringae* infection. Bmc Plant Biology **6:** -

VIII. Appendices

VIII.A. Sample Calculations

VIII.A.1. PAL Activity in Dry Weight Basis (µmol/hr.g dry wt)

Absorbance reading @290nm of 15mins incubation:
Absorbance @290nm of sample 1 = 0.969
Absorbance of control = 0.357
Corrected absorbance = 0.969 – 0.357 = 0.612

Dry weight of sample 1:
Fresh weight of sample 1 = 4.00g
Moisture content of sample 1 = 80.394% (wb)
Dry weight of sample 1 = 4.00g * (1-0.80394) = 0.784g

Dry weight of sample 1 added
= 0.784g/extraction buffer added * vol. subject to PD-10column / vol. collected from the PD-10 column * vol. added to PAL assay
= 0.784g/8000µL*2500µL/3500µL*200µL
= 0.014g

From *trans*-Cinnamic Acid Standard Curve (Figure 1):
Absorbance @290nm = 0.0159(µM of *t*-CA produced) + 0.2476

Therefore in sample 1,
Corrected abs = 0.612 = 0.0159 (µM of *t*-CA produced) + 0.2476
µM of *t*-CA produced = 22.918 µM

PAL Activity in Dry weight basis (µmol/hr.g dry wt):
= 22.918 µM/1000/0.25hr (15mins incubation time)/0.014g (dry wt loaded)
= 6.548 µmol/hr.g dry wt

VIII.A.2. PAL Activity in Protein Basis (μmol/hr.mg protein)

Protein Concentration in Sample 1:
Absorbance @595nm   = 0.380

From the BSA Assay Standard Curve,
Absorbance @595nm   = 0.5971 (protein conc. mg/mL) + 0.3061
Therefore,   0.380   = 0.5971 (protein conc.) + 0.3061
Protein conc. of 20x diluted sample 1 = 0.124 mg/mL
Original protein conc. of sample 1   = 2.475 mg/mL of filtrate

Total proteins extracted,
= 2.475mg/mL*8.0mL (extraction buffer added)*(3.5mL/2.5mL)
= 27.72mg protein

Amount of protein with respect to dry weight of sample 1,
= 0.784g (dry wt) / 27.72mg protein
= 0.0283g (dry wt)/mg protein

PAL Activity in protein basis (μmol/hr.mg protein):
= PAL activity in dry wt basis * 0.0283mg protein
= 6.548 μmol/hr.g dry wt * 0.0283g dry wt/mg protein
= 0.185 μmol/hr.mg protein

## VIII.B. Amino Acid Composition of 7-day old Red Spring Wheat Seedlings

Amino Acid(s)	Concentration of amino acid (μmol/g dry wt)		
	Intract Sprout	Leaf	Root
Phenylalanine	**50.97 ± 4.60**	**69.03 ± 3.30**	**41.35 ± 1.74**
Aspartiac acid	235.78 ± 36.37	425.49 ± 61.11	308.66 ± 18.66
Glutamic acid	238.07 ± 60.23	259.99 ± 33.56	70.50 ± 8.50
Serine	77.94 ± 3.33	98.12 ± 12.04	107.63 ± 14.83
Glycine	121.30 ± 2.21	180.57 ± 38.36	30.24 ± 2.40
Histidine	33.39 ± 1.56	45.16 ± 8.83	57.81 ± 5.80
Arginine	67.32 ± 1.92	100.22 ± 21.45	89.58 ± 11.40
Proline	80.26 ± 4.86	120.84 ± 23.42	147.15 ± 14.72
Tyrosine	133.11 ± 7.09	211.54 ± 35.16	31.12 ± 3.98
Valine	97.11 ± 4.48	143.50 ± 22.70	95.07 ± 10.01
Methionine	21.64 ± 0.45	31.27 ± 3.85	21.66 ± 2.26
Cysteine	3.15 ± 0.57	2.35 ± 0.43	2.35 ± 0.27
Isoleucine	63.30 ± 3.42	89.11 ± 9.49	62.35 ± 5.14
Leucine	106.77 ± 5.85	150.47 ± 15.46	97.51 ± 9.00
Lysine	62.71 ± 18.26	94.91 ± 14.65	58.37 ± 5.29

VIII.C. Standard Curves Figures

VIII.C.1. *Trans*-Cinnamic Acid Standard Curve for Chemical Analysis of PAL activity

$y = 0.0159x + 0.2476$

$R^2 = 0.998$
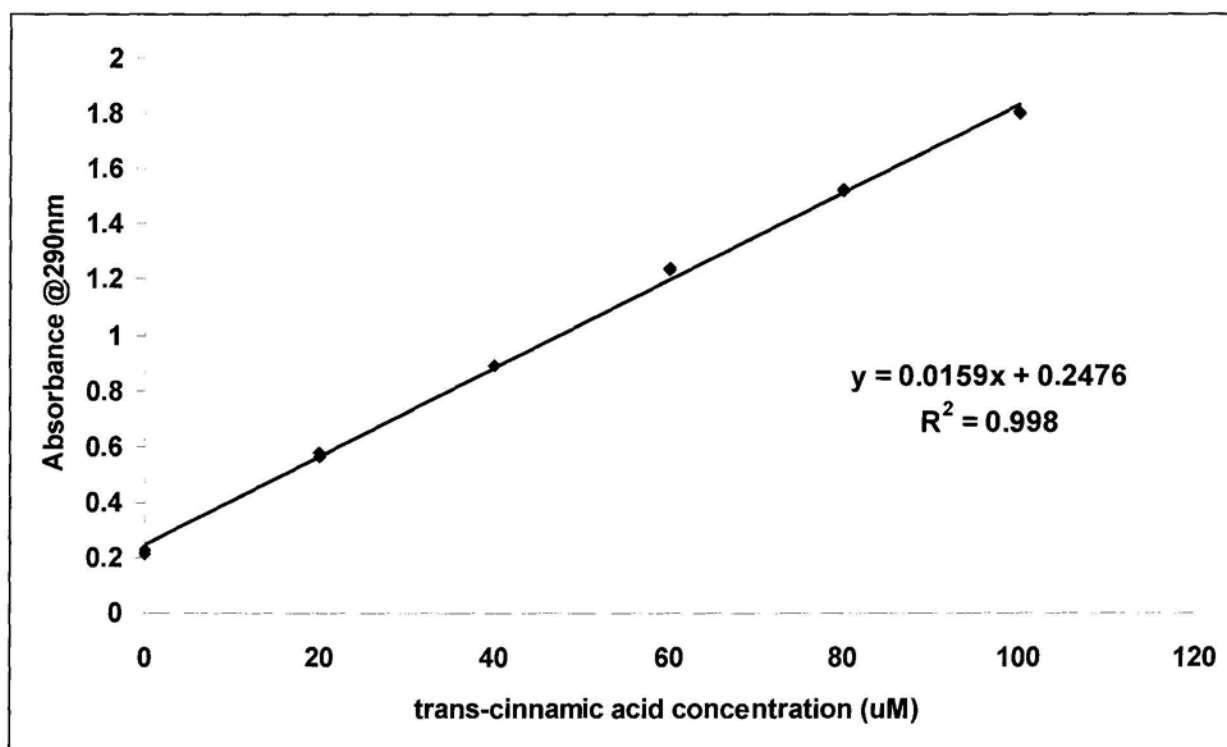
VIII.C.2. BSA Assay Standard Curve for Protein Concentration Determination

$$y = 0.5971x + 0.3061$$
$$R^2 = 0.9874$$
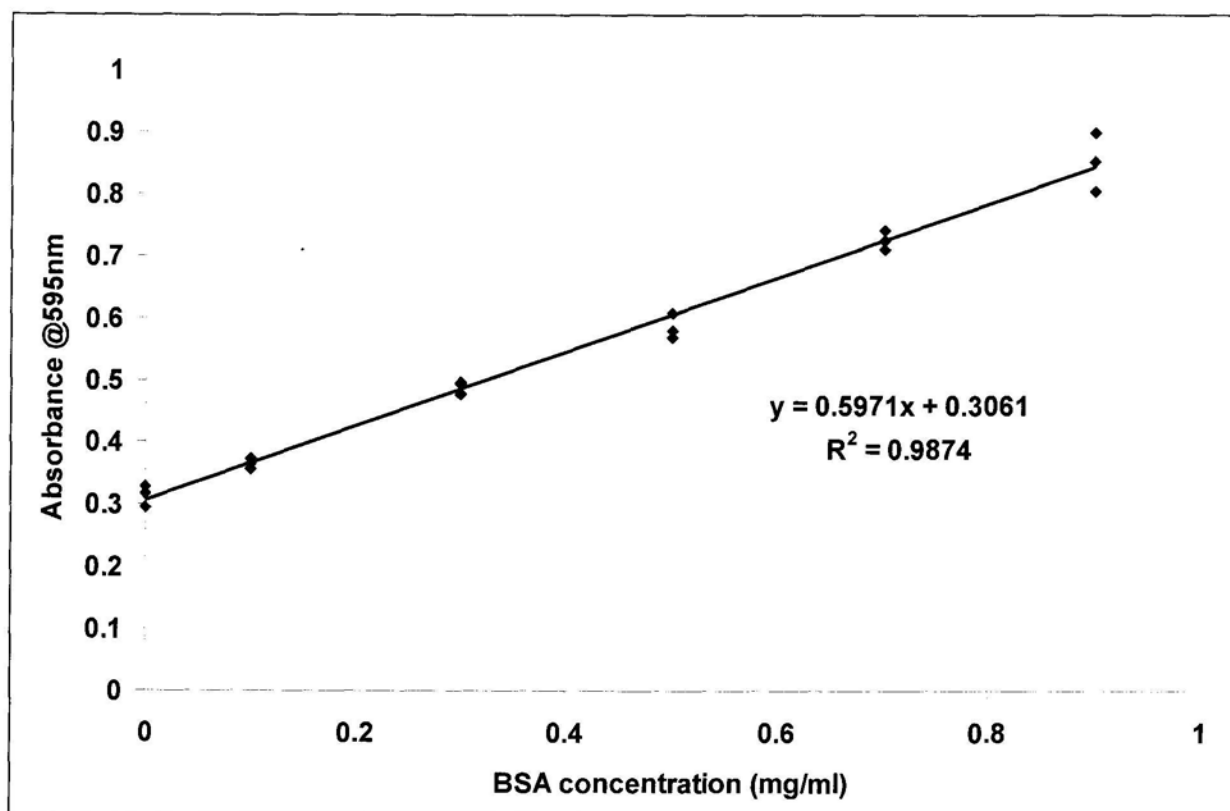
VIII.D. VAK 3.0R Pouch Specification

Intended for use up to 150°F (65°C)

Composition: 0.80mil Nylon/2.2 mil EVA copolymer

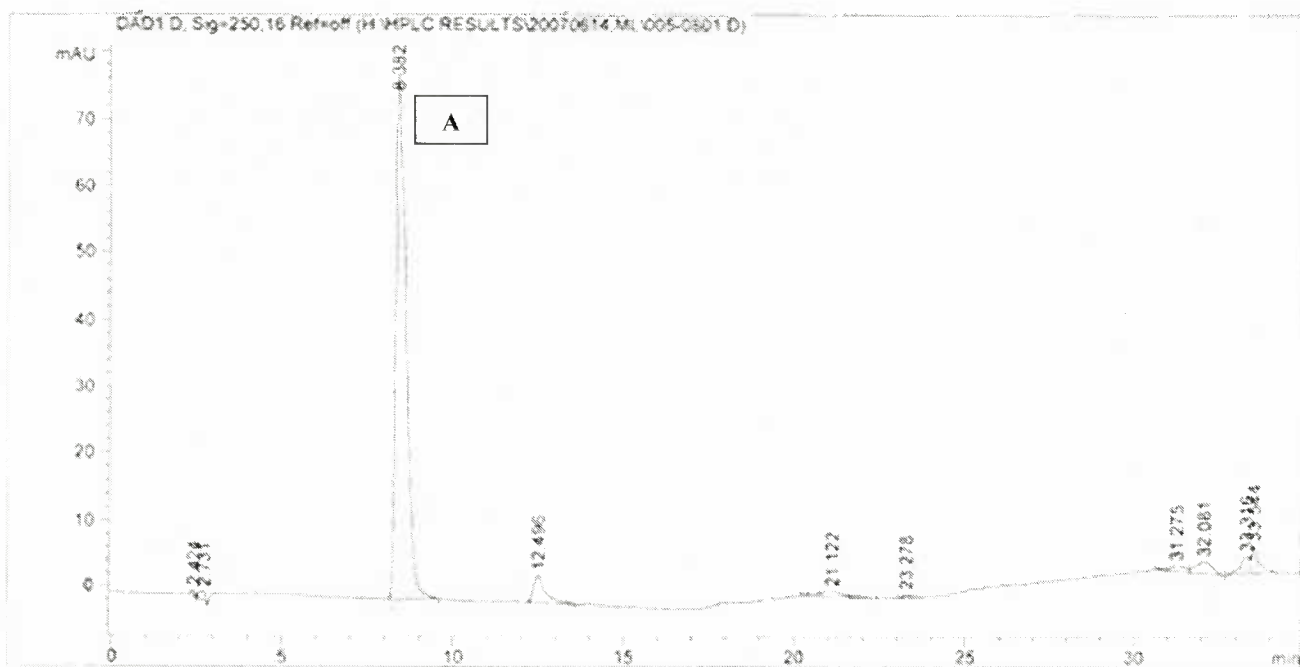| FILM PROPERTIES	UNITS	TYPICAL VALUES
Thickness	Microns	75
	Mils	3
Tensile Strength		
MD	lbs/sq. in	6100
	N/sq. cm	4200
TD	lbs/sq. in	4200
	N/sq. cm	2900
Elongation		
MD	%	410
TD	%	410
Puncture,	lbs.	2.4
Pointed Probe	N	11
Flexcrack @ 900 cycles	# of pinholes	4
Oxygen Transmission		
24 hours	cc/sq. m	5.2
23°C dry	cc/100 sq. in.	3.3
Moisture Vapor Transmission		
24 hours	g/sq. m	8.0
37.8°C @ 90% Rel. Humidity	g/100 sq. in.	0.50

(Provided by West Coast FoodPak System Ltd.)

VIII.E. Standard Curve of Phenylalanine for HPLC Analysis

$y = 39886.782 \, x - 2.999$

$R^2 = 1.000$
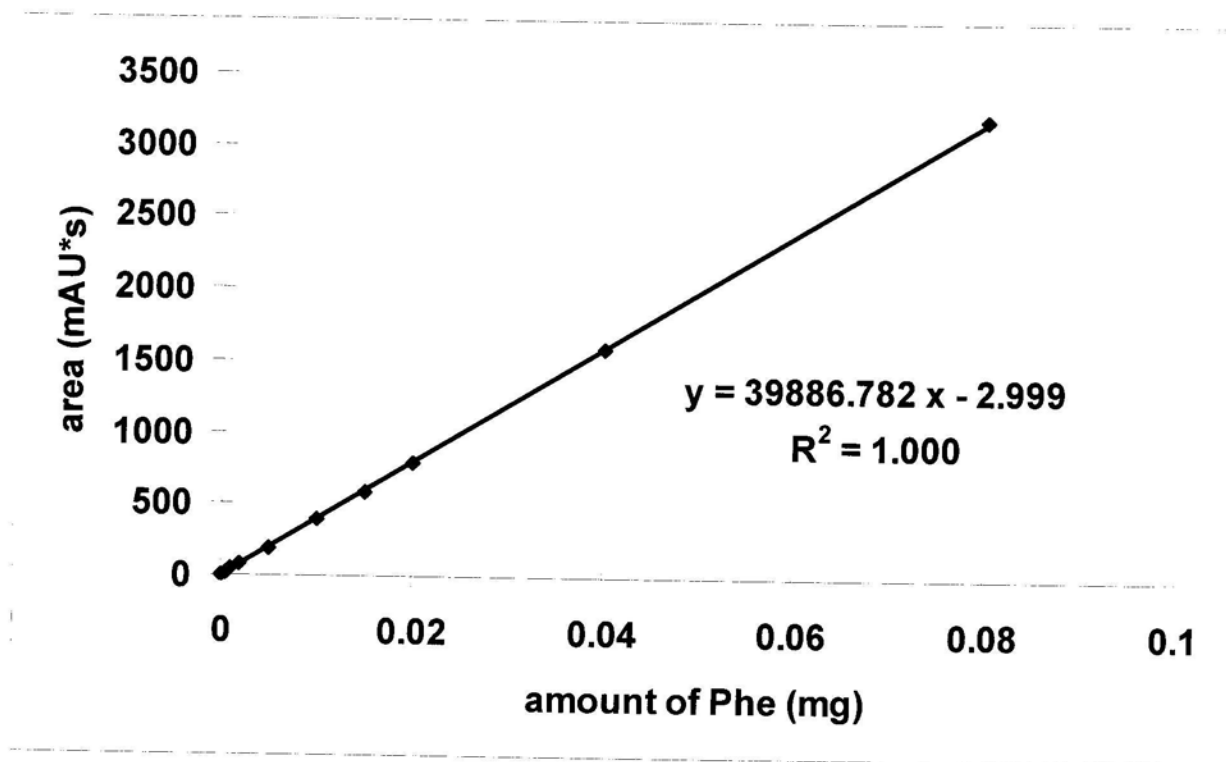
VIII.F. Reverse Phase HPLC Chromatogram of Phenyalanine Standard

Reverse phase HPLC profile of phenylalanine standard. Phenylalanine peak (A) was detected at 250nm at ~8.4 minutes.