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Retention of Phenylalanine Ammonia-lyase Activity in Wheat Seedlings during Storage and in Vitro Digestion

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The retention of phenylalanine ammonia-lyase (PAL) activity in Red Spring wheat seedlings during storage and in vitro protein digestion was evaluated toward assessing the efficacy of plant PAL as a dietary supplement for patients suffering from the metabolic disease, phenylketonuria. Retention of PAL activity in freeze-dried wheat seedling tissues following three months of storage at −20 °C ranged from 62% in the leaf to 89% in root/residual seed tissues. After a 3-h two-stage ("gastric-intestinal") in vitro digestion, 36% and 42% recovery of PAL activity was associated with chopped fresh leaf and root/residual seed tissues respectively; however, no activity was recovered from freeze-dried tissues. High performance liquid chromatographic analysis of the residual phenylalanine (Phe) after in vitro digestion confirmed that the fresh tissues effected a significantly higher conversion of exogenous Phe than freeze-dried tissues. These results demonstrate that the plant cell walls provide protection of PAL during in vitro digestion. In cases where exogenous Phe (100 mg; 24 mM) was supplied to the tissues, the product of the reaction, *trans*-cinnamic acid, may have exerted a significant inhibitory effect on PAL activity.

KEYWORDS: Phenylalanine ammonia-lyase activity; Red Spring wheat seedlings; freeze-drying; in vitro digestion; protein stability; natural encapsulation; phenylketonuria

INTRODUCTION

Phenylketonuria (PKU) and non-PKU-hyperphenylalaninemia (HPA) are autosomal recessive inborn errors of phenylalanine (Phe) metabolism that are caused by mutations in the gene encoding phenylalanine hydroxylase (PAH) (EC 1.14.16.1). The complete or near-complete deficiency of the PAH enzyme results in elevation of blood levels of phenylalanine (Phe) and urinary excretion of phenylketones. If left untreated from birth, patients develop profound irreversible mental retardation which is presumed to be a consequence of the toxic effects of the amino acid on brain development (*1*). The Guthrie test for newborn screening of PKU and related diseases and the implementation of dietary treatment were first introduced in 1953. Dietary treatment 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 concentrations above the recommended level is less than 30% for those younger than age 10, but is almost 80% for patients aged 15 years or older (*2*). The consequences of adults straying from their diet are significant and can include reduced cognitive abilities, neuropsychological dysfunction and emotional disturbances. There are further complications for pregnant mothers, due to the teratogenic effects of maternal PKU (*3*). Because of the problems with dietary noncompliance, alternative therapeutic methods for treating the condition are being investigated, including those that have targeted the cofactor of PAH, tetrahydrobiopterin (*4, 5*). Oral enzyme therapy that uses recombinant phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) in combination with a controlled (i.e., low protein) diet has been suggested as a plausible alternative to the current therapy for PKU (*3*). PAL catalyzes the nonoxidative deamination of L-Phe to yield *trans*-cinnamic acid and ammonia. In the body, *trans*-cinnamic acid is further converted to benzoic acid and rapidly excreted in urine as hippurate (*6*). PAL requires no cofactors and is stable over a wide temperature range (*7*), making it especially suitable as the basis of an oral therapeutic.

Proof of concept of the efficacy of oral PAL therapy has been established using a PKU mutant mouse model (*8*). However, one significant challenge has been the development of efficient methods for protecting the recombinant PAL enzyme from proteolysis and/or denaturation (*9*). Different methods to address this problem have been investigated such as PAL immobilization in semipermeable microcapsules, the entrapment of PAL in silk fibroin, and the immobilization of recombinant PAL in expres-

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sion cells such as *Escherichia coli* or *Lactobacillus lactis* (*8, 9, 13*). An injectable form of PAL is being explored (*12*); however, an immune response occurs after repeated subcutaneous injection of PAL. The efficacy of oral PAL is limited by its susceptibility to intestinal proteases (*8, 14*). Hence, a major challenge of using PAL as a therapeutic is to develop methods that either avoid the generation of immune responses to the protein (injectable PAL), or protect it from enzymatic proteolysis or pH-mediated denaturation, while still allowing it access to enteric Phe (oral PAL).

We hypothesized that PAL, naturally encapsulated within the plant cell wall, would meet the requirements of an oral therapeutic. From previous work, Red Spring wheat (*Triticum aestivum* L.) seedlings were found to be an excellent source of PAL (*15*). The use of seedlings provides a source of PAL that is renewable, readily available, and can be easily managed. In this study, the potential of 7-day old Red Spring wheat seedlings with high PAL activity as a dietary supplement to metabolize Phe under in vitro digestion conditions was evaluated. The retention of PAL activity in freeze-dried tissues was determined during a 3-month storage period. The effects of environmental pH and proteases on PAL stability in wheat seedling tissues, and on the ability of PAL to metabolize Phe were also evaluated. The results from this work provide insights for future development of a plant preparation that can be used as an oral supplement for PKU/HPA patients to improve their diet, allowing them to partake of some Phe-containing foods without negative consequences.

MATERIALS AND METHODS

Plant Materials and Germination/Growth Conditions. Red Spring wheat seeds were purchased from Quality Seeds West (Vancouver, BC, Canada). Seeds were soaked in 2.625% sodium hypochlorite (50% (v/v) chlorine bleach) solution for 15 min, then rinsed with sterile deionized water. Germination of seeds and subsequent growth of seedlings was conducted in Freshlife Automatic Sprouters (Tribest Corp., model 2000) maintained at ~25 °C, which provided intermittent hydration with distilled water every 15 min. After 7 days of germination/growth, seedling leaf tissues were separated from residual seed/root tissues for further analyses. Residual seed is defined as the starchy endosperm, aleurone, pericarp, and seed coat.

PAL Enzyme Activity Assays. PAL specific activity was measured as described in Goldson et al. (*15*). Briefly, plant samples (fresh and freeze-dried) were homogenized with a mortar and pestle in extraction buffer, 50 mM Tris-HCl, pH 8.8, 1 mM EDTA, 10 mM 2-mercaptoethanol, containing 2.5% (w/v) polyvinylpolypyrrolidone (PVPP) in a 1:2 ratio (sample fresh weight:buffer). After homogenization, samples were kept at 4 °C for 1 h, and then centrifuged at 23,500*g* for 15 min at 4 °C. Aliquots of supernatant were desalted using a PD-10 column (GE Healthcare, Montreal, QB, Canada). A 200-μL aliquot of the PD-10 filtrate (or 200 μL deionized water blank) was added to 400 μL of reaction buffer (100 mM Tris-HCl, pH 8.8) and 200 μL of substrate (40 mM L-Phe, 100 mM Tris-HCl, pH 8.8) and incubated at 37 °C for 15 min. Trichloroacetic acid (200 μL of 25% w/v) was added to terminate the reaction, and samples were centrifuged at 13000*g* for 15 min. Absorbance at 290 nm was used to determine the amount of *trans*-cinnamic acid produced, calculated using a standard curve (r^2 = 0.998).

PAL Activity after Freeze-Drying and the Stability of PAL Activity in Freeze-Dried Stored Samples. Freeze-dried (FD) leaf and root/residual seed tissues were prefrozen at −18 °C overnight and freeze-dried for 24 h under reduced pressure (0.027 kPa). After drying, samples were stored in desiccators and assayed for PAL activity within 3 h (t = 0) 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.; Vancouver, BC, Canada). Packed samples were stored at either 4 °C or −20 °C and assayed for PAL activity after 2, 4, 8, and 12 weeks of storage. FD samples assayed within 3 h after hydration were used as the control to calculate the percent recovery of PAL activity in stored samples. The recovery of PAL as a result of FD is greater than 90% (i.e., in comparison to fresh, immediately assayed tissues) (*15*). Experiments were carried out in duplicate in which each replicate represents two to six determinations of PAL activity for each treatment.

Stability of PAL Activity under in Vitro Digestion Conditions. In vitro conditions to mimic gastric and intestinal digestion were generated. Four grams of fresh leaf/root tissue or the equivalent dry mass of FD leaf/root samples (compensated for moisture) was cut into ~1−2 cm lengths and incubated with 25 mL of 4% pepsin (weight enzyme/fresh weight basis) (Sigma, Mississauga, ON, Canada; 800−2500 units/mg protein) adjusted to pH 2.5 with 1 N HCl for 1 h 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) was added and the incubation continued for 2 h. At 30 min intervals, aliquots were removed and the reaction terminated by raising the pH to 7.5 as described above after the gastric phase (first hour) or by the addition of 4-(2-aminoethyl)benzenesulfonyl fluoride (Sigma) and EDTA to achieve final concentrations of 0.1 mM and 1 mM, respectively. Wheat seedling tissues were removed from the reaction solution and ground in extraction buffer, and the supernatants were assayed for PAL activity spectrophotometrically. PAL activity after the in vitro digestion was calculated as a percentage of the PAL activity associated with tissues that had not been subjected to digestion from the same batch.

Phe Conversion During in Vitro Digestion. Four grams of fresh or FD leaf and root/residual seed samples, ground with a mortar and pestle or intact, was subjected to in vitro digestion as described above with the addition of 100 mg of L-Phe (equivalent to 24.2 mM). At 30 min intervals, samples were removed, boiled for 10 min, and centrifuged at 13000*g* for 10 min. Supernatants of samples were diluted 40 times and passed through 0.22 μm filter units. A blank control was performed without the addition of wheat tissue samples, and a sample control was performed without incubation. The residual Phe after the in vitro digestion was measured by HPLC analysis. The amount of Phe converted by the PAL associated with the wheat seedling tissues was calculated after subtracting the sample control.

Stability of PAL Activity at Extreme pH (pH 2.5 and pH 9.5). Ground samples or intact samples cut into ~1−2 cm lengths were incubated with extraction buffer/PVPP in a 1:2 ratio at pH 2.5 or 9.5 adjusted with 1 N HCl or 1 N NaOH, respectively. After 1 h incubation at 4 °C, samples were adjusted to pH 8.8, intact samples were ground, and all extracts were assayed using the spectrophotometric assay. Percent recovery of PAL activity was calculated with respect to samples from the same batch that had not been subjected to the incubation.

Effect of Protease Inhibitors on the Stability of PAL Activity at pH 8.8. Four grams of fresh leaf and root/residual seed samples was separated, cut into ~1−2 cm lengths, and incubated with 25 mL of water adjusted to pH 8.8 using 1 N NaOH for 3 h at 37 °C with shaking at 100 rpm. 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 samples from the same batch that had not been subjected to the incubation.

Phe Conversion at pH 8.8. Four grams of fresh leaf and root samples were cut into ~1−2 cm lengths or ground with a mortar and pestle, and added to 25 mL of water adjusted to pH 8.8 with 1 N NaOH, containing 100 mg of Phe (24.2 mM final concentration) for 2 h at 37 °C with shaking (100 rpm). At 30 min intervals, samples were removed and the reaction was terminated by boiling for 10 min. Ground tissues were then centrifuged at 13000*g* for 10 min. Supernatants were diluted 40 times and passed through 0.22 μm filter units. A blank control was performed without the addition of sample, and a sample control was performed without incubation. The amount of Phe remaining was measured by HPLC analysis, as described below, and used to calculate the amount of Phe digested after subtracting the sample control that had not been subjected to incubation.

Analyses of Water Activity and Moisture Content. The water activity of all fresh and FD samples was determined using the Aqualab

Table 1. Solvents and Gradients of HPLC Analysis

	solvent gradient (%)	
time (min)	solvent A: 100% 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

(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. Measurements were done in duplicate for each sample.

High Performance Liquid Chromatography Analysis of Phe. 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. (*16*). A Hewlett-Packard HPLC system with an autosampler and a diode array UV/vis detector was used with a reverse-phase Zorbax SR-C18 column (4.6 mm × 150 mm; 5 μm particle size). Flow rate was 1.0 mL/min with a column temperature of 40 °C. The UV detector was set to monitor the eluant at a wavelength of 250 nm. An aliquot of 20 μL of the diluted samples was injected. **Table 1** shows the elution method. A standard curve was generated, and the amount of Phe in the samples was calculated accordingly.

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

RESULTS AND DISCUSSION

PAL Activity of Freeze-Dried Tissues under Various Storage Conditions. The use of wheat seedlings as the basis of a dietary supplement/therapeutic requires an evaluation of the shelf stability of the plant preparation. A previous study (*15*) showed that freeze-drying (FD) is the best method to retain PAL activity in both leaf and root/residual seed tissues of wheat seedlings. In this work, FD samples were further analyzed to determine the "shelf stability" of PAL activity by storing the samples under various conditions.

PAL activity of stored FD leaf and root/residual seed samples followed different trends. PAL activity in FD leaf tissues was retained at higher levels at −20 °C than at 4 °C for most samples except week 2 and week 4, vacuum packed (**Table 2**); presumably the lower temperature reduced chemical and enzymatic changes over time. There was a tendency for samples to retain higher activity under non-vacuum packaging, although this was not consistent. Hence, the stability of PAL activity in leaf tissues was more sensitive to storage temperature than to the package conditions.

PAL activity of FD root/residual seed samples decreased during storage at the higher storage temperature (4 °C) for all samples except those from week 4, non-vacuum packaged, and the root/residual seed samples exhibited a tendency for higher PAL activity under the vacuum packaging condition (**Table 2**). Similar to leaf samples, PAL activity in root/residual seed samples was more sensitive to storage temperature than to the packaging conditions. In general, PAL activity was best retained when FD samples were stored at −20 °C and vacuum-packed; this was followed by −20 °C storage with non-vacuum packaging.

In summary, PAL activity was best retained in stored FD wheat seedling tissues at −20 °C. Vacuum packaging did not increase PAL stability during the storage period.

Stability of PAL Activity during in Vitro Digestion. Another consideration of generating a dietary supplement with high PAL activity is the degree of stability of the enzyme under in vitro conditions that mimic gastric and intestinal digestion. The stability of PAL activity in fresh and FD Red Spring wheat seedling tissues during a dual ("gastric-intestinal") phase of an in vitro digestion was investigated. Leaf and root/residual seed tissues (fresh and FD) were incubated with pepsin at pH 2.5 for 1 h and, subsequently, pancreatin at pH 7.5 for 2 h; PAL activity was determined every 30 min during the in vitro digestion. The fresh tissue samples (leaf and root/residual seed) exhibited similar patterns of PAL activity over the 3-h incubation (**Figure 1**). The decline in PAL activity after the 1-h "gastric" phase was approximately 30% and 50%, for leaf and root/residual seed samples, respectively; the "intestinal" phase was associated with a further decline of 34% and 8%, respectively. After incubation times of 1.5 h and longer, the activities associated with the wheat seedling tissues were relatively stable. The main factors that potentially have a significant impact on PAL stability during the in vitro digestion are proteolysis by endogenous and exogenous proteases, and protein denaturation due to the pH conditions. The acidic pH associated with the gastric phase is very likely a significant factor. The reported pH optimum of PAL depending on the plant source is pH 8.0 − 9.5 (*5*). In addition, wheat seedlings contain endogenous proteases with acidic pH optima (*17*) that could act in concert with the exogenous pepsin.

At the end of the in vitro digestion, recovery of PAL activity associated with fresh leaf and root/residual seed tissues was 36% and 42%, respectively, which is equivalent to 4.3 and 3.7 μmol/h/g dry wt (**Figure 1**). This suggests that the PAL activity within fresh wheat seedling tissues was protected to some degree from the harsh in vitro digestion environment. These results are more favorable than those associated with PAL of permeabilized *Rhodotorula glutinis* cells, in which the enzyme is completely inactivated by duodenal juice after 30 min due to its susceptibility to proteases (*14*).

While FD is the best method for retention of PAL activity in seedling tissues (*15*), the FD tissues were very sensitive to the gastric phase of digestion. In FD leaf samples, over 50% of the PAL activity decreased after the first 30 min of digestion and almost no activity was recovered after 3 h (**Figure 1**). FD root /residual seed tissues did not show any activity after 30 min of digestion (data not shown). The differences in PAL recovery between fresh and FD samples are likely due to the increased porosity of cells during lyophilization (*18*), and/or a destruction of cell membrane integrity (*19*). A compromised cellular/subcellular integrity of wheat tissues likely caused PAL to be more susceptible to the exogenous proteolytic enzymes.

Phe Conversion during in Vitro Digestion. Another measure of PAL preservation in the wheat seedling tissues during in vitro digestion is to determine the capacity of the tissue to turnover (metabolize) Phe. This was quantified using reverse-phase HPLC to determine the residual Phe remaining after digestion. It was not possible to verify the production of *trans*-cinnamic acid by HPLC or spectrophotometric analysis because of the detection limits of these methods and the complex reaction mixture. For all samples, there were no differences in the conversion of Phe among the different time intervals, suggesting that the enzyme is active during the first 30 min of the incubation (**Tables 3** and **4**). Interestingly, ground and intact samples within

Table 2. Effects of Temperature and Packaging on the Stability of PAL Activity during Storage of Freeze-Dried Samples

		PAL activity (μmol/h/g dry wt)[a]			
		vacuum-packed		non-vacuum-packed	
	storage duration	4 °C[b]	−20 °C[b]	4 °C[b]	−20 °C[b]
leaf samples[c]	week 2	3.36 ± 0.74 xz	3.90 ± 0.54 x	2.53 ± 0.56 z	5.84 ± 0.17 y
	week 4	1.17 ± 1.60 xy	0.57 ± 0.72 y	0.19 ± 0.61 y	2.56 ± 0.95 x
	week 8	0.58 ± 0.21 x	2.53 ± 0.56 y	0.81 ± 0.28 x	3.22 ± 0.19 z
	week 12	0.31 ± 0.12 x	5.05 ± 0.52 y	1.76 ± 0.31 z	4.44 ± 0.24 w
% retention[d]		4.06	66.2	23.1	58.2
root/residual seed samples[e]	week 2	1.29 ± 0.85 x	4.53 ± 0.66 y	0.71 ± 0.20 x	3.64 ± 0.74 z
	week 4	0.74 ± 0.31 x	3.66 ± 0.88 y	2.54 ± 1.10 z	2.41 ± 0.80 z
	week 8	1.65 ± 1.32 x	5.86 ± 0.78 y	1.33 ± 1.09 x	4.04 ± 0.59 z
	week 12	2.91 ± 0.67 x	6.00 ± 1.94 y	1.63 ± 0.44 z	6.59 ± 0.98 y
% retention[d]		41.0	84.6	23.0	92.9

[a] For samples within the same week and tissue, treatments denoted by different letters are significantly different ($p < 0.05$). [b] Storage temperature. [c] PAL activity of FD leaf tissues at $t = 0$ was 7.63 ± 0.52 μmol/h/g dry wt. Values represent the mean of 6 determinations ± standard deviation. [d] Percent retention of PAL activity after 12 weeks as compared to the activity at $t = 0$. [e] PAL activity of FD root/residual seed tissues at $t = 0$ was 7.09 ± 2.37 μmol/h/g dry wt. Values represent the mean of 12 determinations ± standard deviation.

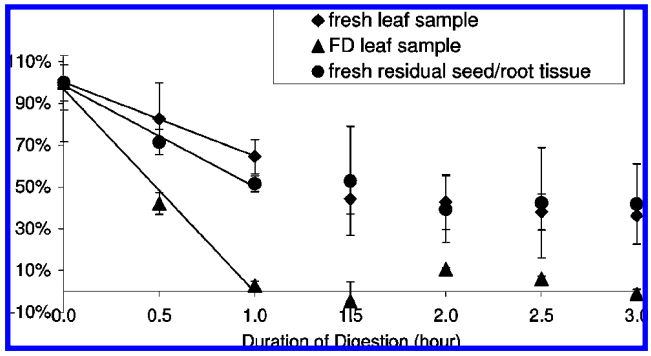

Figure 1. Percent recovery of PAL activity in fresh and freeze-dried wheat seedling tissues during in vitro digestion. Fresh and FD tissues (leaf and root/residual seed) were subjected to 1 h "gastric" digestion and 2 h "intestinal" digestion. Values represent the mean of 6−8 determinations ± SD. Linear regressions were extrapolated on the first 3 digestion intervals in fresh leaf samples (percent recovery of PAL activity = −0.37(duration of digestion) + 1.01; $R^2 = 1.00$); on the first 3 digestion intervals in FD leaf samples (percent recovery of PAL activity = −0.97(duration of digestion) + 0.97; $R^2 = 0.99$); 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 3. Conversion of Phe during in Vitro Digestion Associated with Ground and Intact Fresh and Freeze-Dried Root/Residual Seed Tissues

	conversion of phenylalanine (mg)[a,b]			
	fresh residual seed/root		freeze-dried residual seed/root	
duration of digestion (h)	ground x[c]	intact x[c]	ground y[c]	intact y[c]
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

[a] Conversion of Phe by four grams of tissues calculated in fresh weight basis. [b] Values represent a single determination (due to limited sample availability) or the mean of 3 determinations ± S.D. [c] Different letters (x, y) denote significant differences between ground and intact tissue for each treatment ($p < 0.05$).

each preparation method (fresh or freeze-dried) were not significantly different. Grinding did release some PAL into the reaction solution. Assay of only the fluid extract released from grinding leaf and residual seed/root tissues in extraction buffer) showed that 6.3 mg and 6.1 mg of Phe were converted,

Table 4. Conversion of Phe During in Vitro Digestion Associated with Ground and Intact Fresh and Freeze-Dried Leaf Tissues

	conversion of Phe (mg)[a]			
	fresh leaf[b]		freeze-dried leaf[c]	
duration of digestion (h)	ground x[d]	intact x[d]	ground y[d]	intact y[d]
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)

[a] Conversion of Phe by four grams of tissue, calculated on a fresh weight basis. [b] Values represent the mean of 3 determinations ± standard deviation. Different letters denote significant differences between treatments ($p < 0.05$). [c] Values represent a single determination (due to limited sample availability), or the mean of 2 determinations (range). [d] Different letters (x, y) denote significant differences between ground and intact tissue for each treatment ($p < 0.05$).

compared to 18.3 and 13.5 mg of Phe conversion when both the tissue and fluid extract were assayed. This suggests that not all of the PAL was released into the fluid extract during grinding, and the activity remaining in the plant tissue may have been protected. Conversion of Phe by FD samples was significantly less than fresh samples, again suggesting that the cells of FD samples were more sensitive to the digestive environment. In order to better understand the factors affecting stability of plant PAL during digestion, the effects of endogenous proteolysis and pH were examined independently.

Stability of PAL Activity at pH 2.5 and 9.5. After incubation at pH 2.5 for 1 h, both intact leaf and residual seed/root samples exhibited a high retention of PAL activity, with no significant differences to the control (**Table 5**). Since PAL in fresh leaf and residual seed/root samples lost 35 and 50% activity after 1 h of gastric digestion (**Figure 1**), this suggested that exogenous proteolytic activity was responsible for the majority of the PAL degradation under these conditions. Ground leaf and root/residual seed samples incubated at pH 2.5 lost approximately half of their PAL activity, which indicates that the cell walls of the wheat seedling tissues did offer a significant degree of protection to PAL from the acidic environment.

At pH 9.5, ground root/residual seed samples lost approximately 38% of their original PAL activity, while the activity in ground leaf samples was fully retained. In general

Table 5. Percent Recovery of PAL Activity in Fresh Leaf and Root/ Residual Seed Samples Incubated at pH 2.5 or 9.5 for 1 h

	PAL activity (% control)[a,b,c]	
treatment(s)	leaf	residual seed/root
control	100 ± 5.4 x	100 ± 10.0 x
incubation		
intact, pH 2.5	90.9 ± 17.7 x	83.0 ± 18.6 x,y
ground, pH 2.5	52.3 ± 6.4 y	48.5 ± 26.1 z
ground, pH 9.5	99.5 ± 3.6 x	61.7 ± 19.0 y,z

[a] Percent recovery of PAL activity was calculated with respect to activity of control. [b] Values represent the mean of 4 to 8 determinations ± standard deviation. [c] Within the same tissue, treatments denoted by different letters are significantly different ($p < 0.05$)

Table 6. Stability of PAL Activity in Fresh Leaf and Root/Residual Seed Tissues after a 3-h Incubation at pH 8.8 (37 °C)

	PAL activity (% fresh sample)[a,b,c]	
treatment(s)	leaf	residual seed/root
control[d]	100.0 ± 4.3 x	100.0 ± 13.2 x
incubation		
3 h, pH 8.8, 37 °C	68.7 ± 2.5 y	31.5 ± 2.3 y
3 h, pH 8.8, 37 °C with protease inhibitors[e]	42.8 ± 9.8 z	31.8 ± 3.0 y

[a] Percent recovery of PAL activity was calculated with respect to activity in control samples (dry weight basis). [b] Values represent the mean of 6 determinations ± standard deviation. [c] Within the same tissue, treatments denoted by different letters are significantly different ($p < 0.05$). [d] Control samples were assayed using the PAL activity assay method without incubation. [e] 0.5 mM PMSF and 5 mM of EDTA (final concentration) were added before incubation.

then, PAL in leaf samples was more stable than PAL in root/ residual seed samples at the two pH extremes, and the lower pH resulted in a greater loss of activity compared to pH 9.5.

Stability of PAL Activity at pH 8.8. Further experiments were carried out to evaluate the effect of endogenous proteolysis on PAL during incubation at the optimal pH (for the PAL enzyme) of 8.8, and in the presence/absence of protease inhibitors. A significant decrease in PAL activity was noted after the 3-h incubation in both leaf and root/ residual seed tissues (**Table 6**). Residual seed/root tissues were more sensitive to the incubation, which might be due to the fact that they have the highest specific protease activity on a fresh weight basis (*13*). These acidic proteases are expressed during the early stages of plant development and are reported to be very stable over a wide temperature range (*13*). The addition of protease inhibitors PMSF and EDTA did not improve the retention of PAL activity, as would be expected for acidic proteases.

Phe Conversion at pH 8.8. Ground and intact leaf and root tissues were subjected to a 2-h incubation at pH 8.8 to determine the conversion of Phe by the PAL enzyme at its optimal pH. Significant differences were found among treatments but no differences were found among the different incubation times (**Table 7**), suggesting that most of the conversion occurred within the first 30 min. The conversion of Phe in ground tissues was significantly less than in intact tissues.

A general trend in all the assays was that incubation of plant tissue without Phe yielded a significant retention of PAL activity, even after 3 h of incubation under in vitro digestion conditions. However when the enzyme was incubated with Phe, even under optimal pH conditions of 8.8, activity was limited to the first 30 min of the incubation.

PAL has long been known to be tightly regulated by a feedback mechanism involving *trans*-cinnamic acid, the product of the reaction (*20*). In the common bean (*Phaseolus vulgaris),*

Table 7. Conversion of Phe by Fresh Leaf and Root/ Residual Seed Tissues over 2 h at pH 8.8 (37 °C)

	conversion of Phe (mg)[a]			
	leaf		residual seed/root	
duration of incubation (h)	ground w[b]	intact x[b]	ground y[b]	intact z[b]
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)

[a] Values represent the mean (range) of determinations. Values without range were single determinations (due to limited sample availability). Conversion of Phe by four grams of tissue calculated on a fresh weight basis. [b] Different letters (w, x, y, z) denoted difference between ground and intact samples within each tissue.

trans-cinnamic acid is reported to reduce PAL activity by both inducing the synthesis of a protein inhibitor of PAL, and by preventing the induction of PAL enzyme activity (*21*). In addition, *trans*-cinnamic acid, or a downstream metabolite of *trans*-cinnamic acid, down-regulates PAL gene transcription at 0.1 mM (*22*). In this work, there was potential for the formation of at least a 10-fold higher concentration of *trans*-cinnamic acid. The lowest Phe conversion was 4 mg, equivalent to approximately 1 mM in the assay. Product inhibition by *trans*-cinnamic acid may be an important factor to overcome in the therapeutic application of PAL immobilized in plant cells. This would depend on the in vivo timing of release of PAL from the plant cells, its half-life, and whether the trans-cinnamic acid has a chance to accumulate, or if it is efficiently converted to benzoic and then hippuric acid.

ABBREVIATIONS USED

EDTA, ethylenediaminetetraacetic acid; FD, freeze-dried; HPA, non-PKU-hyperphenylalaninemia; HPLC, high performance liquid chromatography; PAH, phenylalanine hydroxylase; Phe, phenylalanine; PAL, phenylalanine ammonia-lyase; PKU, phenylketonuria; PMSF, phenylmethylsulfonylfluoride; PVPP, polyvinylpolypyrrolidone.

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