

Biochemical Evaluation of Phenylalanine Ammonia Lyase from Endemic Plant *Cyathobasis fruticulosa* (Bunge) Aellen. for the Dietary Treatment of Phenylketonuria

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Summary

Enzyme substitution therapy with the phenylalanine ammonia lyase (PAL) is a new approach to the treatment of patients with phenylketonuria (PKU). This enzyme is responsible for the conversion of phenylalanine to *trans*-cinnamic acid. We assessed the PAL enzyme of the endemic plant *Cyathobasis fruticulosa* (Bunge) Aellen. for its possible role in the dietary treatment of PKU. The enzyme was found to have a high activity of (64.9±0.1) U/mg, with the optimum pH, temperature and buffer (Tris-HCl and L-phenylalanine) concentration levels of pH=8.8, 37 °C and 100 mM, respectively. Optimum enzyme activity was achieved at pH=4.0 and 7.5, corresponding to pH levels of gastric and intestinal juice, and NaCl concentration of 200 mM. The purification of the enzyme by 1.87-fold yielded an activity of 98.6 U/mg. PAL activities determined by HPLC analyses before and after purification were similar. Two protein bands, one at 70 and the other at 23 kDa, were determined by Western blot analysis of the enzyme. This enzyme is a potential candidate for serial production of dietary food and biotechnological products.

Key words: *Cyathobasis fruticulosa*, endemic plant, enzyme purification, phenylalanine ammonia lyase (PAL), phenylketonuria (PKU)

Introduction

Phenylketonuria (PKU) is the most widely inherited disorder of amino acid metabolism, in which mutations affecting phenylalanine hydroxylase (PAH) lead to the inhibition of the hydroxylation of the essential amino acid phenylalanine (Phe) to tyrosine (1), leading to accumulation of the former in blood and tissues (2). Currently, the only way to prevent the disease from progressing and causing various sequelae is to adhere to a strict diet poor in Phe throughout the life of an individual, a task which is difficult and not entirely protective from the detrimental effects of low Phe levels (3). Hence there is a great demand for pharmacological approaches that can be used in the treatment of PKU today. One of them is alternative LNAA (large neutral amino acid) treatment, which aims

to prevent Phe from entering into the brain and hopefully lower its cerebral amount by making LNAAs compete with Phe to cross blood-brain barrier (4).

Tetrahydrobiopterin (BH₄) acts as the cofactor of PAH, mainly by increasing the residual PAH enzyme activity and stabilizing the enzyme tetramer in a multifactorial manner. Orally administered sapropterin dihydrochloride, a synthetic form of BH₄, acts like BH₄ in pharmacological doses in approx. one third of PKU patients (5). In recent years, studies on gene therapy using viral and other vectors have been conducted without any success due to difficulties in gene delivery and maintaining a stable expression. Plant phenylalanine ammonia lyase (PAL; EC 4.3.1.24) enzyme has been suggested as a new oral therapy for the treatment of PKU (6).

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PAL acts by transforming Phe into a metabolically inert substance called *trans*-cinnamic acid and trace amounts of ammonia. *trans*-Cinnamic acid is then converted to benzoic acid, which is renally excreted as hippurate. The enzyme does not use any cofactor (7). Although certain fungi and higher plants possess PAL activity, no animal has been shown to have this enzyme yet (8). Being one of several commercially available non-hydrolytic enzymes, plant PAL has been successfully utilized to treat certain neoplasms in mice as well as to measure serum levels of Phe in humans with PKU and diets low in Phe (9). Modest reductions in Phe levels have been attained in early trials of oral administration of PAL to humans. Some mouse models used a recombinant *Anabaena variabilis* PAL produced in *Escherichia coli*, which was subjected to genetic modification to achieve high protease resistance and conjugated with polyethylene glycol (PEG) to achieve a lower immunogenicity (rAvPAL-PEG) (10). Research efforts are ongoing to find ways to orally administer PAL or encapsulate the enzyme in red blood cells (5).

In the secondary metabolism of plants, PAL initiates the biochemical phenylpropanoid biosynthetic pathway, in which it is one of the most challenging enzymes (11). Its activity is increased by some physical and chemical factors such as light, salt, excision, fungal cell surface elicitors and phytohormones (12), and its levels and activity are expected to rise under stress. Enzymatic reaction produces cinnamic acid, which acts as a precursor of many secondary substances including lignins, flavonoid pigments, UV protectants, plant hormones and phytoalexins; it is also a major source of total carbon in plants (13,14).

Cyathobasis fruticulosa (Bunge) Aellen. is a monotypic single species/single genus plant belonging to the genus *Cyathobasis* that is endemic to salt soils of Central Anatolia in Turkey. It is included in the tribe Salsoleae of the family Chenopodiaceae comprising a large variety of plants that contain alkaloids (15).

This study aims to evaluate the potential of a plant PAL enzyme to be used in biotechnology products for the treatment of PKU patients without any side effects. With high durability at environmental pH and temperature, and resistance to gastric and intestinal pH levels, these preparations may strengthen the effect of dietary therapies when added to the diet of PKU patients. They may also have a role as orally administered preparations. Another objective of this study is to report the endemic *Cyathobasis fruticulosa* (Bunge) Aellen. (Chenopodiaceae) as a natural product possessing a unique, high PAL activity.

Materials and Methods

Plant material

Cyathobasis fruticulosa naturally growing in fields rich in saline salts in Beypazarı, Ankara, Turkey, was collected in August 2011. The plant taxonomy was confirmed by Dr. Zeki Aytaç (Gazi University, Ankara, Turkey). A voucher specimen was deposited at the Herbarium of Gazi University, Ankara, Turkey (voucher ID: ZA-10439). The plants were stored at -80°C until use.

Extraction of PAL enzyme

The leaves were weighed, frozen in liquid nitrogen, and ground in a mortar. The final pulverized powder was extracted in 50 mM Tris (Sigma-Aldrich, Steinheim, Germany)-HCl (Merck, Darmstadt, Germany) buffer (pH=8.8) containing 10 mM 2- β -mercaptoethanol (Merck, Hohenbrunn, Germany), 1 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich) and 2.5 % polyvinylpyrrolidone-40 (PVP-40; Sigma-Aldrich). The mixture was centrifuged at 21 180 \times g for 20 min and the clear supernatant was desalted in aliquots using an Amicon Ultra-15 Centrifugal Filter Units with a membrane nominal mass limit of 50 kDa (Merck Millipore, Tullagreen Carrigtwohill, Ireland) and assayed for PAL activity under standard conditions. The aliquots were stored at -20°C until use (16).

PAL activity assay and biochemical property analysis

Protein concentrations were determined by a dye-binding Bradford method using Bradford reagent (Sigma-Aldrich) and bovine serum albumin (Amresco, Cleveland, OH, USA) as the protein standard (17). PAL activity was assayed by measuring the *trans*-cinnamic acid formation at 290 nm using a UV-1800 UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan) and calculated using a standard *trans*-cinnamic acid, $\geq 99\%$ (Sigma-Aldrich) curve. The enzyme reaction mixture contained 100 mM Tris-HCl, 40 mM L-phenylalanine, $\geq 98\%$ (Sigma-Aldrich), and an aliquot of the enzyme in a total volume of 1 mL at pH=8.8. The reaction was carried out at 37°C for 30 min and terminated by the addition of 50 μL of 4 M HCl. PAL activity was expressed in U/mL and PAL specific activity was expressed in U/mg (16).

Determination of pH, temperature and buffer concentration

To determine the optimum pH, assays were performed at 37°C for 30 min using a buffer (100 mM Tris-HCl and 40 mM L-phenylalanine) with various pH values (pH=2.0–12.0). For optimum temperature determination, assays were performed at pH=8.8 for 30 min at various temperatures (10– 70°C). To determine the optimum buffer concentration, assays were performed at 37°C and pH=8.8 for 30 min using Tris-HCl buffer (40 mM L-phenylalanine) and various concentrations (5–500 mM) of Tris-HCl (18).

Simulated gastric and intestinal digestion

In vitro conditions simulating gastric and intestinal digestion were created. The reaction mixture was incubated with 3 g/L of pepsin (Sigma-Aldrich), pH=2.0 or 4.0, for 30 min at 37°C to mimic the gastric phase. The reaction mixture was incubated with 1 g/L of pancreatin (Sigma-Aldrich) and 0.30 % bile salt (Difco, Franklin Lakes, NJ, USA), pH=5.5, 7.5 or 9.5, for 30 min at 37°C to simulate the intestinal phase (19).

Determination of PAL specific activity under different NaCl concentrations

The PAL activity was determined in the presence of different concentrations (50–300 mM) of NaCl (Sigma-Al-

drich, Hunterdon, NJ, USA) in the reaction mixture (9). Assays were performed at 37 °C and pH=8.8 for 30 min using 100 mM Tris-HCl buffer and 40 mM L-phenylalanine.

Storage stability of PAL extract

PAL extract was stored at either room temperature or at 4 °C for 1–5 days and its activity was assayed. It was also stored at –20 °C for 1–12 months and then its activity was determined. Assays were performed at 37 °C and pH=8.8 for 30 min using 100 mM Tris-HCl buffer and 40 mM L-phenylalanine. The relative activity (in %) of the extract was determined as a ratio of PAL specific activity in test conditions and PAL specific activity under optimum conditions (pH=8.8, 37 °C and 100 mM Tris-HCl).

HPLC separation of PAL

The concentration of *trans*-cinnamic acid (PAL product) formed was determined using different volumes of PAL enzyme (100–300 µL), different concentrations of substrate (40–160 mmol/L) and different reaction times (30–90 min). The percentage of conversion of L-phenylalanine to *trans*-cinnamic acid was also determined under the following conditions: 300 µL of enzyme, 160 mmol/L of substrate and reaction time 60 min. The HPLC separation of the sample components was achieved both before and after the enzyme purification. *trans*-Cinnamic acid detection was performed on an Agilent Technologies HPLC 1200 series (Santa Clara, CA, USA) equipped with a quaternary pump, a manual sampler and an ultraviolet/visible (UV-Vis) detector. The column used was a 5 µm C₁₈ (250 mm×4.6 mm i.d.) from Advanced Chromatography Technologies (ACE, Aberdeen, Scotland, UK). The column was operated at 25 °C. The mobile phase consisted of 35 % H₂O acetic acid and 65 % methanol (both from Merck, Darmstadt, Germany). The injection volume of all samples was 20 µL. Simultaneous monitoring was performed at 280 nm and a flow rate of 1 mL/min (20).

Partial purification of PAL

The enzyme was partially purified in a two-step procedure involving ammonium sulfate (Merck, Darmstadt) precipitation and dialysis (21,22). The enzyme extract was brought to 60 % saturation by gradual addition of solid ammonium sulfate while stirring at 4 °C. After 20 min of equilibration, the precipitated protein was obtained by centrifugation at 10 000×g at 4 °C for 30 min. The pellet was resuspended in 100 mM ice-cold Tris-HCl (pH=8.8). Enzyme activity and protein content were determined after separation of each fraction. The pretreated Slide-A-Lyzer™ G2 Dialysis Cassettes, molecular mass cut-off of 20 000 (Thermo Fisher Scientific, Rockford, IL, USA), were used for dialysis of the enzyme collected and suspended after ammonium sulfate precipitation. The enzyme was dialyzed against 100 mM Tris-HCl (pH=8.8) at 4 °C for 1–2 h. The buffer was changed, and the enzyme was dialyzed for an additional 1–2 h. In the last step, dialysis was continued overnight at 4 °C in 100 mM Tris-HCl (pH=8.8) reaction buffer (more than 200-fold of the sample volume). The dialysate was centrifuged at 21 180×g for 20 min

using an Amicon Ultra-15 Centrifugal Filter Unit (Merck Millipore). The partially purified sample was assayed for protein content and enzyme activity.

SDS-PAGE of PAL

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels containing 4–10 % acrylamide (Merck, Hohenbrunn) were used and stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich) to visualize the PAL protein. The stacking gel (4 %) consisted of: 29.2 % acrylamide and 8 % *N,N'*-methylene-bis-acrylamide (Sigma-Aldrich), 1.5 M Tris-HCl (pH=8.8), 10 % sodium dodecyl sulfate (SDS; Sigma-Aldrich), 10 % ammonium persulfate (APS; Sigma-Aldrich), *N,N,N',N'*-tetramethylethylenediamine (TEMED; Sigma-Aldrich) and the separating gel (10 %) consisted of: 30 % acrylamide-*N,N'*-methylene-bis-acrylamide, 0.5 M Tris-HCl (pH=6.8), 10 % SDS, 10 % APS and TEMED. PageRuler™ Prestained Protein Ladder (10 to 170 kDa; Thermo Fisher Scientific, Vilnius, Lithuania) was used as a size standard (23).

Electrophoresis and Western blotting

SDS-PAGE was performed on 4–12 % Bis-Tris gels (NuPAGE®; Thermo Fisher Scientific, Canoga Park, CA, USA) using a mini-cell gel system (XCell SureLock™; Invitrogen, Carlsbad, CA, USA). Following electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250 and destained with dye-destaining solution. After separation, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (0.2 µm pore size; NuPAGE®) using an iBlot® device (120 V, 1 h, room temperature; Invitrogen). All subsequent steps were carried out with a WesternBreeze® kit (Invitrogen) according to the manufacturer's instructions. The kit contained blocking solution, the primary antibody diluents, secondary antibody (anti-rabbit) diluents, chromogenic substrate and washing solutions. The membrane was blocked for 30 min in the concentrated saline buffer solution containing detergent and the Hammerstein casein solution, then incubated for 1 h with primary polyclonal PAL antibody (Tübitak MAM, Gebze, Kocaeli, Turkey), washed with the concentrated saline buffer solution and incubated for 30 min with alkaline phosphatase-conjugated anti-rabbit IgG antibody. The excess of the secondary antibody was washed away three times and incubation continued with the 5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt (BCIP) and nitroblue tetrazolium chloride (NBT) substrate solution for determination of alkaline phosphatase until purple bands developed on the membrane. The colour development was completed in 1 to 60 min. The membrane was rinsed twice with double distilled H₂O for 2 min and dried. The chromogenic band was visualized with UVP BioSpectrum 410 (Upland, CA, USA) imaging system (24,25).

Statistical analysis

All experiments were performed three times, and the mean value of each measurement was calculated. All results were reported as mean value±standard deviation (S.D.).

Results and Discussion

Enzyme therapy with phenylalanine ammonia lyase (PAL) is complicated because it requires large amounts of purified, highly specific PAL and the activation of immune system with resultant immunological reaction against plant PAL. As such, repeated PAL administration leads to antibody production against it, resulting in both rapidly vanishing serum PAL activity and potentially detrimental allergic reactions. It was reported that in order to maintain therapeutic efficacy, PAL enzyme should stay in blood for an extended period (26). This issue has been formerly addressed by multitubular enzyme reactors with immobilized PAL (from *Rhodotorula glutinis*), which enabled to remove 77 % of blood phenylalanine (Phe) rapidly, without the enzyme entering circulation (27). Novel plant PALs displaying unique properties are still being actively sought.

The PAL activity of *Cyathobasis fruticulosa* is reportedly higher ((64.9±0.1) U/mg) than those of other plants, including *Bambusa oldhamii* with a PAL activity of 0.19 U/mg (25), *Fragaria × ananassa* 44.5 U/mg (28), *Cucurbita pepo* 26.6 U/mg (9), and *Glycine max* 0.43 U/mg (29). Plants are able to utilize light, carbon dioxide and various inorganic substances in an efficient manner for their relatively simple vital demands. Hence, they produce a variety of important therapeutic substances for humans. Mass cultivation allows some plants with high nutritional potential and nonpathogenic composition to be grown in huge amounts as part of commercial production (30). Hence, plants could also be a very valuable commercial source of PAL enzyme; however, this enzyme should be further optimized for use in human body.

PAL enzyme derived from *C. fruticulosa* has a specific activity of (54.9±0.1) U/mg at its optimum pH=8.8. A similar pH range of 7.5–10.6 was also reported for PAL from other plants such as *Ustilago maydis* (31), *Nicotiana tabacum* (32), *Hordeum vulgare* and *Medicago sativa* (33). Our study demonstrated a significantly lower enzymatic activity (Fig. 1a) in acidic medium (pH=2.0, (45.3±0.0) U/mg), while enzymatic activity was improved at a slightly alkaline pH and it was still present at pH=12 ((50.4±0.0) U/mg). Hence, it was concluded that isolated *C. fruticulosa* PAL is an alkaline enzyme. The enzyme lost its activity at 60 °C or above, and had activities of (44.5±0.1) and (40.9±0.1) U/mg at 10 and 50 °C, respectively. Its optimum temperature was 37 °C with the activity of (54.9±0.1) U/mg (Fig. 1b). Previous reports have indicated a temperature range of 25 to 60 °C for other PAL enzymes derived from *Musa sp.* (34), *Triticum aestivum* (19), *Ephedra sinica* (35) and *Vitis vinifera* (36). Activities of (39.1±0.1) and (44.6±0.1) U/mg were recorded at 5 and 500 mM buffer concentrations, respectively. PAL enzyme derived from *C. fruticulosa* had an optimum buffer concentration of 100 mM Tris-HCl ((54.9±0.1) U/mg) (Fig. 1c), which is similar to what has been reported for other plant PAL enzymes, including those of *Cucurbita pepo* (9), *Medicago sativa* (21) and *Glycine max* (29).

The enzyme is susceptible to degradation by gastrointestinal proteases when orally administered, necessitating the advancement of novel ways to protect it from enzymatic proteolysis or acidic denaturation and to bring it

into contact with enteric Phe (19). Gastric pH has a range between 1.5 and 2, and intestinal pH between 6 and 7.5, depending on dietary factors (37). As Phe enters entero-circulation, enzyme development efforts should give priority to oral formulations containing entrapped PAL, which enable the release of Phe from bloodstream into the gastrointestinal tract where it is metabolized but not reabsorbed (38). We therefore measured PAL activity of *C. fruticulosa* in artificial gastric and intestinal fluids. The relative activity was not different between pH=2.0 and 4.0 in the simulated gastric phase and pH=5.5, 7.5 and 9.5 in the simulated intestinal phase (Fig. 2a). In a previous report, PAL of permeabilized *R. glutinis* cells was completely inactivated by proteases found in duodenal juice within 30 min (39); thus, our results indicated a more favourable stability of enzyme activity.

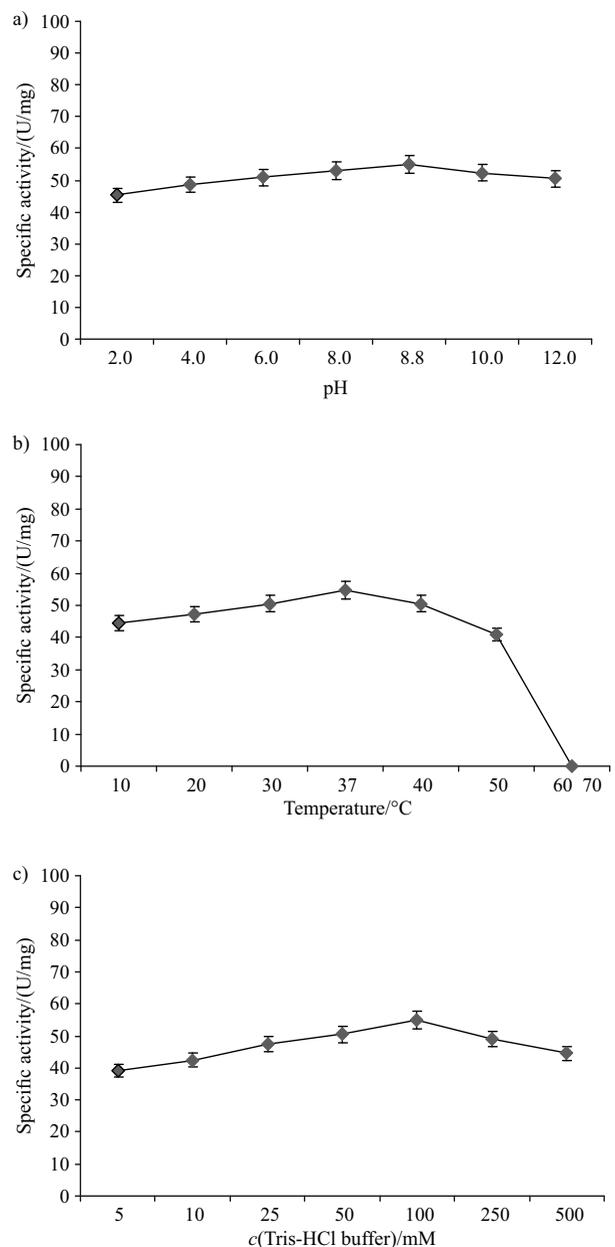


Fig. 1. Effect of: a) pH, b) temperature and c) Tris-HCl buffer concentration on phenylalanine ammonia lyase (PAL) activity

Above optimal concentrations, NaCl may give rise to hysteresis followed by reduction of the enzymatic activity. Shielding of negatively charged amino acid residues and increasing hydrophobic interactions within the protein molecule are responsible for the stability of proteins (9). Thus, various NaCl concentrations were studied for their impact on PAL activity of *C. fruticulosa* at a pH=8.8 (Fig. 2b). According to this analysis, the optimum NaCl concentration was determined to be 200 mM, similar to what was reported by Guo and Wang (40) and El-Shora (9). As NaCl concentration was increased to a range of 250–300 mM, the relative activity was reduced (Fig. 2b).

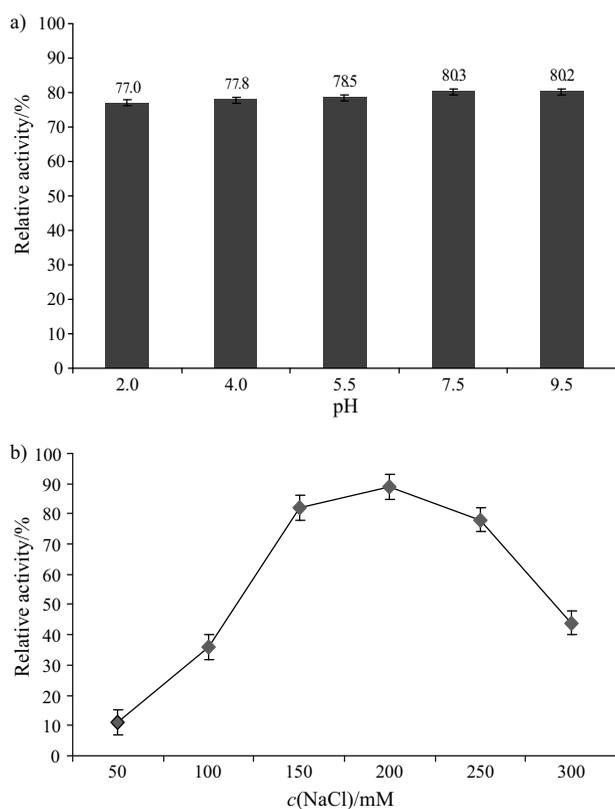


Fig. 2. Effect of: a) simulating gastric pH (2 and 4) and intestinal phase pH (5.5, 7.5 and 9.5), and b) NaCl concentration in the reaction mixture on phenylalanine ammonia lyase (PAL) activity

Enzyme denaturation and reaction rates are altered by storage temperature. As shown in Fig. 3, enzyme activity of *C. fruticulosa* PAL was absent at 4 °C whereas it was stable at –20 °C and maintained so for 12 months. This result was in contrast to several reports that reported a 38 % loss in enzyme activity in 3 months at –20 °C (19), an almost 100 % loss in 7 days at 6 °C (29), and a 25 % loss in 3.5 months at –10 °C (33).

The amount of formed *trans*-cinnamic acid was also determined by HPLC to measure the activity of *C. fruticulosa* PAL. A range of concentrations of 40–160 mmol/L produced mass concentrations of *trans*-cinnamic acid ranging between 3.3 and 3.7 ng/mL. A 90-minute reaction time and a volume of enzyme of 300 µL produced the greatest mass concentrations of *trans*-cinnamic acid (6.2 and 8.4 ng/mL, respectively) determined by HPLC (Fig. 4).

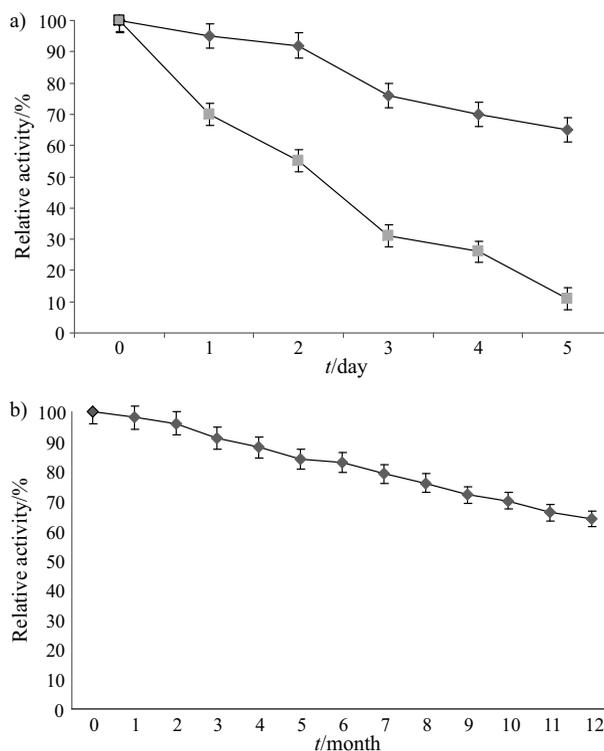


Fig. 3. The storage stability of phenylalanine ammonia lyase (PAL) at: a) room temperature (■) and 4 °C (◆), and b) at –20 °C

Pre-purification results showed that before and after enzymatic reaction, the L-phenylalanine was detected at concentrations of (160.0±2.3) and (124.0±1.8) mmol/L, respectively, which corresponded to the conversion rate of L-phenylalanine to *trans*-cinnamic acid of (22.5±1.3) % and a post-reaction *trans*-cinnamic acid mass concentration of (12.3±1.0) ng/mL. Chromatographic separation of the reaction mixture was completed within less than 10 min. L-Phenylalanine and *trans*-cinnamic acid had retention times of 2.7 and 5.6 min, respectively (Fig. 5).

Purification yield of *C. fruticulosa* PAL from crude extract was 1.87-fold, which is lower than that of *Medicago sativa* (21). The final specific activity of *C. fruticulosa* was 98.6 U/mg (Table 1), while lower values have been previously obtained for other species such as *Cucurbita pepo* (26.60 U/mg; 9) and *Oryza sativa* (4.625 nkat/mg; 41). SDS-PAGE of partially purified PAL produces a single protein band corresponding to a molecular mass (*M*) of about 70 kDa (Fig. 6). Other values of molecular masses were 80 kDa for *Bambusa oldhamii* reported by Hsieh *et al.* (25) and 84 kDa for *Oryza sativa* reported by Sarma and Sharma (41).

The *trans*-cinnamic acid mass concentration yielded by the partially purified *C. fruticulosa* PAL was measured with HPLC. A substrate concentration of 40–160 mmol/L yielded a *trans*-cinnamic acid mass concentration of 3.1–3.3 ng/mL. A 90-minute reaction time and an enzyme volume of 300 µL yielded the highest *trans*-cinnamic acid concentration (6.9 ng/mL) (Fig. 4).

After partial purification, L-phenylalanine was detected at concentrations of (160.0±2.6) and (122.0±2.2) mmol/L before and after the enzymatic reaction, respectively, which

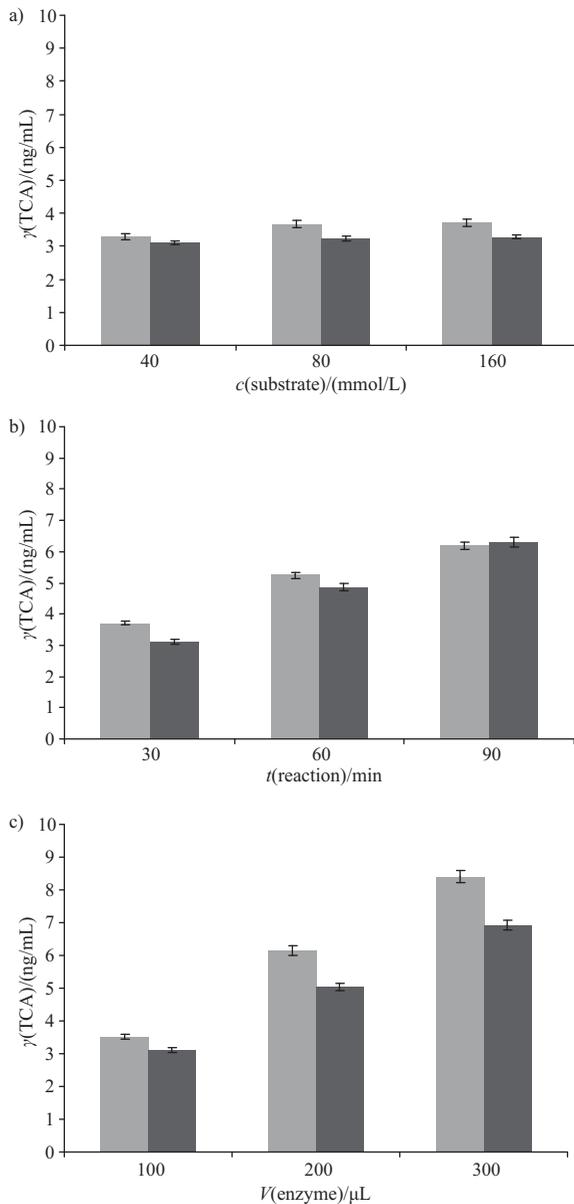


Fig. 4. Effect of: a) substrate concentration, b) reaction time, and c) enzyme volume on mass concentration of *trans*-cinnamic acid (TCA)

Table 1. Partial purification of phenylalanine ammonia lyase (PAL) from *Cyathobasis fruticulosa*

Purification step	$m(\text{total protein})$	Total activity	Specific activity	Purification	Y
	mg	U/mL	U/mg	fold	%
Crude extract	0.8±0.0	40.8±0.0	52.6±0.1	1	100
(NH ₄) ₂ SO ₄ precipitation	0.6±0.0	40.2±0.0	63.4±0.1	1.21	98.7
Dialysis	0.4±0.0	39.8±0.0	98.6±0.2	1.87	97.6

is equal to the *L*-phenylalanine to *trans*-cinnamic acid conversion rate of (23.8±1.5) % and a post-reaction *trans*-cinnamic acid mass concentration of (13.0±1.2) ng/mL. Chromatographic separation of the reaction mixture was completed within less than 10 min, with the retention times of *L*-phenylalanine and *trans*-cinnamic of 2.7 and 5.6 min, respectively (Fig. 5). Pre- and post-purification PAL activities measured by HPLC were in accordance with each other.

Western blot analysis of *C. fruticulosa* PAL was done by SDS-PAGE. The blotted protein extracts of *C. fruticulosa* were probed by anti-rabbit PAL antibodies, and a major band was spotted in the region corresponding to the molar mass (*M*) of the *C. fruticulosa* PAL subunit of 70 kDa, as well as a minor band in the region corresponding to the *M* of the *C. fruticulosa* PAL subunit of 23 kDa (Fig. 6).

Plants possess PAL activity that is regulated by a variety of PAL-encoding genes which may be uniquely encoded only in some regions of plants or under certain environmental conditions (9). It has formerly been shown that various plants contain PAL enzymes with a heterotetramer structure (42). For instance, PAL from *Rhizoctonia solani* has an α -chain of 70 kDa and a β -chain of 90 kDa (43), while that from *Triticum aestivum* possesses a heterotetramer structure with an α -chain of 75 kDa and a β -chain of 80 kDa (29). We also detected a minor band corresponding to a $M \approx 23$ kDa, suggesting that the *C. fruticulosa* PAL enzyme also possesses a heterotetramer structure. A protein analysis will accurately establish such a structure.

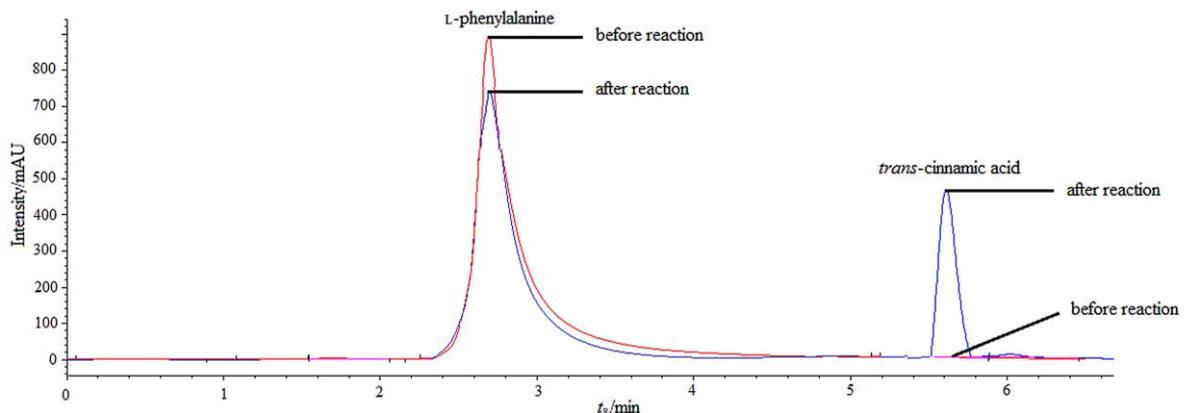


Fig. 5. Comparison of *L*-phenylalanine and *trans*-cinnamic acid amounts before and after enzymatic reaction, as a result of phenylalanine ammonia lyase (PAL) activity as determined by HPLC

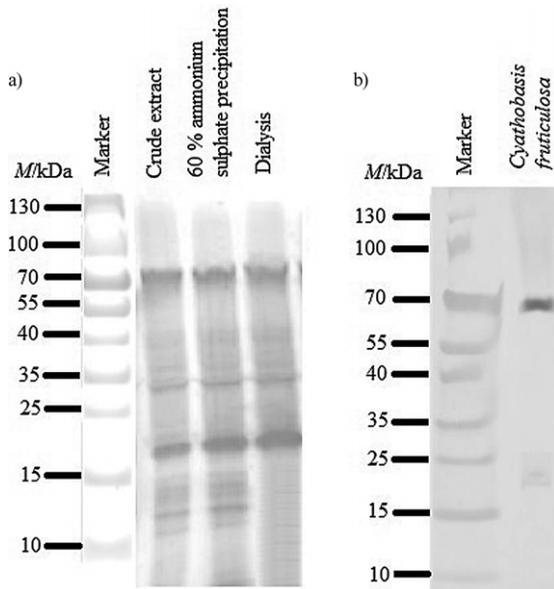


Fig. 6. SDS-PAGE of phenylalanine ammonia lyase (PAL) at various stages of partial purification (a) and Western blot of PAL from *Cyathobasis fruticulosa* (b)

Conclusion

Blood phenylalanine (Phe) level of phenylketonuria (PKU) patients should be adequately lowered to non-toxic concentrations by early initiated, strict dietary measures, which effectively averts neurological and functional sequelae, although this measure does not usually suffice alone to keep Phe levels low for a lifetime. Hence, search for alternative therapies for PKU patients is ongoing, such as large neutral amino acids (LNAA), sapropterin, gene therapy, phenylalanine ammonia lyase (PAL), and others. Replacement of the culprit enzyme is an attractive solution, but the relationship between repeated dosage, immunogenicity, and treatment efficacy should be meticulously studied before the administration of the enzyme in humans. As a candidate enzyme, PAL derived from *Cyathobasis fruticulosa* has a high activity that is not lost under a variety of physical and chemical conditions such as differing pH, temperature, and NaCl concentration. The advantage of the enzyme in question is that it is stable at body temperature (*i.e.* 37 °C) and immune to degradation by artificial gastric juices and intestinal fluids. Hence, it appears that it would be a useful candidate for mass production of dietary products by food industry and of biotechnological products by pharmaceutical companies that develop treatments for PKU patients.

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