CHARACTERIZATION OF PHENYLALANINE AMMONIA LYASE PURIFIED FROM LOCALLY CULTIVATED GRAPE SEEDS (*Vitis vinifera L.*)

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ABSTRACT

This study was conducted to investigate certain characteristics of the purified Phenylalanine ammonia lyase (PAL). The results revealed that the molecular weight was 180 kDa by gel filtration and Native-PAGE. The optimal pH and temperature for enzyme activity were 7 and 40 °C respectively. The enzyme activity was stable against pH values range of 6-8. The enzyme retained 79% of its total activity after incubation for one hour at 50 °C and retained 7% (lost 93%) of its activity at 70 °C. The entire activity was completely lost when the enzyme was incubated for 1 hour at temperatures over 70 °C. Carbohydrate content of the purified enzyme was estimated to be 24.61%. Km, Kcat and Vmax were estimated with phenylalanine as substrate, the values were 0.021 mM, 20.67 sec^{-1} and 4.13 mM/min respectively.

Key words: PAL, optimal condition, enzyme kinetics.
***Part of Ph.D. dissertation of the 1st author.**

دراسة صفات الفنيل الانين امونيا لايز المنقى من بذور العنب المزروعة محلياً

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المستخلص

أجريت الدراسة الحالية للتحري عن بعض خصائص الفنيل الانين امونيا لاييز المنقى. كان الوزن الجزيئي 180 بالترشيح الهلامي و Native–PAGE. وكانت درجة الحموضة ودرجة الحرارة المثلى لنشاط الإنزيم 7 و 40 درجة مئوية على التوالي. أظهر الإنزيم ثباتًا عند االرقم الهيدروجيني يتراوح 6–8. احتفظ الإنزيم بنسبة 79٪ من نشاطه بعد حضنه لمدة ساعة واحدة على 50 درجة مئوية واحتفظ بنسبة 7٪ (فقد 93%) من نشاطه الكلي لدى حضنه على 70 درجة مئوية لمدة 1 ساعة. فقد الانزيم نشاطه بالكامل لدى حضنه على درجة حرارة تزيد عن 70 درجة مئوية. تم تقدير محتوى الكربوهيدرات في الإنزيم المنقى وكانت بواقع 24.61٪. ثم قدر كلاً من Km و Kcatو Vmax باستخدام الفنيل الانين كمادة اساس و كانت 0.021 ملي مولار و 20.69 ثانية$^{-1}$ و4.13 ملي مولار / دقيقة على التوالي.

الكلمات الافتتاحية: فنيل الانين امونيا لايز، بذور العنب، توصيف الانزيم، حركيات الانزيم.
البحث جزء من أطروحة الدكتوراه للباحث الاول

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INTRODUCTION

Phenylalanine ammonia lyase: PAL (E.C. 4.3.1.5) is a crucial and regulating enzyme in the biosynthesis of phenylpropanoids and their derivatives. PAL catalyzes the non-oxidative deamination (forward) and hydroamination (reverse) reactions using phenylalanine as a substrate. The forward process involves the deamination of L-phenylalanine (L-Phe) to trans-cinnamic acid (t-Ca) and ammonia, which is the initial step in the biosynthesis of phenylpropanoid-derived molecules. Whereas the reversing reaction involves the biotransformation of trans-cinnamic acid to L-phenylalanine (5, 18). The enzyme was later discovered in higher plants, algae, ferns, fungus, yeast, actinomycetes, a few cyanobacteria, bacteria, and even extremophiles but not found in animals (19, 36). The majority of identified PALs have natural molecular masses ranging from 300 to 340 kDa. Masses of 152 kDa in Ocimum basilicum (15), 226 kDa in the bacteria Streptomyces (7), 250 kDa in Helianthus annuus (20), 266 kDa in Fragaria ananassa (12), 320 kDa in Ustilago maydis (22), and 560 kDa in Alternaria (30) are instances of outliers. The Km values of PAL from various sources varied significantly (37) L-phenylalanine Km values have been reported to vary from 0.011mM (29) to 1.7mM (23). Despite these differences, the catalytic activity of the majority of PALs is highest in the alkaline zone, with an optimum pH range from 8.5 to 9.5 (4). PAL activity varies considerably according to developmental stage, plant type, and environmental circumstances (28). PAL is typically a tetrameric protein with four identical subunits, a feature shared by homotetrameric proteins and hetero-tetrameric PAL as a complex comprising two hetero-dimers has been described from *H. annuus* (2 58 kDa and 2 68 kDa) (12) and *Rhizoctonia solani* (2 70 kDa and 2 90 kDa) (21). Monomer pairs combine to produce a protomer with a single active site. (7) The pH optimum for PAL is typically between 8.2 and 9.0 (19, 30). In tobacco (29), sunflower (6), and Rhizoctonia (21), the temperature optimum for PAL has been reported to be 35°C, 55°C, and 44°- 46°C, respectively. Plant PAL enzymes are typically susceptible to repeated freezing and thawing, and their activity declines when the temperature approaches 60 degrees Celsius. Fungal PAL, on the other hand, is also more thermally stable (21). When maintained at 60^{o}C. PLA is an enzyme that regulates the production of polyphenol compounds from phenylalanine. PLA is present in a large number of higher plants and is now used in a wide range of industries and medical fields. High PAL activity is related to the buildup of phenolic compounds in fruit tissues of many species. After carbohydrates and fruit acids, phenols are the third most prevalent component in grapes. Grapes contain a wide range of phenolic compounds. The composition of phenolics in a single grape variety varies on whether the extraction is conducted on entire grape pulp, peel, or seeds (1, 2, 27). The total extractable phenolics in grape are only approximately 10% or less in the pulp, 60–70% in the seeds, and 28–35% in the skin. The phenol content of seeds can range between 5% and 8% by weight. The present study deals with the purification and characterization of PAL from locally cultivated grape seeds, which showed very high PAL activity in our preliminary examination (35).

MATERIALS AND METHODS

Purification of phenylalanine ammonia lyase: PAL was extracted and purified according to Hadeel and Khalida (16)

Enzyme assay

A 0.2 ml aliquot of the sample was added to 0.5 ml of reaction potassium phosphate buffer (100mM, pH 7) and 0.25 ml of substrate (40mM L- phenylalanine, 100mM potassium phosphate buffer, pH 7) and incubated at 37 °C for 30 min. A 50 µl aliquot of 4M HCl was added after incubation to terminate the reaction, and samples were centrifuged at 15490 ×g for 15 min. The absorbance at 290nm was used to determine the amount of cinnamic acid produced. One unit of PAL enzyme activity was defined as the amount of enzyme required to cause a 0.001 change in absorbance under experiment conditions, according to the method described by (13) with some modification.

Characterization of the purified enzyme

Determination of molecular weight by gel filtration: The molecular weight of PAL

purified from grape seeds powder was determined by gel filtration on Sephadex G-200 column (1.5 × 70 cm) equilibrated with (100mM, pH 7) potassium phosphate buffer, the column calibrated with protein standards (Collagen 270 kDa, Alcohol dehydrogenase 150 kDa, Uricase 125 kDa and Bovine serum albumin 66 kDa), then the molecular weight of grape seeds PAL was determined from the calibration curve.

Determination of PAL molecular weight by electrophoresis: To determine the molecular weight of PAL Native -polyacrylamide gel electrophoresis (Native -PAGE) gels containing acrylamide (4–10 percent) were employed. The stacking gel included 29 percent acrylamide and 8 percent N,N'-methylene-bis-acrylamide, 1.5MTris-HCl (pH=8.8), 10 percent ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), and the separating gel had 30 percent acrylamide-N,N'-methylene-bis-acrylamide, 0.5MTris-HCl (pH=6.8), 10 percent APS and TEMED, the sample was loaded to the gel using micropipette with gel loading. After electrophoresis was completed, the gel was stained with Coomassie Brilliant Blue (R-250). Protein ladder (10-180 KDa, Thermo Fisher Scientific) was used as a size standard, according to the method described by (25) with some modification.

Determination of the optimal pH for activity: PAL activity was investigated with a range of pH values (pH 3.0- 9.0). The following buffers were used: 0.01 M Citrate buffer for pH range (3.0-6.0), 0.01 M Phosphate buffer for pH range (6.5-8.0) and 0.01 M Tris-HCl buffer for pH range (8.5-9.0), then determined enzyme activity.

Determination of the optimal pH for stability: Purified grape seeds PAL was incubated for 45 min at 37°C, with pH ranging (3.0- 9.0) buffer solutions, immediately after incubation they were cooled. After assaying enzyme activity as described above, the remaining activity percent was calculated as a percentage of the maximum value of enzyme activity.

Determination of the optimal temperature for activity: PAL activity was tested after incubation of the reaction mixture (substrate and enzyme) under a range of temperatures (25, 30, 35, 40, 45, 50, 55, 60 and 65) °C. Then, the PAL activity was estimated for each temperature.

Determination of the optimal temperature for stability: Purified PAL was incubated for 1 hour at temperatures ranging from 25 to 80 °C, at the optimum pH for stability, then cooled and tested for enzyme activity as described above.

Determination of carbohydrate content

The carbohydrate content of the purified PAL was determined according to Dubois *et al.*, (8). To prepare the standard curve, test tubes were prepared and the following volumes of glucose standard solution (glucose 8 mg/100 ml water), 0.1, 0.3, 0.5, 0.7 and 1 ml were added to them and the volume was completed to 1 ml with distilled water (8.0-80.0 µg/ml glucose), then 1 ml of phenol (5%) was added and mixed well by a vortex mixer, then 5 ml of sulphric acid, (Analytical grade 98%) was added, the mixture was let to cool, then the absorbance was determined at 490 nm. The first tube (contains 1 ml of water) was used as blank, then a standard curve was plotted, the determinations were performed in duplicate. Following the procedures above, the carbohydrate content of the purified enzyme was determined, and the carbohydrate concentration was computed using the glucose standard curve.

Estimation of kinetics constants

Michaelis constant (K_m) and maximal velocity (V_{max}) were estimated by using different concentrations of phenylalanine (10, 20, 30, 40, 50, 60 and 70 mM) in 100mM potassium phosphate buffer, pH 7 at 40 °C for 45 min., KM and Vmax were determined by method Lineweaver- Burk plot (33). The turnover number (Kcat) of PAL was calculated on the basis of on active site per subunit

$$Kcat = \frac{V\,max}{Et}$$

Et: Enzyme concentration

RESULTS AND DISCUSSION

Characterization of the purified PAL

Molecular weight determination: The molecular weight of the purified PAL was estimated by two techniques: gel filtration and Native-PAGE electrophoresis. Native molecular mass of the purified PAL was determined by gel filtration using a column of Sephadex G-200 (1.5 × 70 cm.) calibrated in

100mM potassium phosphate buffer, pH 7 and then calibrated with gel filtration standard molecular weight: Collagen (Mw 270 kDa), Alcohol dehydrogenase (Mw 150 kDa), Uricase (Mw 125) and Bovine serum albumin (Mw 66 kDa), the column void volume was estimated with blue dextran (Mw 2000 kDa). V_e/V_o for the standard proteins were 2.5, 3.75, 4.5 and 5.25 respectively, then a standard curve was plotted according to the relationship between the ratios of (V_e/V_o) and logarithm of the molecular mass of each standard protein, native enzyme was found to be 185 kDa estimated by gel filtration (Fig. 1).

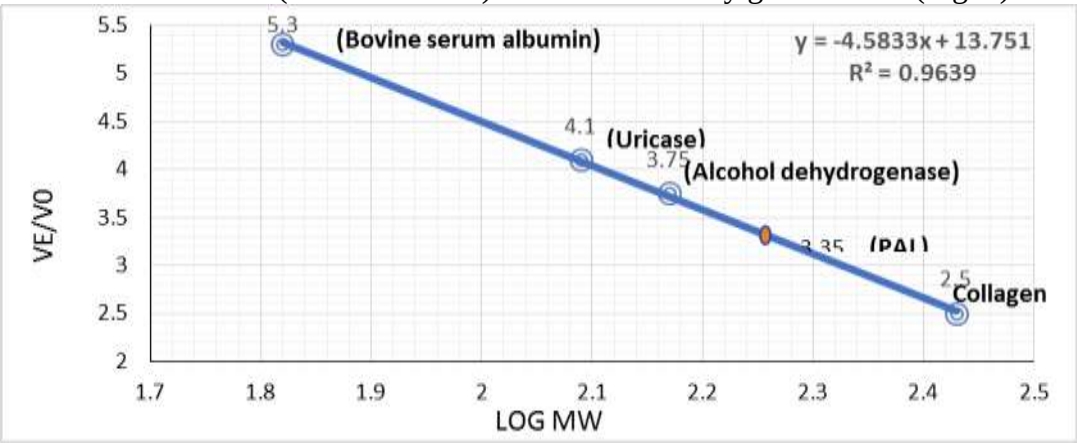

Figure 1. Native molecular mass of grape seeds PAL by gel filtration (Sephadex G-200)

The second method was used to quantify the molecular mass of pure PAL by electrophoresis since the molecular mass was estimated to be 180 kDa (Fig. 2) and the PAL preparation formed a single protein band on electrophoresis after staining.

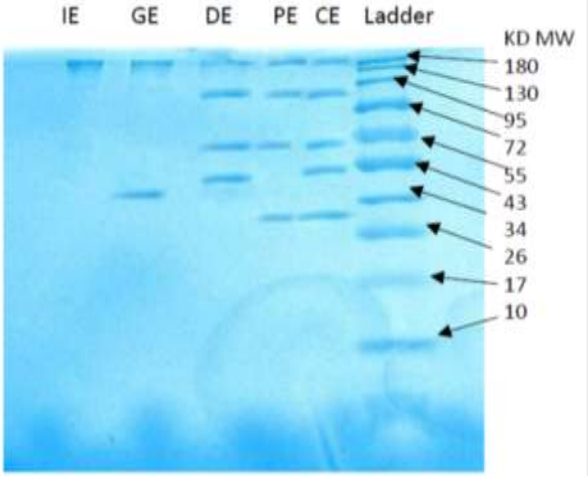

Figure 2. Electrophoresis of the purified PAL, CE, crude enzyme; PE, Precipitation with ammonium sulfate; DE, dialysis; GE, gel filtration; IE, ion exchange

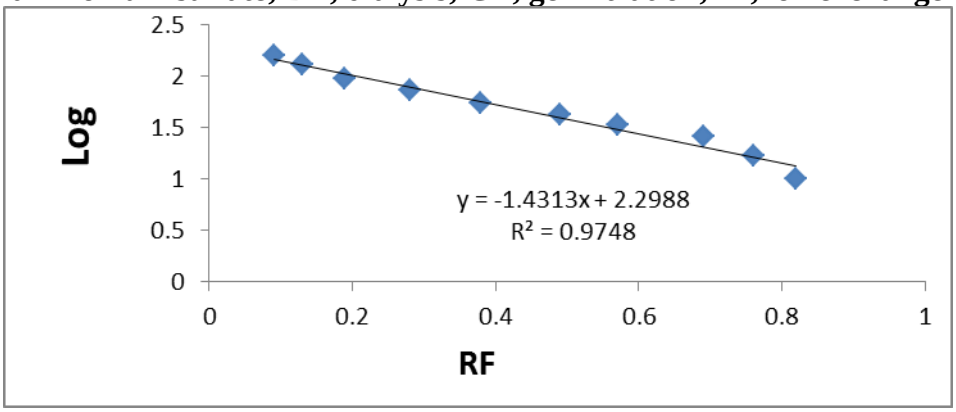

Figure 1. Native molecular mass of grape seeds PAL by electrophoresis

These findings are close to that Lim (26), who reported that the molecular weight (Mw) of leaf mustard PAL estimated with gel filtration 170 kDa and leaf mustard PAL 4 subunit Mw

estimated with SDS-PAGE was 40 kDa, Hao *et al*., (17) found that the molecular weight of PAL purified from aves of *Ocimum basilicum* by gel filtration was 153 KDa. The majority of identified PALs have natural molecular masses ranging from 300 to 340 kDa. Masses of 226 kDa in the bacteria Streptomyces (10), 250 kDa in Helianthus annuus (20), 266 kDa in Fragaria ananassa (22), 320 kDa in Ustilago maydis (22), and 560 kDa in Alternaria (30) are instances of outliers.

Optimal pH for activity

This experiment was carried out in order to identify the optimal pH for PAL activity. The optimal pH experiment was performed with a wide pH values range of 3.0- 9.0. The purified PAL from grape seeds powder had the highest activity at pH 7.0, with a value of 3.66 U/ml, and the lowest activity at extreme pH 3.0 and 9.0, with values of 0.33 and 0.55 U/ml, respectively (Fig. 4).

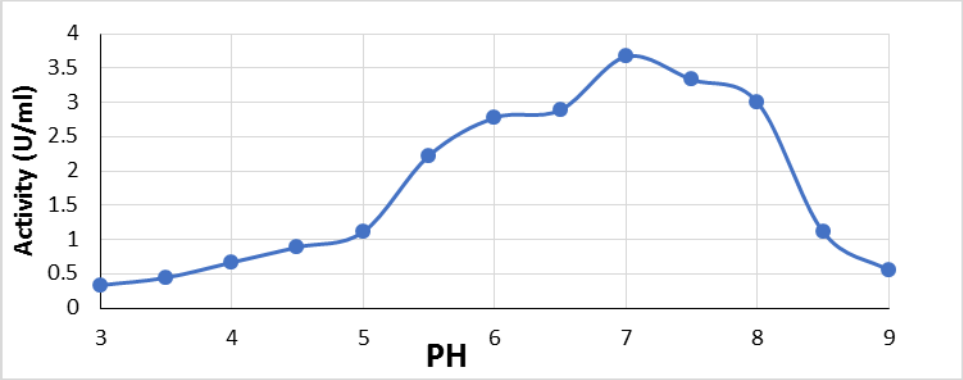

Figure 3. The optimal pH for the activity of the purified PAL from grape seeds

Goldson Barnaby *et al*., (15) found that optimum activity of *Cissus sicyoides* berries PAL was at pH 7.5. Rahmatabadi *et al*., (32) reported that optimal pH for activity of *Lotus japonicus* PAL was at 8.5. Our findings were lower than other related studies which confirmed that PAL activity has reached its maximal values at pH 8.8, (23, 30)

Optimal pH for stability

The impact of pH on PAL stability was determined experimentally by pre-incubating the enzyme at various pH levels ranging from 3.0 to 9.0. Results in Fig. (4) showed that the purified PAL from grape seeds powder was stable over a range of pH values from 6.0-8.0. The enzyme retained 90-100 of its activity at pH 7.5-8.0, while 81% of its activity was retained at pH 7.0. Purified PAL was not stable at extreme pH; it retained 18, 23, 39, 31 and 15% of its entire activity at pH 3.0, 3.5, 4.0, 8.5 and 9.0 respectively.

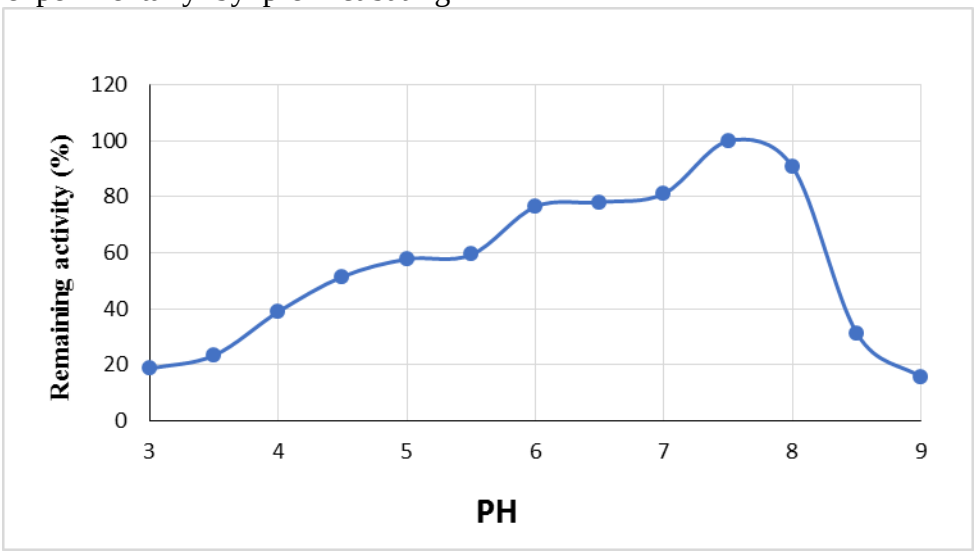

Figure 4. The optimal pH for the stability of the purified PAL from grape seeds

These observations agreed with Eissa and Ibrahim, (9) who reported that the purified PAL from banana fruit was stable over a range of pH values from 6.0 -8.0.

The optimal temperature for activity

This experiment was carried out to identify the optimal temperature for PAL activity. The PAL assay was performed at a range of temperatures (25, 30, 35, 40, 45, 50, 55, 60 and 65) °C. The purified PAL from grape seeds displayed optimum activity 6.22 unit/ml at 40 °C. Adversely, enzymatic activity was decreased over and below this temperature, while enzymatic activity was totally lost at temperatures over 80 °C (Fig. 5).

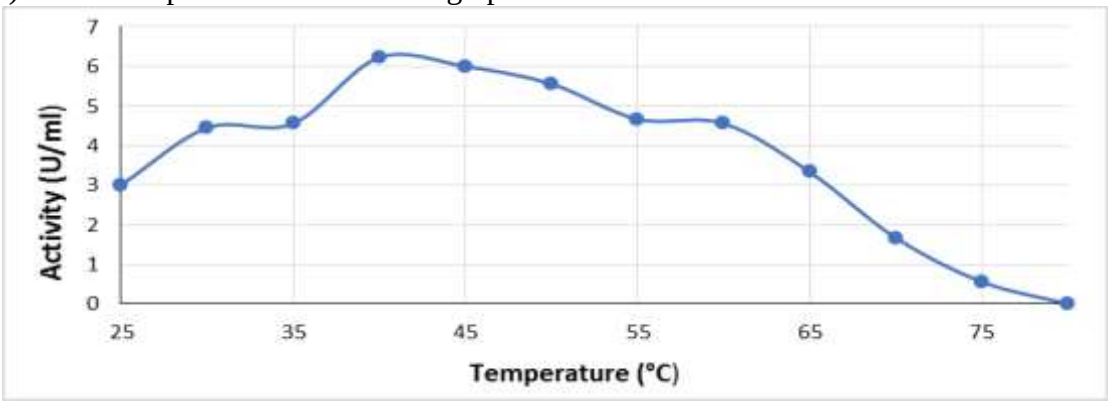

Figure 5. The optimal temperature for activity of the purified PAL from grape seed

Similar findings were reported by other related studies. Eissa and Ibrahim, (9) observed that optimum temperature for the activity of banana fruit PAL was at 30 °C. Rahmatabadi *et al*., (32) reported that optimal temperature for activity of *Lotus japonicus* PAL was at 50°C. Babaoglu Aydas *et al*., (3) demonstrated that optimum temperature for activity of PAL produced from *Centaurea depressa* was at 37 °C. While Goldson-Barnaby and Scaman,(13) found that *Trichosporon cutaneum* PAL had an optimum temperature for its activity at 32°C.

Optimal temperature for stability

Thermal stability is an important element in determining the temperature at which an enzyme keeps its efficiency and activities while avoiding those that negatively impact it, and it is an important approach for selecting acceptable temperatures for industrial enzyme applications (37). Results in Fig. (6) show that purified PAL retained 79% of its original activity after incubation for one hour on 50 °C, while 7% of its activity was retained at 70 °C. Enzyme total activity was lost at temperatures over 75 °C.

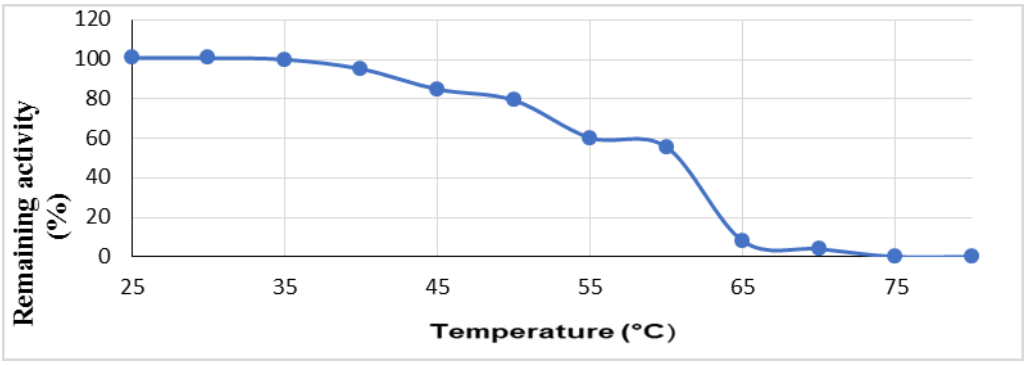

Figure 6. The optimal temperature for stability of the purified PAL from grape seed

Eissa and Ibrahim, (9) found that PAL from banana fruit had the highest activity at 20 °C to 40 °C, thereafter, PAL activity decreased with increasing temperature, lowest activity was recorded at 60 °C and there was no activity above this temperature.

Carbohydrate content

CHO content of purified PAL was 24% as determined according to the method of Dubois *et al*., (1956).

Estimation of kinetics constants

For kinetic studies, the initial velocity of the sample was measured at various concentrations of the substrate L-phenylalanine ranging between (10, 20, 30, 40, 50, 60 and 70 mM). From Lineweaver-Burk plot, the Vmax for the enzyme was 4.13 µM /min, while the apparent value of affinity constant (Km) was 21.8 µM (Fig. 7). The turnover number (Kcat) of the enzyme was calculated to be 20.67 Sec^{-1}.

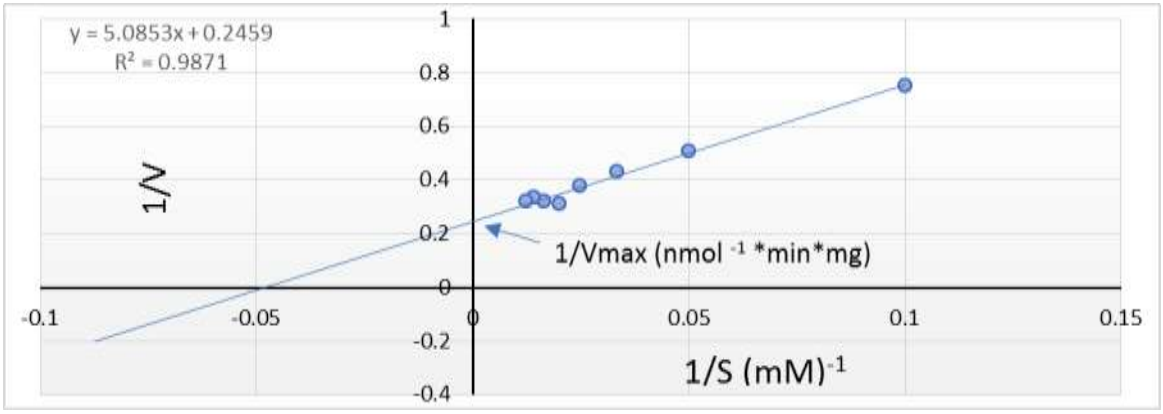

Figure 7. Lineweaver-Burk plots of PAL from grape seeds

These results agreed with other studies, which reported that Km values of the *Populus trichocarpa* PAL: 21.3–32.6 µM and Km for *Arabidopsis thaliana* PAL: 64–2560 µM (6), *S. viminalis* PAL: 32.3–88.5 µM, *N. tabacum* PAL: 36.4–59.8 µM (30), and Vmax: *S. viminalis* PAL: 7.3–27.2 mM/min, *N. tabacum* PAL: 9.8–19.6 mM/min (30), *Arabidopsis thaliana* PAL: 0.4–10.5 nkat/mg (6). The turnover number (kcat) for *S. viminalis* PAL (2.3– 29.0 Sec^{-1}), on the other hand, is an order of magnitude higher than those reported for most other PALs (*N. tabacum* PAL: 0.8–1.5 Sec^{-1} (30), *Populus trichocarpa* PAL: 1.1–1.4 Sec^{-1} (33), *Arabidopsis thaliana* PAL 0.1– 3.2 Sec^{-1} (4), with the exception of maize (10.6 Sec^{-1}) (27).

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