# OPTIMUM CONDITIONS FOR PHENYLALANINE AMMONIA LYASE EXTRACTION AND PURIFICATION FROM LOCALLY CULTIVATED GRAPE SEEDS

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**ABSTRACT**

**In the present study, Phenylalanine ammonia lyase (PAL) activity was detected in different parts of grape plant (leaves, pulp, whole seeds and defatted seeds) extracts. The results revealed that the whole seed possess highest PAL activity (69.44 U), followed by defatted seed (44.22 U), whereas extracts of leaves and pulp gave the lowest activity values (22.77 and 5.11 U, respectively). The optimum conditions for crude PAL extraction achieved using 50mM potassium phosphate buffer (pH 7) at mixing ratios of (1:7) for 1 h. extraction time. PAL was shown to precipitate out at the saturation range between 20-90%. For further purification, gel filtration chromatography using Sephadex G-200 column (1.5×70) and ion exchange chromatography using DEAE- Cellulose (DE 52) column (3.2× 10) were applied. The specific activity, number of folds and yield of purified PAL was 1157 U/mg, 19.94 and 15.32 % respectively.**

**Key words: Phenylalanine ammonia lyase, grape seeds, optimization.**
***Part of Ph.D. dissertation of the 1st author.**



## تحديد الظروف المثلى لاستخلاص وتنقية انزيم الفنيل الانين امونيا لايز من بذور العنب المزروعة محلياً

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**المستخلص**

**في هذه الدراسة، تم الكشف عن نشاط فينيل ألانين أمونيا لايز (PAL) في مستخلصات أجزاء مختلفة من نبات العنب (الأوراق واللب والبذور بالزيت والبذور بدون زيت). أظهرت النتائج أن اعلى فعالية للانزيم كانت في البذور الكاملة (69.44 وحدة) ، تليها البذور المزال منها الزيت (44.22 وحدة) ، بينما كان لمستخلصات الأوراق واللب نشاط اقل وبواقع (22.77 وحدة ، 5.11 وحدة على التوالي). كانت الظروف المثلى لاستخلاص PAL الخام من بذور العنب بأستعمال محلول فوسفات البوتاسيوم 50 ملي مولار ذي اس هيدروجيني مقداره 7 بنسب خلط (1: 7) وفترة استخلاص 1 ساعة. تم ترسيب انزيم PAL في نطاق التشبع بين 20–90٪. ولغرض تنقية اكثر لـPAL تم تطبيق، كروماتوكرافي الترشيح الهلامي باستخدام عمود Sephadex G–200 (1.5 × 70) والتبادل الأيوني باستخدام عمود DEAE– Cellulose (DE 52) (3.2× 10) لتصبح الفعالية النوعية للانزيم وعدد مرات تنقية الانزيم والحصيلة الانزيمية 1157 وحدة / مغم ، 19.94 و 15.32% على التوالي.**

**الكلمات الافتتاحية: فنيل الانين امونيا لايز, بذور العنب ,تنقية انزيم.**

**البحث جزء من أطروحة الدكتوراه للباحث الاول**

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## INTRODUCTION

Grape (*Vitis vinifera L.*) is an important fruit crop worldwide, with numerous varieties ranging in features like as flavor and color, with or without seeds, which are related with the amount of phenolic compounds and therefore with antioxidant capability (28, 33). Approximately half of all grapes are used to make wine, one-third as fresh fruit, and the remainder are refined into foods such as jam, juice, grape seed extract, jelly, grape seed oil, dried grapes (raisins), and vinegar (3, 29). Grapes are abundant in carbs (17 g/100 g), have a high caloric content (65 kcal/100 g), and have a low glycemic index (GI). Grapes are an excellent source of manganese (Mg) and potassium(K), as well as vitamins B6, C, thiamine, and are among the greatest sources of phenolic compounds. When compared to other fruits and vegetables, grapes are one of the most abundant sources of phenolic compounds (37). Grape seeds which are grape by-product are a rich source of biologically active compounds (27, 38). Tang and colleagues (37) discovered that the total flavonoid concentration of grape seeds is up to 10 times that of grape peel, highlighting the significance of this grape by-product. Anthocyanins, flavan-3-ols, flavonols, stilbenes, and phenolic acids are the most important grape seed phenolic constituents (27, 36). The total phenolic component concentration of grape seeds ranges from 55 to 964 mg per 100 g, with an average of 380 mg per 100 g dry mass (35). The aromatic amino acid phenylalanine was used to create phenolic compounds, which were obtained from flavonoid compounds. The most significant enzyme in regulating the production of phenolic compounds from phenylalanine is phenylalanine ammonia-lyase (PAL), which is produced de novo in various plant tissues by UV radiation and mechanical injury (11). Phenylalanine ammonia lyase (PAL) belongs to the lyase family and has the EC number4.3.1.5 (35). Phenylalanine ammonia-lyase (PAL) is the first enzyme in the phenylpropanoid pathway, converting phenylalanine to cinnamic acid, which is a precursor to a range of secondary metabolites found in higher plants and certain other organisms and is mostly engaged in defensive processes (17). PAL is presently being tested in humans as an enzyme replacement therapy for the treatment of phenylketonuria (PKU), a hereditary metabolic disorder caused by the loss or impairment of phenylalanine hydoxilase, an enzyme that metabolizes L-phe (24). Since there is no local or regional studies about grape seeds PAL, present study was aimed to investigate the optimum conditions for extraction and purification of phenylalanine ammonia lyase from locally cultivated grape seeds

## MATERIALS AND METHODS

**Sample collection:** Locally cultivated grape (*Vitis vinifera L.*) was purchased from local market in Baghdad- Iraq. The grapes were peeled and separated into skins and seeds, the seeds were washed and dried at 40± 5 °C for 6 hour.

**Extraction of PAL**

Phenylalanine ammonia lyase extractions and activity assay were performed similarly to as described by Goldson *et al.* (12) with some modifications. Grapes (leaves, pulp, seeds with oil and seeds without oil) were ground into a fine particle and were homogenized in extraction Tris- HCL buffer (50mM, pH 8,8) containing 1mM EDTA, 10mM 2β-mercaptoethanol, and 2.5%(w/v) polyvinylpyrrolidone-40 (PVP-40) in 1:4 ratio (sample weight: buffer). After 1 hour, samples were centrifuged at 21180 ×g for 20 min at 4°C. Supernatant was used as the crude enzyme.

**PAL activity assay**

A 0.2 ml aliquot of the sample was added to 0.5 ml of reaction Tris-HCl buffer (100mM, pH 8.8) and 0.25 ml of substrate (40mM L-phenylalanine, 100mM Tris-HCl, pH, 8.8) and incubated at 37 °C for 30 min. A 50 µl aliquot of 4M HCl was added after incubation to terminate the reaction, and sample 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.

**Testing different extraction solutions:** Different extraction solutions were tested for extracting phenylalanine ammonia lyase from

grape seeds. 50 mM Tris- HCl (pH 8.8), 50mM sodium borate buffer (pH 8.8), 50mM potassium phosphate buffer (pH 7), sodium phosphate buffer (pH 7), phosphate buffer $Na_2HPO_4$/ $KH_2PO_4$ (pH 7) and water (pH 6.5)were tested as extraction solutions. Subsequently, the PAL activity was determined as mentioned above. The extraction solution with the high PAL activity was chosen for further experiment in this study.

**Testing different extraction time:** For extracting phenylalanine ammonia lyase from grape seeds, different extraction times were evaluated (1,6, and 12 hours). As a result, the PAL activity was calculated as previously stated, then extraction time for highest PAL activity was adopted in the next experiment.

**Testing different mixing ratios:** Several mixing ratios (1:3), (1:4), (1:5), (1:6), (1:7), and (1:8) (w/v) were tested for extraction of phenylalanine ammonia lyase from grape seeds. The mixing ratios, which gave the highest PAL activity was selected for the following experiment.

**Purification of phenylalanine ammonia lyase**

**Crude enzyme extraction:** Extraction of Phenylalanine ammonia lyase from grape seeds was conducted according to Goldson *et al.* (12) with some modifications. Grapes seeds were ground into a fine powder and were homogenized in extraction solution (potassium phosphate buffer (50mM, pH 7) containing 1mM EDTA, 10mM 2β-mercaptoethanol, and 2.5%(w/v) polyvinylpyrrolidone-40 (PVP-40) in 1:7 ratio) (sample weight: buffer). After 1 hour, samples were centrifuged at 21180 ×g for 20 min at 4°C. The supernatant was used as the crude extract.

**Precipitation with ammonium sulfate**

Precipitate extract ammonium sulfate saturation ratio (20, 30, 40, 50, 60, 70, 80 and 90%) were set up to precipitate the fractions with enzyme activity, considering increasing the salt saturation level from 20% to higher saturation ratio. Solid ammonium sulfate was added to the supernatant with continues stirring for 1 hours in ice bath, then incubation for 3 hours. All samples centrifuged (at 9390 ×g for 20 min) and pellet resuspended in a small volume of extraction buffer, then tested for PAL activity.

**Dialysis of the enzyme**

The ammonium sulfate suspension was put into a dialysis tub that had been previously wetted and placed in a beaker of 50mM potassium phosphate buffer (pH 7). The ammonium sulfate suspension was dialyzed for 24 hours against three changes of the same dialysis buffer. The whole process was accomplished in a cold condition at 4°C.

**Gel filtration chromatography:** Sephadex G-200 was used to prepare the gel filtration column (1.5 × 70 cm). Sephadex G-200 (4 g) is suspended in 100 mL of distilled water and it was submerged into water bath at 85- 95 °C with stirring for 3 hours, and then cooled until reached room temperature. Decant the supernatant once the swelling is complete. Add 100 mM potassium phosphate buffer (pH 7) to make a 75% suspension. The slurry degassed and stirred with a glass rod to make a homogeneous suspension free from aggregates then packed into column (1.5 × 70). The column was equilibrated using the same buffer at a flow rate of 5 ml/ 15 min. after column preparation, a certain amount of the enzyme solution was added gently to column surface and eluted using 100 mM potassium phosphate buffer (pH 7) with a flow rate 5 ml/20 min. optical density (at 280nm), and enzyme activity were determined for each fraction. Fractions with PAL activity were pooled and kept at 4°C for the next purification step (16).

**Ion exchange chromatography**

**Preparation of medium for use:** DEAE-Cellulose (DE 52), column (3.2 × 10) was prepared according to Peterson and Sober (1, 30), where 10 gm of dry powder was suspended in 1 N NaOH ~ (thus avoiding the occlusion of air); the suspension is then filtered six times and washed with 1 N NaOH until no more color is lost. After that, enough 1 N HCI is added to create a highly acid suspension, which is filtered and rinsed free of acid with water very immediately. The filter cake is suspended in 1 N NaOH again, filtered, and washed with water to remove alkali before being adjusted to the pH (7) of the 100 mM potassium phosphate buffer (pH 7) (starting buffer). The slurry was poured down the column in one continuous motion while holding a glass rod against the column's wall. The column was then immediately filled with

starting buffer and equilibrated at a flow rate of 5 ml/ 7 min. After equilibration of the column, concentrated enzyme was gently poured over the surface of the DEAE-Cellulose beads. To allow unbinding protein to flow through, the column was rinsed with 100 mM potassium phosphate buffer (pH 7). Elution is accomplished by employing a constantly rising salt gradient to liberate the other protein attached to the column; NaCl (0.1- 0.5M) dissolved in 100 mM potassium phosphate buffer (pH 7) was employed for this purpose. The optical density (at 280nm) and enzyme activity of each fraction were measured. Fraction represent PAL activity were pooled and kept at 4°C for following studies.

**Native- PAGD of PAL:** Native - polyacrylamide gel electrophoresis (Native -PAGE) preformed according Laemmli system (23) with some modification, Native -PAGE gels containing acrylamide (4–10) % were used and stained with Coomassie Brilliant Blue (R-250) to visualize the phenylalanine ammonia lyase protein. The stacking gel 4 % consisted of: 29.2 % acrylamide and 8 % N ,N'-methylene-bis-acryl amide ,Tris-HCl (1.5 M , pH=8.8), ammonium persulfate (APS) 10 %, N,N,N',N'-tetramethylethylenediamine (TEMED ) and the separating gel 10 % consisted of: (30 %) acrylamide-N,N'-methylene-bis-acrylamide, Tris-HCl (0.5 M , pH=6.8), 10 % APS and TEMED, the sample was loaded to the gel using pipet with gel loading.

**Statistical analysis:** The collected data were statistically analyzed using analysis of variance (ANOVA). Differences among treatment means were compared using Least Significant Difference (LSD) ≤ 0.05 probability level.

## RESULTS AND DISCUSSION

Phenylalanine ammonia lyase activity in different parts of grape (*Vitis vinifera L.*) plant illustrated in Table 1. It has been noticed that there were significant differences in PAL activity among the tested parts, however the grape seeds extract appeared to be the most promising source since it gave the highest units for PAL activity. The statistical analysis indicated significant different between all parts under study.

**Table 1. PAL activity in the extract of plant parts of grape (*Vitis vinifera L.*)**

| Source | PAL activity (U/ml) |
|---|---|
| Grape (seeds with oil) | 69.27 A |
| Grape (seeds without oil) | 43.83 B |
| Grape(leaves) | 22.77 C |
| Grape(pulp) | 4.88 D |

**The results represent an average of two replicates, the different letters indicate a significant difference ( P< 0.05)**

Other finding mentioned that a variety of plant species, fungi, and bacteria have been used to isolate and describe PAL, such as Seedlings (22), branches (7), leaf-sheath (10), cell culture (8), fruit (2), mycelium (15, 34), and prokaryotic cells (13). PAL was isolated, purified, and characterized from barley seedlings (Hordeum vulgare L. var. Sravat) by Koukol and conn (21).

**Testing different extraction solutions**

In order to identify the optimum solution for PAL extraction from grape seed, several solutions were utilized. These solutions were: 50mM potassium phosphate buffer (pH 7), 50 nm phosphate buffer $Na_2$ $HPO_4$/ $KH_2PO_4$ (pH 7), water (pH 6.9), sodium phosphate buffer (pH 7), 50mM sodium borate buffer (pH 8.8), and 50 mM Tris- HCl (pH 8.8). The statistical analysis revealed that there were a significant different between 50mM potassium phosphate buffer (pH 7) and the rest buffer, since it gave the highest value for enzyme activity (21 U/ml). The value of enzyme activity for other extract solutions were 17.44, 15.33, 13.44, 12.66 U/ml respectively.

**Table 2. Effects of extraction solution on PAL activity**

| Extraction Solution | PAL activity (U/ml) |
|---|---|
| Phosphate buffer ($K_2$ $Hpo_4$/$KH_2po_4$) (pH 7) | 20.00 A |
| Phosphate buffer ($Na_2$ $Hpo_4$/$KH_2po_4$) (pH 7) | 17.44 AB |
| Water (pH 6.9) | 14.88 BC |
| Phosphate buffer ($Na_2$ $Hpo_4$/$NaH_2po_4$) (pH 7) | 13.33 CD |
| Borate buffer (8.8) | 12.60 CD |
| Tris- HCl (pH 8.8) | 12 D |

**The results represent an average of two replicates, the different letters indicate a significant difference (P< 0.05)**

Arafa *et al.* (4) found that the best solution for PAL extraction from the algal cells was Tris-HCl buffer (100 mM and pH 8.9), and addition (0.2%) Na2-EDTA and (125 µl) β-mercaptoethanol, this solution gives the high enzyme activity, and Goldson Barnaby *et al.* (13) using Tris-HCl buffer (50 mM and pH 8.5) for extraction PAL enzyme from *Cissus sicyoides* berries. Babaoglu Aydas *et al.* (5) effectively extracted PAL from cyanobacteria using 50 mM potassium phosphate (pH 7), with 2% (w/v) polyvinylpyrrolidone (PVP), Triton X- 100 % 0,1 (v/v), and EDTA (1 mM). While [23] demonstrated that using of 50 mM phosphate buffer (Na2HPO4/KH2PO4) pH 7.0 containing (PVP (5%), Na ascorbate (50 mM), mercaptoethanol (18 mM and Triton X-100 0.1%) for extracting PAL from The skin of apples. From the leave of pepper (*Capsicum annuum*), PAL has been extracted by sodium borate buffer (0.1 M, pH 8.8) with (mercaptoethanol 5 mM and tetrapyrrolidone 5 % (m/v) (29). However, Kim and Hwang, (19) applied 100 mM phosphate buffer pH 6.0 consist of 2 mM EDTA, dithiothreitol (4 mM), and polyvinylpyrrolidone (2% (w/w)) for extracting of PAL from pepper and *Arabidopsis* leaves.

**Testing different extraction time**

In order to identify the optimal extraction time for PAL from grapes, different extraction time were tried in this study. These extraction times were 1, 6, and 12 hours. The results revealed that the optimal extraction time was 1hour which gives the highest enzyme activity 119.66 U, while extraction time (6 and 12 hours) give 59.66 and 24.55 U respectively (Table 3)

**Table 3. Optimum period for extraction of PAL from grape seeds powder**

| Extraction time | PAL activity (U) |
|---|---|
| 1 hour | 119.71 A |
| 6 hours | 59.60 B |
| 12 hours | 24.49 C |

**The results represent an average of two replicates, the different letters indicate a significant difference ( P< 0.05)**

Gonzalez-Mendoza *et al.* (14) stated that the best extraction time for PAL from leave tissue of *Prosopis glandulosa*, was 96 hours. Goldson Barnaby (13) depended 1 hour as extraction time for getting PAL from *Cissus sicyoides* berries.

**Optimum mixing ratios**

Different mixing ratios were applied to evaluate their impact on the activity of the enzyme in order to find optimal mixing ratios for extracting PAL from grape seeds powder. Different mixing ratio 1:3, 1:4, 1:5, 1:6, 1:7, and 1:8 (w/v) were used. It has been noticed that the mixing ratios (1:7 w/v) was the most efficient one, since it gave the highest value for enzyme activity (119.66 U/ml), whereas the rest extraction ratios gave 14.22, 24.88, 50.44, 71.44 and 63.11 U, respectively (Table 4).

**Table 4. Effect of extraction ratios on PAL activity**

| Extraction ratios (w/v) | PAL activity (U) |
|---|---|
| (1:3) | 14.05 F |
| (1:4) | 24.77 E |
| (1:5) | 50.44 D |
| (1:6) | 71.44 B |
| (1:7) | 119.21 A |
| (1:8) | 62.99 C |

**The results represent an average of two replicates, the different letters indicate a significant difference ( P< 0.05)**

The mixing ratio for exteacted PAL from *Cissus sicyoides* berries was (1:2) w/v (3). In another study, the mixing ratio (1:2.5) w/v used for extraction of PAL from leave tissue of *Prosopis glandulosa* (14) In this regard, Campos *et al.* (6) found that the 1:4 was the optimum mixing ratio for PAL extraction from Romaine lettuce leaves. Adversely, Babaoglu Aydas *et al*, (5) obtained high enzyme activity by 1:10 (w/v) mixing ratio for PAL extraction from cyanobacteria. Whilst, Kosyk *er al.* (20) showed that the highest enzyme activity of lettuce fresh leaves PAL was reached using mixing ratio 1:15.

**Purification of phenylalanine ammonia lyase**

**Precipitation with ammonium sulfate**

Ammonium sulfate precipitation was used as the first step in PAL purification from crude extract. Figure 1 shows that phenylalanine ammonia lyase precipitates between 20% - 90% ammonium sulfate saturation. It has been seen a gradual increase in PAL activity in pallet as the saturation (%) increased up to 60%, this was coincides with gradual decrease

in PAL activity for the supernatant. The partially purified enzyme showed higher enzymatic activity 230.25 U/ml with 60% as ammonium sulfate saturation percentage.

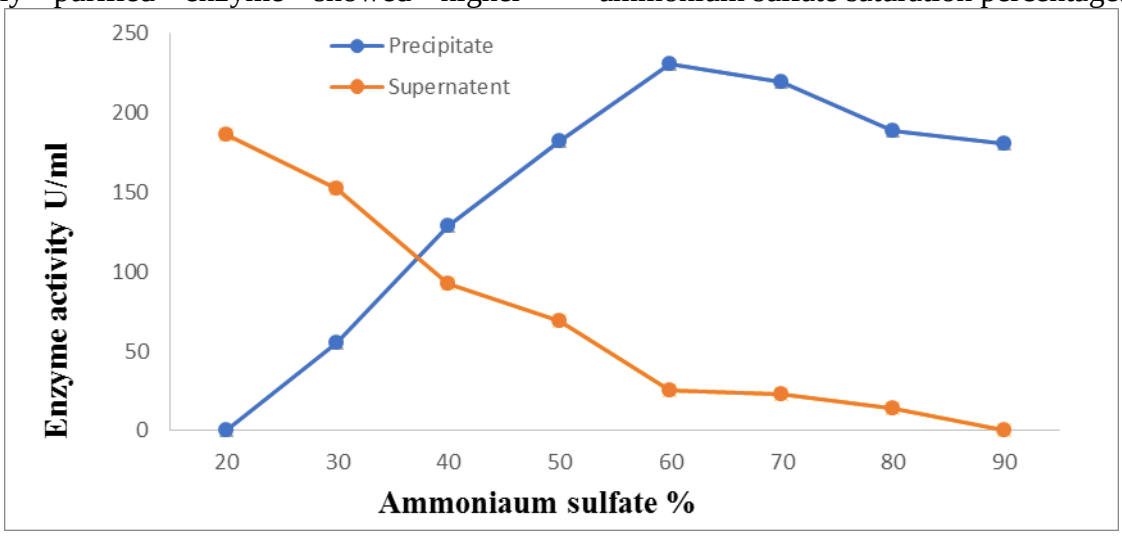


**Figure 1. Ammonium sulfate salt precipitation of PAL**

Ammonium sulfate precipitation of PAL was mentioned by Rao *et al.* (32) who added solid ammonium sulfate (60% saturation) to the crude extract of the cells of Ustijago hordei. In the same context, Lister *et al.* (26) reported that the PAL can be isolated at 80% saturation fraction by fractional precipitation with ammonium sulfate. Another study demonstrated that PAL precipitated out between 40-70% of ammonium sulfate saturation (39).

**Gel filtration chromatography**

Following partial purification of the enzyme using ammonium sulfate and dialysis, the PAL pellet was chromatographed on a Sephadex G-200 (1.5 × 70 cm dimensions) column that was equilibrated with 100 mM potassium phosphate buffer (pH 7) and eluted with the same buffer. The PAL activity test was performed on the A 280 nm peak fraction of Sephadex G-200, then the PAL activity containing fractions were pooled. Figure 2. shows the two protein peaks. After the elution, just one peak represented by tube (13-23) showed PAL activity, these fractions pooled it concentrated using polyethylene glycol. The concentrated fraction was tested for protein concentration and PAL activity. As shown in Table 5 the specific activity and the enzyme yield for this step were 830.5 U/mg and 24.98 % respectively (Figure 2)

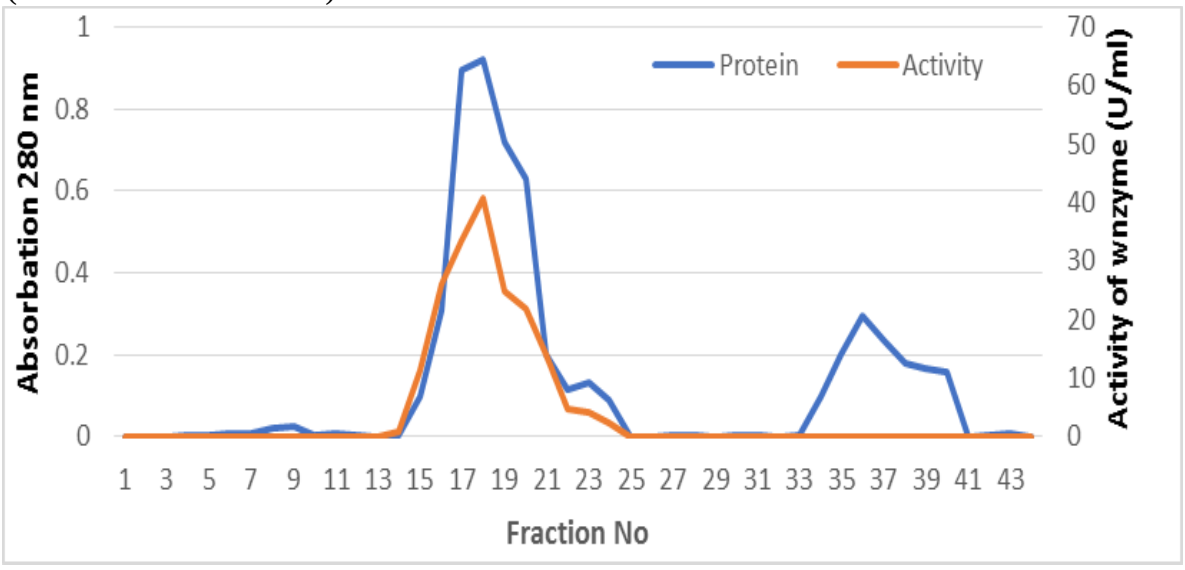


**Figure 2. Gel filtration chromatography using Sephadex G-200 (1.5 × 70 cm) equilibrated by 100 mM potassium phosphate buffer (pH 7) at a flow rate of 5 ml/ 15 min**

Studies revealed that the gel-filtration chromatography is a type of partition chromatography that may be used to separate and purification molecules (enzyme) of

various molecular sizes (29). Lim *et al.* (25) illustrated the purification of PAL produced by leaf mustard using precipitation with ammonium sulfate 50%, dialysis and Sephadex G-200 gel filtration which that PAL activity was eluted in a single peak.

**Ion exchange chromatography**

Ion exchange chromatography was used for further purification using a DEAE- Cellulose (DE 52) column that had previously been equilibrated with 100 mM potassium phosphate buffer (pH 7). Elution was done in a gradient fashion with linear increasing molarity of sodium chloride (NaCl) 0.1- 0.5 M in the 100 mM potassium phosphate buffer (pH 7). The results in figure 7 indicated that the PAL activity was eluted in a single peak after raising the ionic strength of the NaCl up to 0.3M. Activity- containing fractions were pooled and dialyzed against 100 mM potassium phosphate buffer (pH 7). After purification by DEAE- Cellulose Chromatography, the specific activity of PAL was 1157.8 U/mg and the yield was 15.3 %. (Figure 3)

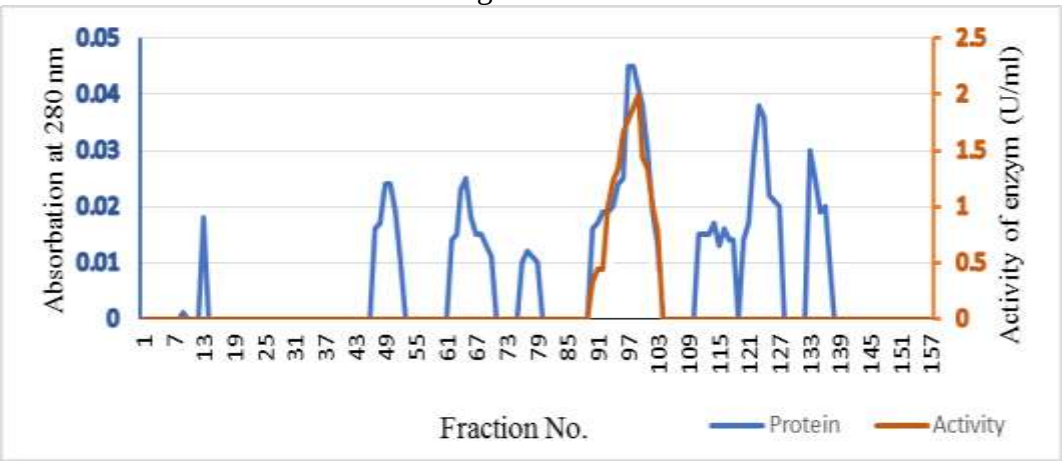


**Figure 3. Ion exchange chromatography using DEAE- Cellulose, equilibrated using 100 mM potassium phosphate buffer (pH 7) at a flow rate of 5 ml/ 6 min**

Ion exchangers can interact with proteins and other biomacromolecules because they expose charged moieties on the surface. Anion exchangers are positively charged. Above its PI, the protein is negatively charged and binds to an anion exchange (18). Rao *et al.* (32) who stated that the purified PAL from Ustijago *hordei* was eluted with phosphate buffer (150 ml, 0.01 M and pH 7) containing 0.1 M NaCl. After a series of steps that included ammonium sulfate fractionation, gel filtration chromatography on Sephadex G-200, and ion exchange chromatography on DEAE-Cellulose, a 19.9-fold purification of PAL from locally produced grape seeds (*Vitis vinifera L.*) was achieved. With each phase of purification, the enzyme's specific activity rose, giving a final yield 15.32 % (Table 5). Because the specific activity was enhanced and a good quantity of activity was preserved, the fold purification and enzyme recovery indicated that the purification process is effective. These effective purification findings might be attributed to the enzyme's low critical temperature (4°C) and steady pH (7) throughout purification operations

**Table 5. Purification steps of PAL from grape seeds**

| Purification step | Enzymatic activity (U/ml) | Volume ml | Protein conc. (mg/ml) | Specific activity (U/mg) | Total activity U | No. of folds | Yield % |
|---|---|---|---|---|---|---|---|
| Crude extract | 30.77 | 700 | 0.53 | 58.05 | 21539 | 1 | 100 |
| Ammonium Sulfate 60% | 254.16 | 60 | 1.34 | 189.67 | 15249.6 | 3.26 | 70.79 |
| Dialysis | 266.7 | 57 | 1.12 | 238.12 | 15201.9 | 4.10 | 70.57 |
| Concentration | 519.8 | 29 | 1.43 | 363.49 | 15074.2 | 6.26 | 69.98 |
| Gel filtration | 102.2 | 55 | 0.17 | 601.17 | 5621 | 10.35 | 26.09 |
| Gel filtration(con.) | 298.98 | 18 | 0.36 | 830.5 | 5381.64 | 14.30 | 24.98 |
| Ion exchange | 110 | 30 | 0.095 | 1157.8 | 3300 | 19.94 | 15.32 |

**Native- PAGD of PAL**

In order to determine the efficiency of the purification procedures, it is critical to test the enzyme purity. It must occur prior to enzyme characterizations since the presence of other components frequently leads to incorrect results. Figure 4. represent the electrophoresis patterns of PAL through the purification steps by visual inspection of native- PAGE gels. The results show the appearance of several protein bands in the crude extract on PAGD was clearly visible, while for gel filtration were two bands and only a single band on a polyacrylamide gel electrophoresis for ion exchange. This means the adequacy of the purification steps.

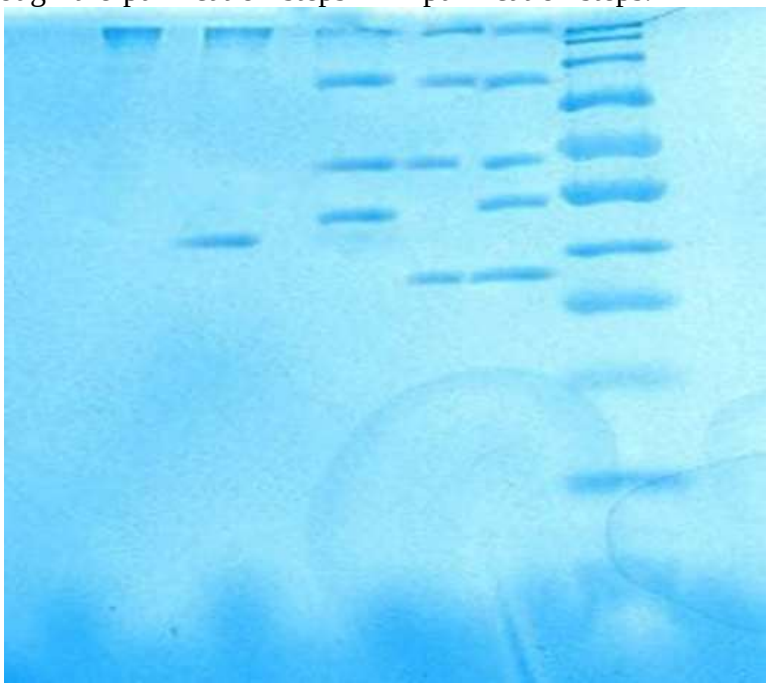

**Figure 4. Electrophoresis of the purified PAL, CE, crude enzyme; PE, Precipitation with ammonium sulfate; DE, dialysis; GE, gel filtration; IE, ion exchange**

Several purifying methods were used to purify phenylalanine ammonia lyase from loblolly pine. The SDS-PAGD of the enzyme preparation from the purification phases revealed that the protein bands appeared to diminish concurrently as the purification process progressed to the final purification steps (39).

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