CHARACTERIZATION OF L-ASPARAGINASE PURIFIED FROM POLE BEANS

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Abstract

The Plant samples of *P. vulgaris* were collected from a glass-house at Michigan State University (USA) and purified to homoginity by several purification steps. The purified enzyme was found to be a homodimer, with a molecular mass of 79.5 ± 2 KDal as estimated by size exclusion chromatography and SDS-PAGE. The optimum pH for enzyme activity and stability were pH 8.5 and 8 respectively, while the optimum temperature for enzyme activity and stability were 37°C and 30°C respectively. The K_m, V_{max} and k_{cat} values for the enzyme were 0.294 mM, 1.09 mM/min and 893.8 Sec^{-1} respectively. This study concluded that beans L-Asparaginase appeared to consist of homodimer subunits with a molecular weight of 40.6 KDal, it is active and stable at basic conditions while it does not endure above 60 C° and loses its activity and stability. It is recommended to studying the amino acid sequence of the L-Asparaginase extracted from beans and alignment with other L-Asparaginases using a protein database for molecular comparison and studying the L-Asparaginase application.

Keywords: L-Asparaginase, Pole beans, Homodimer, characterization, Phaseolus vulgaris L.

***Part of Ph.D. Dissertation of the first author.**

توصيف انزيم الاسباراجينيز المنقى من نبات الفاصوليا

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المستخلص

جمعت عينات الفاصوليا من بيت زجاجي في جامعة ميشيغان الامريكية ونقي الانزيم منها الى حد التجانس باستخدام عدد من خطوات التنقية. وصف انزيم الاسباراجينيز المستخلص من الثمار ووجد بانه يتكون من وحدتي انزيم متماثلة وبوزن جزيئي 79.5±2 كيلودالتون وتم ذلك باستخدام عمليتي الترشيح الهلامي والترحيل الكهربائي . وجد ان افضل اس هيدروجيني لعمل وثبات الانزيم هو 8.5 و 8 على التوالي اما الحرارة المثلى لعمله وثباته فهي 37 و 30 درجة مئوية على التوالي . بينت نتائج تقدير حركيات الانزيم ان قيم كل من ثابت ميكاليس Km والسرعة القصوى Vmax و الثابت الحفزي kcat هي 0.294 ملي مولار و 1.09 ملي مولر / دقيقة و 893.8 دقيقة$^{-1}$. خلصت هذه الدراسة الى ان انزيم اسباراجينيز الفاصوليا يتكون من وحدتين متطابقتين في الوزن الجزيئي لكل منهما 40.6 كيلودالتون كما انه فعال وثابت في الظروف القاعدية لكنه فقد ثباتيته وفعاليته في درجات حرارة فوق 60 م°. توصي هذه الدراسة بدراسة تتابع الاحماض الامينية لاسبارجينيز الفاصوليا ومقارنته مع باقي الاسبارجينيزات الموجودة في قواعد بيانات البروتينات وكذلك دراسة تطبيقات هذا لانزيم.

*بحث مستل من اطروحة الدكتوراه للباحث الاول.

INTRODUCTION

First enzymatic hydrolysis of L-Asparagine to L-Aspartate and ammonia were reported by Lang (15), who noticed L-Asparaginase activity in several beef tissues. In recent years, L-Asparaginase has attracted much attention in both food industrial and pharmaceutical applications. L-Asparaginase (EC.3.5.1.1; asparagine amidohydrolase) catalyses the conversion of L-asparagine into L-aspartate and ammonia and this catalytic reaction is basically irreversible under physiological circumstances (9). In plants, L-Asparaginase is the main nitrogen carrier compound and there is a probability of its accumulation under stress conditions (21). Classification of all known enzymes with Asparaginase activity is based on their amino acid sequences as well as on biochemical and crystallographic data. The known Asparaginase sequences can be divided into three families: the first family is similar to bacterial-type Asparaginases, the second is similar to plant-type asparaginases and the third one is similar to enzymes correspond to *Rhizobium etli* asparaginase (3). In addition, bacterial-type L-Asparaginases are divided into subtypes I and II, according to their extra-/intracellular location, oligomeric arrangement and substrate affinity. Remarkably, bacteria harbor a gene that encodes a plant-type enzyme as well. Plant type L-Asparaginases are developmentally and structurally differed from the bacterial-type enzymes. They function as potassium-dependent or -independent Ntn (N-terminal nucleophile)-hydrolases (17). In food industry, L-Asparaginase was used to reduce acrylamide from different processed foods using pretreatment of L-Asparaginase to degrading L-asparagine, the precursor of acrylamide, prior to baking (30, 6, 8). On the other hand L-Asparaginase used for development of a novel diagnostic biosensor for the detection of L-asparagine in Leukemia cells (27). Moreover, L-Asparaginase is a medicinally important enzyme used in combination with other medicines for treatment in all pediatric regimens and in the majority of adult treatment protocols. It is used in the treatment of acute lymphocytic leukemia (mainly in children), Hodgkin's disease, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma, reticlesarcoma and melanosarcoma (26) (24). Due to the economic importance of the L-Asparaginase this study aimed to studying some of specific characters of L-Asparaginase purified from the beans.

MATERIAL AND METHODS

Pole beans (*Phaseolus vulgaris* L.) pods were collected from a glasshouse at Michigan State University (USA). Healthy looking pods were taken and purified to homogenization.

Enzyme assay

The Protein estimated by BCA protein assay using bicinchoninic acid (BCA). The enzymatic activity determined by Asparaginase activity kit obtained from Sigma – Aldrich Company. The activity was determined by a coupled enzyme assay, which results in colorimetric (570 nm) / fluorometeric (λex = 535 / λem = 587 nm) products proportional to the aspartate generated. One milliunit (mU) of Asparaginase is defined as the amount of enzyme that catalyzes the formation of 1 µmole of aspartate per minute at 25 °C. The arbitrary units (U) of L-Asparaginase were described as the amount of enzyme that gives 0.001 increases in absorbance (A) per minute.

Gel filtration Sephacryl S-200 was used to prepare the gel filtration column (1.5 x 90 cm dimensions), it was prepared according to GE Healthcare instructions. The matrix suspension diluted with 0.2 M MOPS buffer at pH 7.9 to 2 x. the desired packed medium volume and stirred with a glass rod to make a homogeneous suspension free from aggregates. The column was equilibrated using the same buffer at a flow rate of 75 ml / h for 5 hours. After column preparation, 3 ml of the enzyme solution was added gently to column surface and eluted using 0.2 M MOPS buffer pH 7.9 with a flow rate of 25 ml / hr (2ml for each fraction). Optical density (at 280nm), and enzyme activity were determined for each fraction. Fractions represent Asparaginase activity were pooled and kept at 4°C.

Molecular weight estimation

Asparaginase molecular weight was estimated by gel filtration chromatography using Sephacryl S-200. Gel Filtration Markers Kit, provided from Sigma-Aldrich, containing five standard proteins Cytochrome C, Carbonic

Anhydrase, Bovine serum albumin, Alcohol Dehydrogenase and β-Amylase their molecular weight are 12.4, 29, 66, 150, and 200 KDal respectively. They were prepared at a recommended concentration for each as described in the kit technical bulletin. Void Volume (Vo) determined by using the blue dextran. A volume of 3ml of the enzyme sample and 2ml of blue dextran and standard proteins were added individually to the top surface of the gel, and fractions were collected at a flow rate of 25 ml/hour (2 ml for each fraction). The optical density for each fraction was measured for the blue dextran and the proteins at 620 and 280 respectively. Then standard curve was plotted according to the relationship between the log molecular mass of each standard protein and the ratio of Ve/Vo, then Asparaginase molecular weight was estimated from the standard curve.

Electrophoresis

SDS-PAGE provided by Bio-Rad was used in the determination of the Asparaginase molecular weight. Criterion gels were set up according to the Criterion precast gel instruction manual and application guide. The sample was prepared by adding 5 µl of sample to 4.75 µl electrophoresis sample buffer and 0.25 µl β – mercaptoethanol to make a total volume of 10 µl then the diluted sample heated at 70 °C for 10 min. Subsequently the sample was loaded to the gel using a pipet with gel loading tip. Power supply aligning to the lid of the tank and electrophoresis running condition was under 200 V constant voltages for 1 hr. After electrophoresis the gel floated in nanopure water and rinsed 3 times for 5 minutes with 100 ml nanopure water to remove SDS and buffer salts, which interfere with the binding of the dye to the protein and discarded each rinse. The gel stained with enough simply blue safe stain (~20 mL) to cover the gel and allowed to stain for 1 hour at room temperature with gentle shaking. After incubation, the stain discarded and the gel washed with 100 mL of water for 1–3 hours.

Estimation of molecular weight by using the Electrophoresis

Set of MW standards were separated on the same gel with the sample. These standards are Myosin, β – galactosidase, Phosphorylase, Serum albumin, Ovalbumiine, Carbonic anhydrase, Trypsin inhibitor, Lysozyme and Aprotinin, their molecular weight are 200000, 116250, 97400, 66200, 45000, 31000, 21500, 14400 and 6500 KDal respectively. Next a graph of log molecular weight vs. Relative migration distance (R_m) was plotted, based on the values obtained for the bands in the MW standard. The MW of the unknown protein band was then calculated by interpolating using this graph.

Estimation of optimal pH for enzyme activity and stability Different buffer solutions were used to estimate the optimal pH for Asparaginase activity. Sodium acetate, sodium phosphate, Tris – HCl and glycine – NaOH buffers were used to prepare a pH ranged from (3 – 10). The substrate 0.01 M L-asparagine was added to 0.05 M of buffer solutions at stated pH. The Asparaginase activity were measured and plotted against the different pH. Purified enzyme was incubated at different pH values ranging between 3 and 10 at room temperature for one hour. The remaining activity% was measured after assaying enzyme activity as a percentage of the highest value of enzyme activity.

Estimation of optimal temperature for enzyme activity and stability

Asparaginase activity was estimated after incubation of the purified enzyme along with the substrate at different temperatures (20, 25, 30, 35, 37, 40, 45, 50, 55 and 60 °C). Then Asparaginase activity was estimated after each incubated temperature. The purified enzyme was incubated in a water bath at different temperatures ranging between (10 – 90) °C for one hour, then immediately transferred into an ice bath. The enzyme Activity was estimated and the remaining activity (%) of Asparaginase was plotted against the temperature (°C).

Estimation of Enzyme kinetics

For kinetic analyses the initial velocity of the sample was estimated in a varying concentration of the substrate L-asparagine ranged between (0.2 to 4 mM). The *K*m of the enzyme was determined by making the reaction mixture containing fixed volume (0.25 ml) of the partially purified enzyme and the total volume of the mixture was made up to 2 ml with 0.5 M Tris-HCl buffer of pH 8.6 and Asparaginase activity was measured. A

graph of the substrate concentration was plotted against the reaction velocity. *K*m and V_{max} were calculated from Lineweaver-Burk plot (Segal, 1976). The turnover number (kcat) of Asparaginase was calculated on the basis of one active site per subunit (Subunit ~41.6 kDa).

$$Kcat = \frac{V_{max}}{E_t}$$

E_t: Enzyme concentration

RESULTS AND DISCUSSION

The molecular weight of the enzyme was determined using Sephacryl S-200 column. A plot of Ve/Vo against log molecular weight was used to determine the molecular weight of L-asparaginase (figure 1), (figure 2).

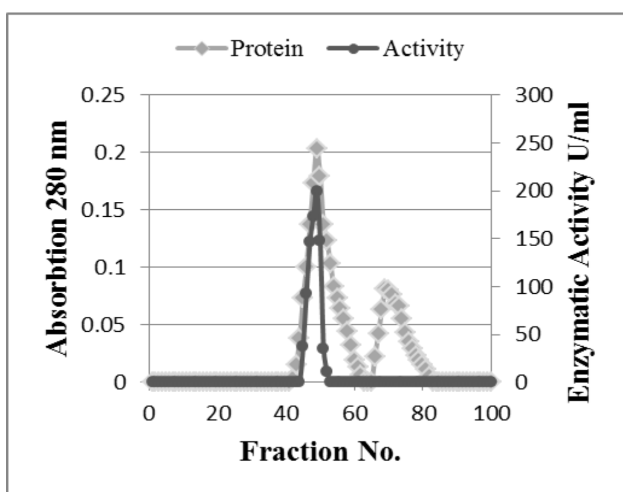

Figure 1. Gel filtration chromatography using Sephacryl S-200 (1.5 x 90 cm) equilibrated with MOPS buffer at pH 7.9, fraction volume was 2ml at a flow rate of 25 ml/hr.

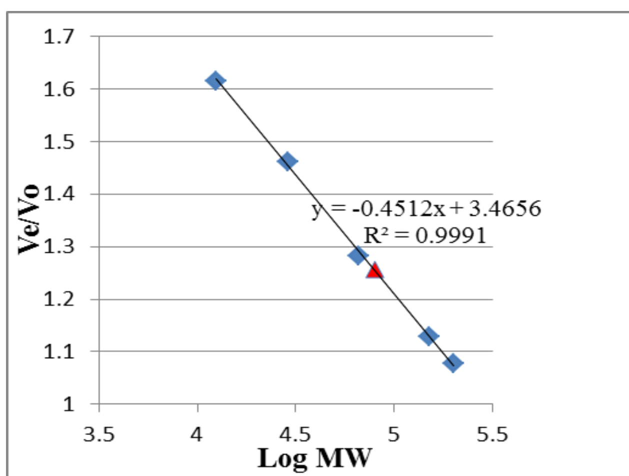

Figure 2. Calibration curve for the determination of molecular weight of L-Asparaginase by gel filtration Chromatography.

The molecular weight of the enzyme from *P.vulgaris* was found to be 79.5 KDa, which sharply differs from the enzymes of guinea pig serum and *E. coli,* which have a molecular weight of around 130 KDa. However, the molecular weight of other higher plant L-Asparaginase was quite similar to that of *P. vulgaris*. Lea *et al*. (16) demonstrated that the m olecular weight of *Withania somnifera* L-Asparaginase is approximately half of that of prokaryotic L-Asparaginase. Some other studies reported that the molecular weight of the plant L-Asparaginase was estimated below 69 KDa (22,12). The subunit molecular mass of the L-Asparaginase from *P. vulgaris* was estimated by SDS-PAGE. A graph of log molecular weight vs. relative migration distance (R_m) was plotted, based on the values obtained for the bands in the molecular weight markers (figure 3). The molecular mass of each subunit were 40.6 KDa as observed from SDS-PAGE separation. This is consistent with a homodimeric enzyme (figure 4). Swain *et al.,*(25) reported that the plant L-Asparaginase is a homodimer, the symmetric unit contains four polypeptide chains, the chains A and C correspond to subunit α while chains B and D correspond to subunit β. In another study, the molecular mass of L-Asparaginase subunit, isolated from *Withania somnifera*, was 36 ± 0.5 KDa corresponding to a homodimeric enzyme as detected by SDS-PAGE separation (18). The pH effect on the activity L-Asparaginase enzyme was studied at 37°C. The results presented in Figure (5) show that the enzyme exhibited maximum activity between pH 7.0 and 8.5, while the enzymatic activity was decreased at low and high pH. Previous studies on plant L-Asparaginase revealed that the pH for maximum activity was found in the range of 8.2 to 8.5 (1, 11, 18, 12). The Optimal pH for L-Asparaginase activity may differ corresponding to the organism genera or species. *E.coli* has an optimum activity at pH 7.4 (13), while the optimum pH for L-Asparaginase activity of Marine Actinomycetes was at pH 7.5 (2). The change in pH affects the ionization of the essential active site amino acid residues, which are involved in substrate binding and catalysis (breakdown of the substrate into a product (20).

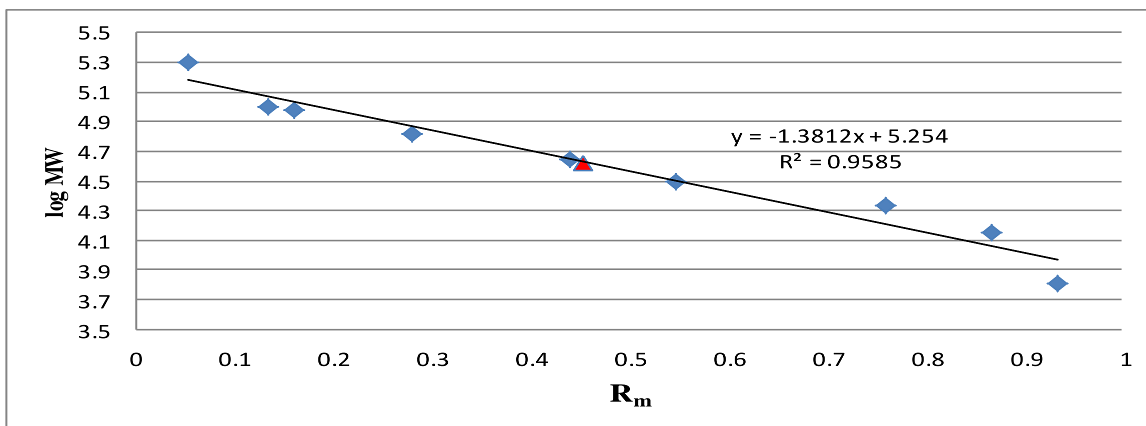

Figure 3. Calibration curve for the determination of molecular weight of L-Asparaginase by SDS/PAGE

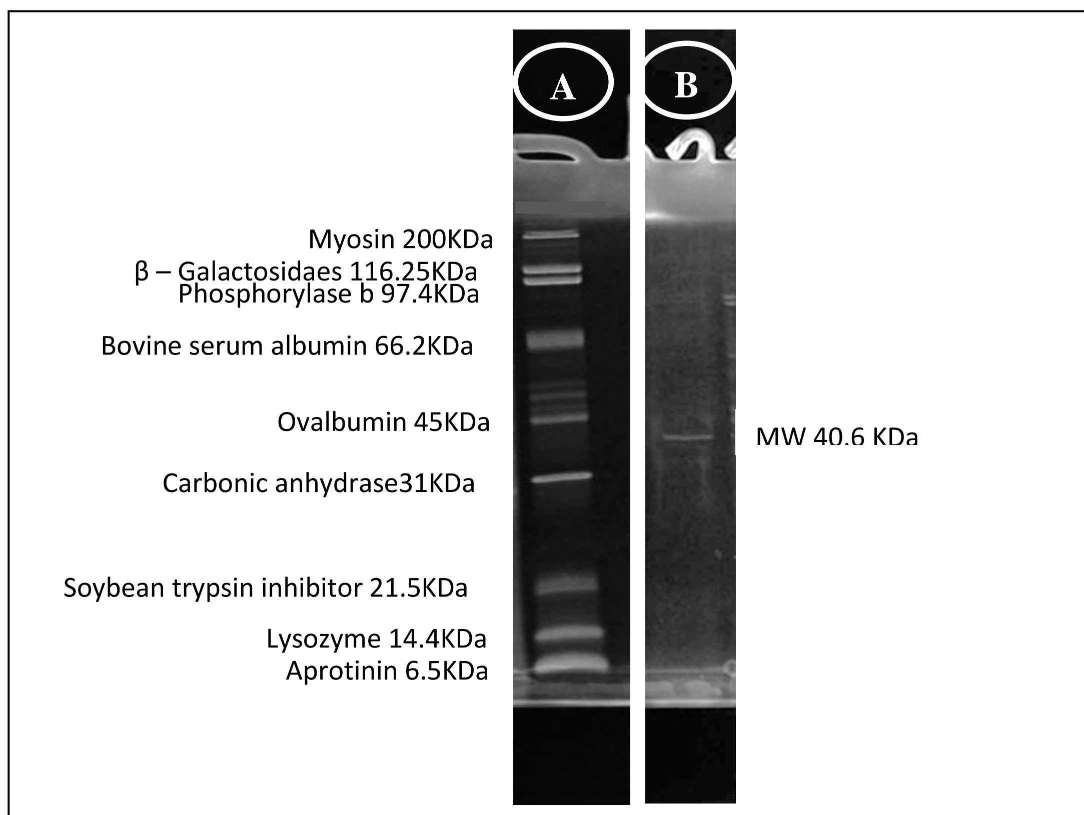

Figure 4. SDS/PAGE of the purified L-Asparaginase from the *P. vulgaris* (A) molecular weight marker proteins, (B) purified L-Asparaginase.

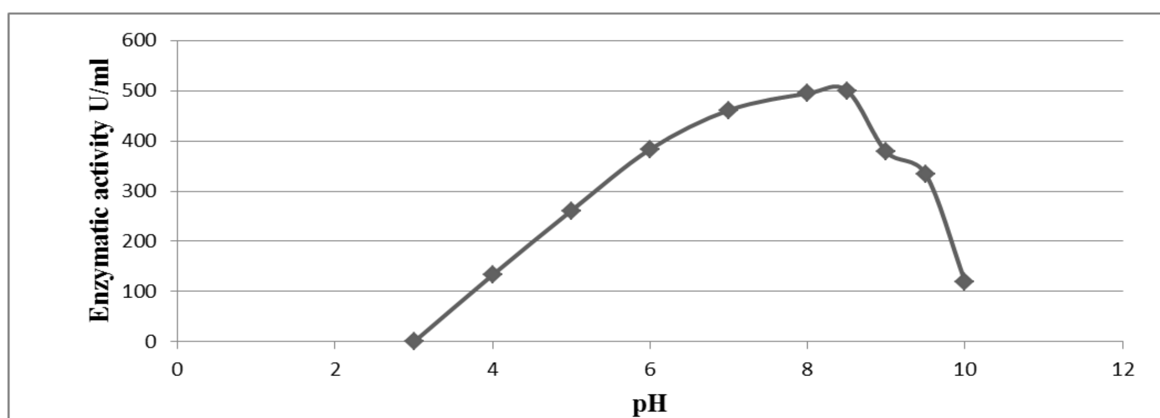

Figure 5. Effect of pH on activity of purified L-Asparaginase from *P. vulgaris*

The optimum pH for L-Asparaginase stability was estimated by incubation of the purified L-Asparaginase at different pH values. The remaining activity was then determined after assaying enzyme activity. Figure (6) shows that the enzyme was more stable at pH 8 and kept 87.5% of its activity at pH 9, while the remaining activity was decreased after incubation at low pH range as well as high range. Most enzymes undergo irreversible denaturation in strong acidic or alkaline conditions (28). Enzymes like other proteins are stable over only a limited range of pH. Outside this range, changes in the charges on ionisable residues result in modifications of the tertiary structure of the protein and eventually cause denaturation (29). Stecher *et al*., (23) studied the stability of the L-Asparaginase and found that the tertrameric form of asparaginase remained stable at pH ranged (4.5-11.5).

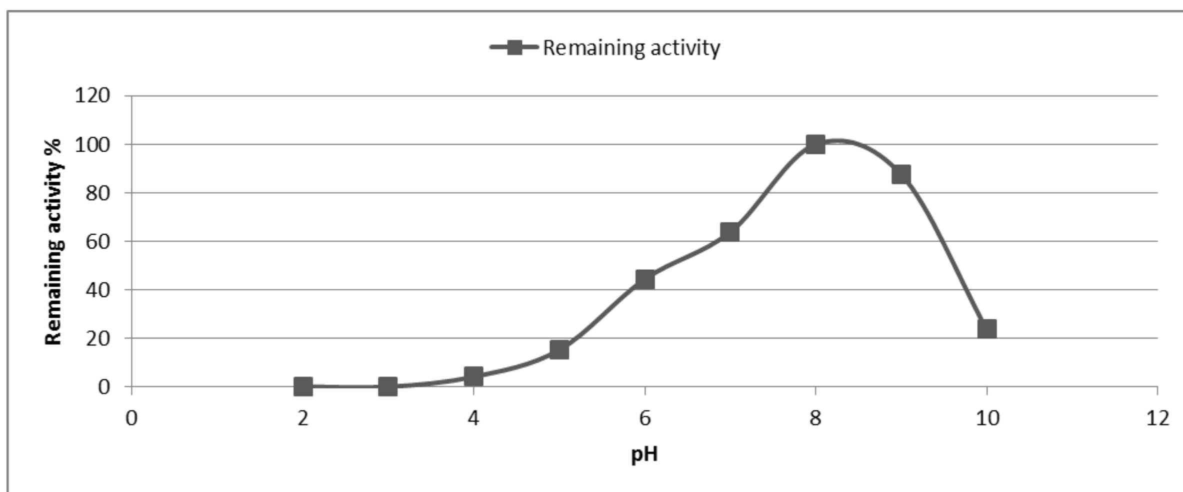

Figure 6. Effect of pH on the stability of *P. vulgaris* L-Asparaginase.

The activity of the enzyme was measured at different temperatures ranging from 20°C to 60 °C, and at the optimum pH. The results placed in a figure (7) indicated that the enzyme functioned optimally at temperature 37°C, on the other side there was a decline in enzyme activity. Raising the temperature increases the kinetic energy of molecules. Increasing the kinetic energy of molecules increases their motion and therefore the frequency with which they collide. This combination of more frequent and more highly energetic and productive collisions increase the reaction rate (19). This observation is corroborated with the earlier studies that were done by Bano & Sivaramakrisnan (1) who reported that Green chili (*Capsicum annum* L.) L-Asparaginase worked optimally at temperature 37°C. Another study revealed that 37°C was the optimum temperature for the activity of *Pisum sativum* L-Asparaginase, while enzyme activity was decreased at less or higher of this temperature (12).

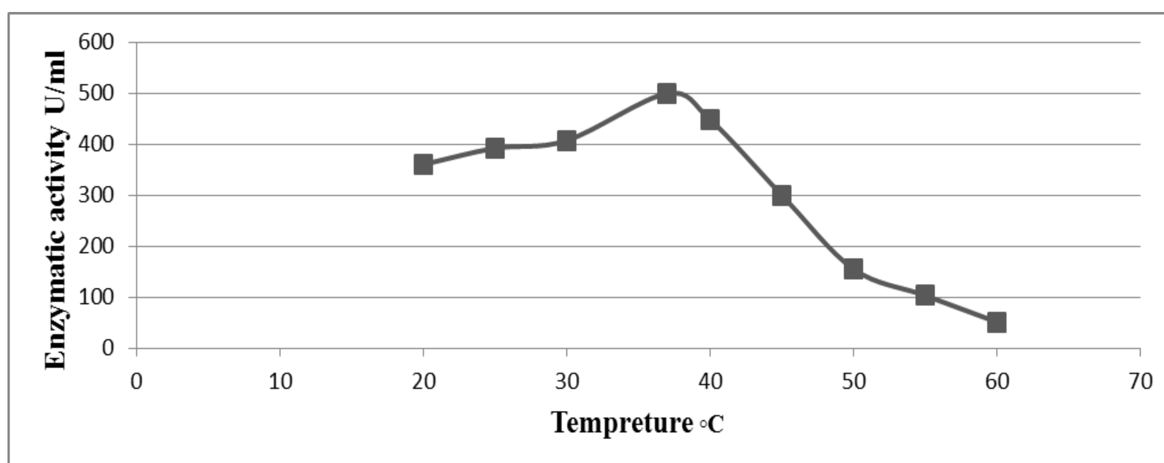

Figure 7. Effect of temperature on activity of purified L-Asparaginase from *P. vulgaris*.

Results in figure (8) indicated that no loss of activity was observed when the enzyme was incubated at 10, 20 and 30 °C for 1 hour. Higher temperatures led to an increasing degree of inactivation. Thus incubation at 70 °C led to complete inactivation. The conformational stability of the enzyme depends upon stabilizing forces arising from a large number of weak interactions. High temperature plays a more important role in degradation reactions in functioning enzymes and the interrelationship between degradation and denaturation (4). The crude and partially purified enzymes are more stable than purified enzymes due to the existence of carbohydrates and other proteins protecting them (20). A number of studies were verified these findings, as in the characterization of *Acinetobacter calcoaceticus* L-Asparaginase by Joner *et al.* (10) who mentioned that there was no loss of activity observed when the enzyme was incubated at 20 °C and 30 °C for 3 hours, while the incubation at 60 °C led to complete inactivation in approximately 5 minutes.

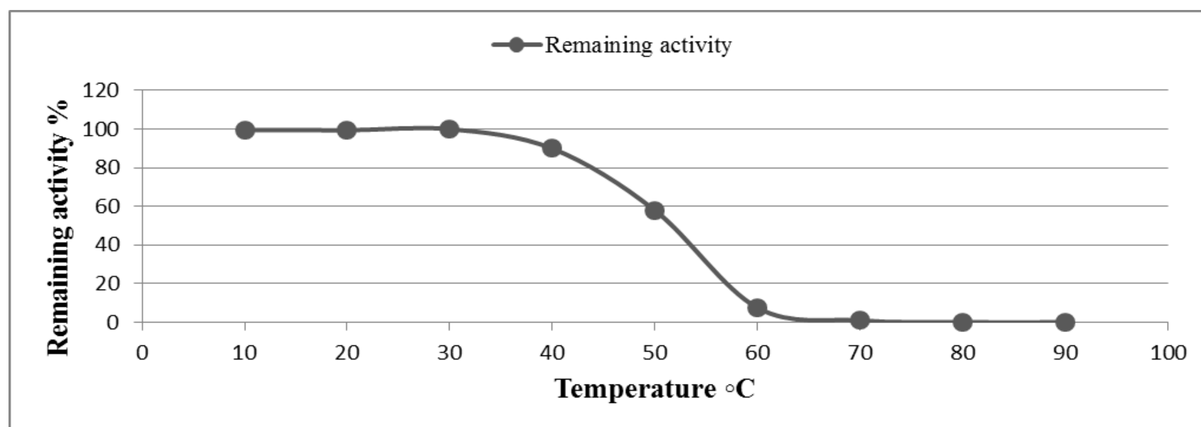

Figure 8. Effect of temperature on the stability of *P. vulgaris* L-Asparaginase

For kinetic analyses the initial velocity of the enzyme was estimated in a varying concentration of the substrate L-asparagine ranged between (0.2 to 4 mM). The turnover number (k_{cat}) of Asparaginase was calculated on the basis of one active site per subunit (Subunit ~41.6 KDa).

$$Kcat = \frac{V_{max}}{E_t}$$

From Lineweaver-Burk plot, the maximum specific activity (V_{max}) for the enzyme was 1.09 mM/min, while the apparent value of affinity constant (K_m) was 0.294 mM (Fig. 9). The turnover number (k_{cat}) of the enzyme was calculated to be 893.8 Sec^{-1}. Earlier investigators reported K_m values between 0.074 and 3.5 mM for L-asparaginases purified from different microbial sources (5) (14). The K_m and V_{max} of purified L-asparaginase from *S. halstedii* were 0.1939 mM and 1.22 mM/min, respectively (7).

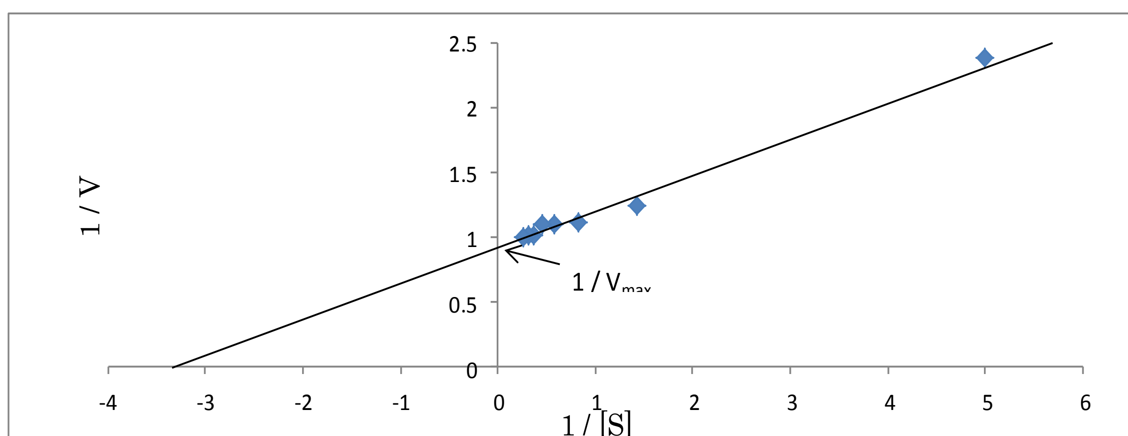

Figure 9. Lineweaver-Burk plot of L-Asparaginase from *P. vulgaris*. V is the reaction rate and S is the concentration of the substrate.

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