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# Purification and Immobilization of Aginase from Fenugreek Plants

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**Abstract**

Enzymes are recorded in all living organisms where they speed up and regulate the chemical reactions that crucial for the life of organisms. L-Arginase (L-arginine urea hydrolase, or amidinohydrolase, EC 3.5.3.1) is hydrolytic enzyme. It is a divalent cation-dependent and plays an essential role in nitrogen metabolism through converting L-arginine to L-ornithine plus urea. This work aimed to isolate, purify and comparing the characteristics of the free and immobilized L-arginase from Fenugreek. L-arginase was purified from fenugreek plants. L-arginase was purified successfully to homogeneity from fenugreek by 80 % ammonium sulphate, DEAE-cellulose and Sephadex G200. L-arginase was immobilized on calcium alginate and chitosan. Reuse of immobilized L-arginase was tested through 7 cycles. The results in the present investigation showed that the optimal pH for the free L-arginase was 8.5 whereas the immobilized enzyme expressed optimal pH at 9.0. On studying the effect of temperature on the free and immobilized enzyme showed that the optimal temperature was 40°C for the free enzyme and shifted to 50ºC for the immobilized form on both Ca alginate and chitosan. The presence of L-arginase in fenugreek suggests that this plant may be a potential plant source for developing the industrial biosynthetic production of L-ornithine.

***Keywords:*** L-arginase, Purification, Characterization, Active site groups, Immobilization, Applications.

## Introduction

L-arginine is a non-protein amino acid that functions as an intermediate metabolite in the urea cycle **[1].** This amino acid performs an important role in humans and is used for the healing of liver diseases to reinforce the heart function for weight loss and to progress immunity. Because of these valuable advantages, L-arginine is widely requested in the health care as well as several pharmaceutical industries. Therefore, this field has called extensive attention because of the huge market **[2].**

Enzymes are recorded in all living organisms where they speed up and regulate the chemical reactions that crucial for the life of organisms **[3].** L-Arginase (L-arginine urea hydrolase, or amidinohydrolase, EC 3.5.3.1) is hydrolytic enzyme. L-arginase is a key enzyme in urea cycle that catalyzes the production of urea in the mammalian liver **[4].** It is a divalent cation-dependent and plays an essential role in nitrogen metabolism through converting L-arginine to L-ornithine plus urea. L-arginase is responsible for the cyclic nature of the urea cycle **[5].** L-arginine is one of the most functionally diverse amino acids in living cells. In addition to serving as a constituent of proteins, arginine is a precursor for the biosynthesis of polyamines, agmatine and proline as well as the cell-signaling molecules glutamate, aminobutyric acid, and nitric oxide **[6].**

Generally, enzyme stability is essential in basic and applied enzymology. This stability is critical to understand the enzyme stabilization through illustrating how the enzyme loss their activity followed by deriving the structure stability relationships existing in enzymatic molecules **[7]**. Thermal stability and pH stability indicate the capability of the enzyme to resist higher temperatures or pH at alkaline or acidic sides before occurring denaturation **[8].**

Storage stability is recognized as an ability of the enzyme to keep its activity under some certain conditions of storage. However, the operational stability does only represent the enzyme function but it represents the durability of the carrier and concentrations of the inhibitor in the solution under assay **[9]**.

L-arginine signifies about 50% of the nitrogen in seed protein, and up to 90% of the free

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nitrogen in vegetative tissues. In numerous plant species such as pumpkin, soy bean, broad bean and lupine nitrogen mobilization throughout the development of seedling is linked with great increases in L-arginase expression **[10].**

Non-availability of necessary nitrogen in general limits plant functioning. Therefore, plants have not only an effective nitrogen uptake system, but also to recycle the available nitrogen **[11].** The present work aimed to: isolate, purify and comparing the characteristics of the free and immobilized L-arginase from fenugreek.

## Materials and Methods

### Growth of fenugreek plants

Seeds of fenugreek were germinated according to **[12].** The seeds of different were surface sterilized in 2% sodium hypochlorite for 10 min, soaked in running tap-water for 24 h, and then germinated between paper towels, moistened with distilled water in sterilized plastic trays. The trays were covered and incubated in the dark at 25°C for 48h. The germinated seeds with well-grown roots were then supported on plastic bowls containing 0.2 mM $CaCl_2$ solution. $CaCl_2$ solution was continuous and vigorously aerated. Seeds were grown for further 48 h in light at 25°C. Seedlings of 10-days old plants were cut with a razor blade and kept on ice to be used for enzyme extraction immediately.

### Extraction of L-arginase

The enzyme was extracted from 10-days old germinated seedlings as described by **[12].** The plant leaves were ground with a pestle and mortar at 0-4°C using 50 mM sodium acetate buffer (pH 6.0). The homogenate was centrifuged at 5000 rpm for 15 min, and the supernatant was collected and represented the crude extract.

### Purification of L-arginase

The crude extract of L-arginase prepared by the extraction method was treated with solid ammonium sulphate. After 1 h it was centrifuged at 5,000 rpm and the crude preparation was adjusted to 80 % saturation of $(NH_4)_2SO_4$ and centrifuged at 5,000 rpm for 30 min. The resulted pellet was re-suspended in the minimum volume of 100 mM acetate buffer (pH 6.0) and dialyzed overnight against the same buffer. The dialyzed preparation was centrifuged for 20 min at 5,000 rpm and then loaded onto DEAE-cellulose column (2×10 cm). The column was equilibrated with 100 mM acetate buffer (pH 0.6) containing 0.2 M NaCl. L-arginase was eluted from the column by a steep gradient of NaCl from (0.1 to 0.5 M) in equilibrating buffer at flow rate of 1.0 ml min-1. The active fractions were combined and dialyzed over night against 100 mM acetate buffer (pH 6.0). The eluted enzyme from DEAE-cellulose column was loaded onto Sephadex G-200 column (2×10cm). The active fractions were collected, concentrated by dialysis as described above and later purified on hyderoxyapatite column (2×10cm) and eluted with a flow rate of 1.0 ml min-1. The active fractions were pooled and considered as the pure enzyme.

### Determination of molecular weight by Gel Electrophoresis

The homogeneity of purified L-arginase was checked using dissociating polyacrylamide gel electrophoresis (SDS-PAGE) and was carried out according to a protocol proposed by **[13].**

### Immobilization of L-arginase on alginate bead

The method was initially adopted from that of **[14].** The pure L-arginase was added to 40 ml sodium alginate (3% w/v). The solution was placed in a separating funnel and suspended over a beaker containing 200 ml $CaCl_2$ (3% w/v). The alginate bead was then prepared by dropping gently the alginate solution through a 200 µl Eppendorf tip into $CaCl_2$ solution at a rate of approximately 30 drops $min^{-1}$. After 3 h stirring gently to allow the bead to harden, the bead was filtered out of the $CaCl_2$ solution and thoroughly washed by the same buffer. The activity of the immobilized enzyme was assayed.

### Determination of protein content

The protein content was determined according to **[15].** One ml of the supernatant was mixed rigorously to 5 ml (1/4 diluted Coomassie Brillant Blue G-250 then the mixture was kept in the dark for 1 min and the absorption of the protein was measured at 595 nm spectrophotometrically. The protein concentration was determined using standard curve of bovine serum albumin (BSA).

### Determination of L-arginase activity

L-arginase activity was measured according to the **[16].** Approximately 0.1 g of the immobilized enzyme was used for enzyme assay as described earlier for the free enzyme.

**Effect of L-arginine concentration on L-arginase activity**

The effect of L-arginine as a substrate on L-arginase activity was studied at various arginine concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mM). The other factors affecting the enzyme activity such as pH, temperature were kept constant.

**Determination of kinetic parameters**

The kinetic parameters $K_m$ and $V_{max}$ values of both free and immobilized enzymes were carried out at various substrate concentrations. The rate of enzymatic hydrolysis of L-arginine was expressed by Lineweaver-Burk double reciprocal plot **[17].**

**Effect of pH on L-arginase activity**

The optimal pH for the enzyme activity was determined within a range of pH (4 to 10). The following buffers were used sodium acetate/acetic pH (4.0 - 5.0), potassium phosphate buffer (6.0 – 7.0), Tris-HCl buffer (8.0 – 9.0) and sodium bicarbonate buffer (10.0). A graph of the enzyme activity against pH was plotted, and subsequently the optimal pH for L-arginase was determined.

**Effect of temperature on L-arginase activity**

The reaction mixture of L-arginase was incubated at different temperatures (10, 20, 30, 40, 50 and 60 °C). The optimal temperature was determined from the graph of enzyme activity against temperature.

**Operational stability of the immobilized enzyme**

The operational stability was tested by repeated batch experiments using the method for activity determination. After each reaction run, immobilized beads were removed and washed with buffer to remove any residual substrate within the beads, then reintroduced to fresh reaction medium.

**Statistical analysis:**

All the data in the present study are expressed as mean of three replicates ± SE obtained from three measurements.

**Results:**

In **Fig.1,** this experiment aimed to investigate the effect of immobilization time (10, 20, 30, 40, 50, 60 and 70 min) on immobilization efficiency of L-arginase on Ca-alginate and chitosan. The immobilization efficiency was calculated every 10 min throughout 70 min. The results revealed that increasing the immobilization time from 10 min to 60 min resulted in continuous increase in the immobilization efficiency where its values were 50% and 74 % for Ca-alginate and chitosan, respectively. However, after 70 min the immobilization efficiency decreased to 46% and 70% for two the supports, respectively. Thus, the period of 60 min represents the optimal time for immobilization of L-arginase on both Ca-alginate and chitosan.

Table 1 : Purification of L-arginase from Fenugreek.

| Purification step | Volume (ml) | Total protein (mg) | Total activity (U) | Specific activity ($Umg^{-1}$) | Purification fold | Yield (%) |
|---|---|---|---|---|---|---|
| Supernatant | 140 | 330 | 196 | 0.59 | 1.0 | 100 |
| Ammonium sulphate precipitation (80%) | 100 | 140 | 172 | 1.23 | 2.1 | 87.8 |
| DEAE-Cellulose | 20 | 13 | 144 | 11.08 | 20.0 | 73.5 |
| Sephadex G-200 | 12 | 1.6 | 98 | 61.25 | 103.8 | 50 |

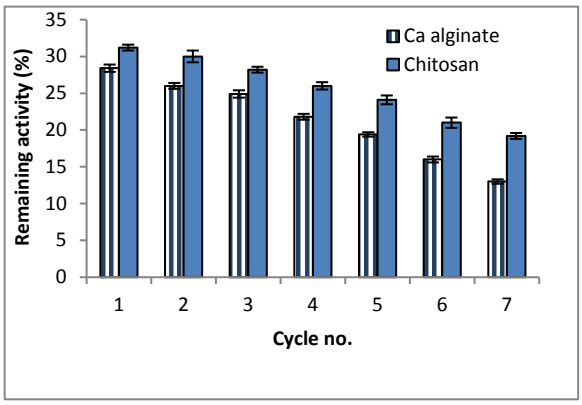


**Fig. 1:** Effect of time course on immobilization efficiency of L-arginase from Fenugreek.

In **Fig.2**, the reusability of immobilized enzyme by entrapment and cross-linking methods was tested throughout 7 cycles. These results indicated that entrapped enzyme retained 13% of its initial activity whereas the cross-linked enzyme retained 19.2 % of its initial activity after the 7$^{th}$ cycles.

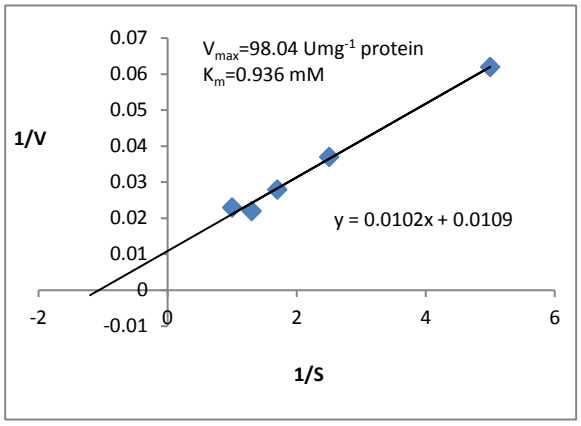


**Fig. 2:** Reusability of alginate immobilized L-arginase from Fenugreek.

The results in **Fig.3** revealed that the activity Plotting 1/V against 1/s for Ca alginate-immobilized L-arginase resulted in straight line and from this figure the values of $V_{max}$ and $K_m$ were calculated these values were 96.15 U mg$^{-1}$ protein and 1.405 mM, respectively.

The results in **Fig.4** showed that Plotting 1/V against 1/s for Ca alginate-immobilized L-arginase resulted in straight line and from this figure the values of $V_{max}$ and $K_m$ were calculated these values were 96.15 Umg$^{-1}$protein and 1.405 mM, respectively.

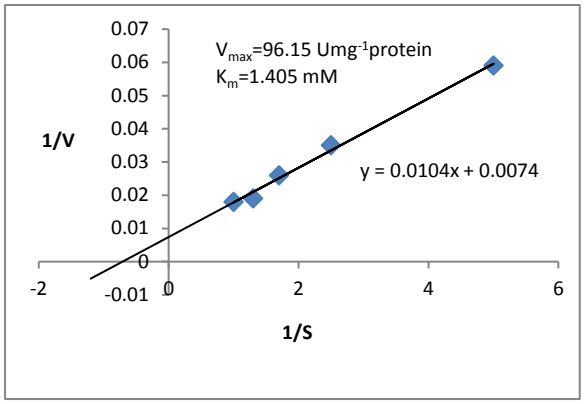


**Fig. 3:** Lineweaver-Burk plot of free L-arginase from Fenugreek.

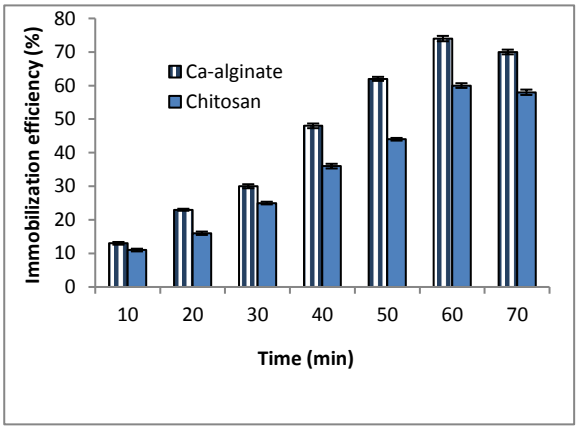


**Fig. 4:** Lineweaver-Burk plot of Ca alginate-immobilized L-arginase from Fenugreek.

The results in **Fig.5** showed that The effect of pH on the activity of free and Ca alginate-immobilized L-arginase was studied at pH values between 5 and 10 and this indicated that there was continuous increase in the enzyme activity from pH 5 to pH 8.5 for the free L-arginase where the highest activity was 28.2 units mg$^{-1}$ protein. After pH 8.5 the activity of the free enzyme declined gradually with increasing the pH value to reach 19.5 units mg$^{-1}$ protein at pH 10. However, the pH optimum of the immobilized L-arginase on Ca-alginate and chitosan was shifted to 9.0 where the activities were 33.0 and 35.0 units mg$^{-1}$ protein, respectively. At the higher pH values over pH 9.0 the activities declined continuously with increasing the pH value to 9 and 10. The effect of temperature on the activity of both free and immobilized L-arginase was investigated at various temperatures (20, 30, 40, 50, 60 and 70 ºC).

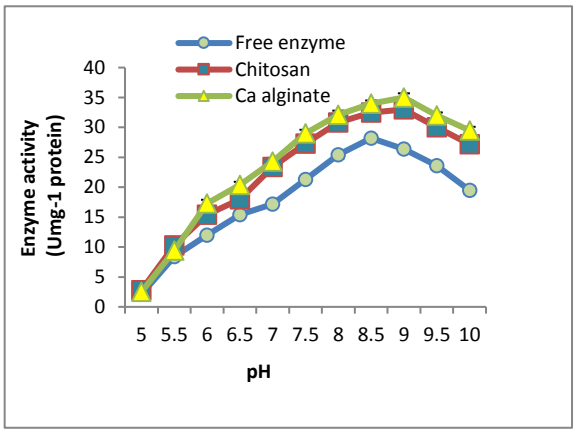


**Fig. 5:** Effect of pH on the activity free and immobilized L-arginase from Fenugreek.

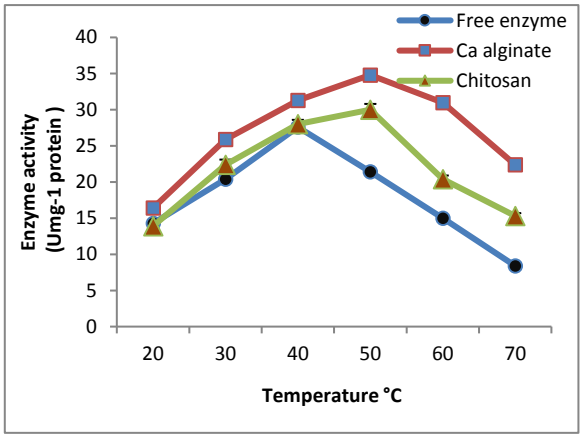


**Fig. 6:** Effect of temperature (°C) on the activity of free and immobilized L-arginase from Fenugreek

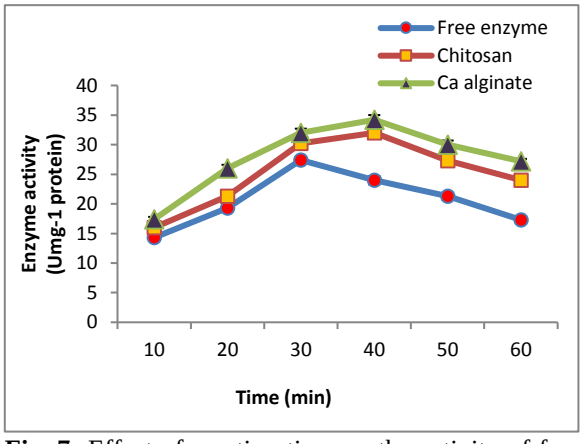


**Fig. 7:** Effect of reaction time on the activity of free and immobilized L-arginase from Fenugreek.

The results in **Fig.6** revealed that there was continuous increase in L-arginase activity with the increase in the incubation temperature up to 40ºC at which the enzyme activity reached 27 units $mg^{-1}$ protein. After 40 ºC the activity decreased continuously with any increase in temperature until it reached 8.4 units $mg^{-1}$ protein at 70 ºC. Therefore, it seems likely that 40ºC is the optimal temperature for the free enzyme.

The results in **Fig.7** revealed that The relation between L-arginase activity and the incubation time (10, 20, 30, 40, 50 and 60 min) was studied, and indicated continuous increase in the enzyme activity with increasing incubation time up to 30 min for the free enzyme and 40 min for the immobilized enzyme. After the optimum time the activity declined gradually with increase in the reaction time.

**Discussion**

L-arginase was purified successfully to homogeneity from fenugreek by 80 % ammonium sulphate, DEAE-cellulose and Sephadex G200. The enzyme was purified with specific activity of 61.3 units $mg^{-1}$ protein and 103.8-fold. L-arginase was purified from soybean by Sephadex G-200, DEAE-sephacel, hydroxyapatite, and arginine-affinity columns with specific activity of 90 units $mg^{-1}$ protein and 152.5-fold. L-arginase was purified from *Bacillus thuringiensis* with specific activity of 589.2 units/$mg^{-1}$ protein and the molecular weight of the enzyme was 33 KDa **[5].**

Enzymes are amphoteric molecule consisting of a large number of acid and basic groups, mainly located on their surfaces. The charges found in these groups differ with pH of the environment. This will influence the total net charge of the enzyme, the reactivity of the catalytically active residues and the distribution of charges on the outer surfaces. These effects are important in the neighborhood of the active sites which will influence the activity and enzyme solubility **[18].**

The increase in the enzyme reaction due to the effect of temperature at certain point becomes equal to the reduction in the reaction rate because of the destruction of tertiary structure **[19].** The activity at this point is described as the maximum and this temperature is often known as the optimum temperature **[20].** The effect of temperature on the free and immobilized L-arginase show that the optimal temperature was 40°C for free L-arginase and shifted to 50ºC for immobilized L-arginase on both alginate and chitosan.

The optimal temperature of 40ºC for the free enzyme in the present investigation is similar with that

reported for the enzyme from *Bacillus thuringiensis* by **[5].**

Also, the optimum temperature of L-arginase coincides with the reported optimum temperature of 30-40 ºC for arginases from other microorganisms **[21-24].** One exception is the optimum temperature of 60 ºC for L-arginase from an extreme thermophile **[25].** This shift in the optimal temperature of L-arginase from 40 ºC for the free to 50 ºC for the immobilized enzyme is a consequence of enhanced thermal stability. The shift in the optimal temperature to higher values after the immobilization was reported for other enzymes **[26].**

The shift may be explained by the immobilization procedure could protect the enzyme in the active conformation from distortion or damage by heat exchange. One of the main reasons for enzyme immobilization is the anticipated increase in its stability to different deactivating forces due to restricted conformational mobility of the molecules following immobilization. It has been reported that, the stability of the enzyme can be increased by immobilization **[27].**

Plotting the reciprocal of substrate concentration against that of the velocity straight line was obtained. The $V_{max}$ and $K_m$ values were 98.04 units $mg^{-1}$protein and 0.936 mM, for the free enzyme, however their values were 96.15 units $mg^{-1}$protein and 1.4 mM, respectively. The Km was 83 mM for L-arginase from soybean **[28]** and 15.6 mM for the enzyme from *Bacillus thuringiensis* **[29].** The lower Km values of the free and immobilized enzyme in the present investigation indicate that the enzyme has a great affinity for L-arginine. The increase in $K_m$ of the immobilized L-arginase on Ca alginate might be due to the lower accessibility of L-arginine as the substrate to the active site of Ca alginate-immobilized enzyme, which could be due to several factors including conformation changes in the enzyme molecule produced after attachment to the alginate, steric and diffusion effects.

Certain benefits result from knowing the $K_m$ value for an enzyme of interest. First, if we can guess whether the cell needs more enzymes or substrate to speed up the reaction. Some enzymes are usually not saturated by substrates **[30-31]**. Second $K_m$ value signifies inverse measure of the affinity of the enzymes that can catalyze reactions with two similar substrates, the substrate for which the enzyme has the lower $K_m$ is the one most commonly acted upon the cell **[32]**. Thirdly, the $K_m$ gives an approximate measure of the cell in which reaction occurs. For example, enzymes can catalyze reactions with the relatively concentrated substrates usually have relatively high $K_m$ values for their substrates and enzymes that react with substrates present in very low concentration have much lower $K_m$ values for their substrates **[30-31]**.

The results in the present investigation showed that optimal pH for the free L-arginase was 8.5 whereas the immobilized enzyme expressed optimal pH at 9.0. L-arginase from *Idomarina sediminium* **[33]** exhibited pH valued of 7.5. **[5]** reported pH of 9 for L-arginase from *Bacillus thuringiensis.* **[29]** found an optimal pH 8.8 for L-arginase from kiwi fruit. Generally, all enzymes are sensitive to the pH value. This is because the pH can affect the enzyme activity through different mechanisms: 1) It can change the ionization of the different groups of the enzyme molecule and this affect the enzyme affinity for its substrate. 2) It can change the ionization of the enzyme substrate complex.3) It changes the ionization of the substrate and this affects the formation of E-S complex. Fourthly, extreme pH may cause changes in the protein structure which alters the stability of the enzyme **[34]**.

Studying the relation between the enzyme activity and the incubation time resulted in appearance of a graph which has two distinct regions. Firstly, in initial period of time, the amount of substrate transformed, seems to be directly proportional with the length of time of the reaction. After the initial period, the rate of reaction decreases and shows that the substrate is present in excess. The explanation of this phenomenon is the reduction of enzyme activity after a period of time. This may be attributed to the effect of heat of incubation on the tertiary structure of the enzyme or to formation of side-product of the reaction, which inhibits the enzyme activity **[35]**.

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**Declaration of interest:**

No conflicts of interest.

**Data Availability:**

The data that supports this work is available upon reasonable request.

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