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Research Article

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Partial Purification and Biochemical Characterization of Horse Gram Arginase

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Abstract : Plant arginase that catalyses the hydrolysis of arginine to ornithine and urea is known to play an important role in nitrogen metabolism. Recently, we reported a highly stable arginase from cilantro and its sensitivity to biotic and abiotic stress. During this investigation, we found horse gram also possessing a stable arginase among legumes. Hence, we partially purified arginase from horse gram seedlings by conventional chromatographic techniques with 869-fold purity, and a specific activity of 13752 nmoles of urea formed/mg of protein/min. The enzyme is relatively heat stable and requires Mn^{2+} for its activity and is sensitive to reducing agents and EDTA similar to cilantro arginase. The optimum pH and temperature for partially purified horse gram arginase was found to be 7.87 and 37° C - 60° C respectively. Arginine-derived polyamines and amino acids can regulate horse gram arginase *in vitro*. Partially purified arginase hydrolyses L-arginine and is incapable of hydrolysing other arginine analogues except L-homoarginine, a property that distinguishes horse gram arginase from cilantro arginase. The Km for partially purified arginase was found to be 5.47 ± 0.34 mM with respect to L-arginine. As plant arginases are not stable and their subunit organization differs from source to source, for further purification and biochemical characterization horse gram serves as an ideal and easily available source.

Key words: Horse gram arginase, Polyamines, Nitrogen recycling, Enzyme modulators, Arginine analogues

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1. INTRODUCTION

The amino acid arginine by virtue of possessing the highest N:C (4:6) ratio among all amino acids plays an important role in nitrogen metabolism among plants. Therefore, maintaining appropriate levels of arginine is critical to plants. Arginase catalyses the hydrolysis of arginine to ornithine and urea. Arginine is also a substrate for two other enzymes namely arginine decarboxylase (ADC) and nitric oxide synthase (NOS)[1]. Nitrogen is a limiting resource for plant growth. In plants, nitrogen is mainly stored in proteins as arginine. Hence, arginase mediated degradation of arginine in plants is thought to be involved in nitrogen re-assimilation [1]. The urea formed by arginase is further hydrolysed by urease to ammonia, which is later assimilated mainly by glutamine synthetase [1]. In many plants, it has been reported that arginase transcript increases with germination with concomitant increase in arginase activity [2-4]. Apart from playing many important role(s) in plant development [5-8], arginase in plants is also reported to perform a defensive role[9]. Moreover, arginase is also sensitive to both abiotic [10-12] and biotic stress [7, 13-15]. In addition, downstream catabolites of the arginase pathway such as polyamines, proline, ammonia, GABA, H_2O_2, nicotine – all are known to perform a variety of functions in stress management [12, 13, 15-22]. However, structural heterogeneity in terms of subunit organization exists among plant arginases [23-27]. For example arginase from *Glycine max* (soybean), has a molecular weight around 240kD with a subunit molecular weight of 60kD, suggesting the homo tetrameric nature of the enzyme [24]. In contrast, arginase from *Panax ginseng* (ginseng), is a homodecamer with the subunit molecular weight being 34.5kD [23]. In *Arabidopsis thaliana*[28] and *Solanum lycopersicum* (tomato) [7], two isoforms of arginase are reported. However, the precise oligomeric state of the native enzyme in these plants is not clear. Even though many studies have been conducted in the past, purification of arginase to its apparent homogeneity and its biochemical properties are studied only from few sources [23-27]. One reason for this is limited enzyme stability. For example, arginase from *Actinidia deliciosa* (Kiwifruit)[29], *Iris hollandica* (iris bulbs) [26], *Helianthus tuberosus* (Jerusalem artichoke tuber) [30] are not stable. Hence, keeping the enzyme stability in mind, we screened several plants for stable arginases. Recently we reported a highly stable L-arginase from cilantro, yet we are uncertain about its subunit organization [12]. Further, commercially antibodies to plant arginases are not yet available. While characterizing stable arginase from cilantro, we found horse gram seedlings possessing stable arginase among legumes. Hence, in the present study, we partially purified the enzyme by conventional chromatographic techniques. The biochemical properties of the partially purified enzyme were studied and compared with other plant arginases including cilantro arginase.

2. MATERIALS AND METHODS

2.1 Plant materials and chemicals

Horse gram (*Macrotyloma uniflorum*) seeds were procured from the local market Mysore, Karnataka, India. L-arginine monohydrochloride, L-canavanine sulphate, L-agmatine sulphate, L-argininamide, dithiothreitol (DTT) and dansyl chloride were purchased from Sigma Aldrich St. Louis, Missouri, USA. D-arginine, diacetyl monoxime (DAMO), thiosemicarbazide, sodium dodecyl sulphate (SDS), guanidine hydrochloride (GuHCl), 2-mercaptoethanol, hydroxylapatite, polyamines (putrescine, spermidine, spermine), were purchased from Sisco Research Laboratories (SRL), Mumbai, Maharashtra, India. Sephadex G-150 was purchased from Pharmacia Fine Chemicals, Piscataway, New Jersey, USA. Arginine separopore 4B was procured from bioWORLD, Dublin, Ohio, USA. DEAE– cellulose and L-homoarginine was obtained from Santa Cruz biotech, Santa Cruz, California, USA. Centricon filters (30 kDa) were from Merck Millipore, Billerica, Massachusetts, USA. *E.coli* DH5α (MTCC#1652) was obtained from Microbial Type Collection Centre (MTCC), Chandigarh, India. Other routinely used laboratory chemicals (analytical grade) were obtained from SRL, Mumbai, Maharashtra, India and RANKEM Gurugram, Haryana, India.

2.2 Arginase assay

The assay for arginase involves estimation of urea formed from arginine, according to the method of Coulombe and Favreau [31] and is described in our previous publication [12]. Briefly, the reaction mixture consists of enzyme (3-3.5 µg), buffered substrate (L-arginine monohydrochloride 130 mM, 50 mM Tris-HCl, 0.5 mM $MnCl_2$ whose final pH was 7.87). The total reaction volume was maintained at 100 µl. The reaction mixture was incubated at 37° C for 30 min. Following incubation, the reaction was arrested by adding 100 µl of 5 % TCA. Then 50 µl of 2 % diacetyl monoxime (DAMO) was added followed by 500 µl of colouring reagent (44 ml of H_2SO_4, 66 ml of orthophosphoric acid +2 g cadmium sulphate and 50 mg thiosemicarbazide- made up to 1000 ml). Then the tubes were immediately transferred to a boiling water bath for 10 min and the colour developed was read at 540 nm. The amount of urea released was determined using urea standard with a linear range between 1-5 µg/100 µl. One unit is defined as the amount of enzyme producing 1 nmole of urea per min.

2.3 Estimation of proteins

Proteins were estimated by Bradford's method using bovine serum albumin (BSA) as standard [32].

2.4.1 Partial purification of arginase from horse gram seedlings

1150 g of 7-8 days old horse gram seedlings were harvested and homogenised in 50 mM Tris-HCl (pH 7.5) containing 0.5 mM $MnCl_2$. The homogenate was filtered using clean muslin cloth and centrifuged at 15,000 xg for 10 min. The supernatant was then saturated with solid ammonium sulphate (50 %) and stirred at 4° C for overnight. The resulting solution was centrifuged at 20,000 xg for 5 min at 4° C. The supernatant was discarded as it was devoid of arginase activity and the pellet was dissolved in 10 mM Tris-HCl buffer pH 7.5 containing 0.5 mM $MnCl_2$ and dialyzed against 2 liters of the same buffer with 3 changes. The dialysate was heat treated at 70° C for 60 min. The heat-treated sample was centrifuged at 20,000 xg for 5 min to remove the heat-denatured proteins. The heat treated sample was concentrated and then applied to a sephadex G-150 column (120 x 1 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM $MnCl_2$. Further, the active fractions from sephadex G-150 were concentrated and applied to DEAE-cellulose anion exchange column (30 x 1.2 cm) previously equilibrated with 10 mM Tris-HCl buffer

(pH 7.5). Proteins were eluted by increasing the concentration of NaCl from 0-1 M in equilibration buffer (10 mM Tris-HCl buffer pH 7.5). 0.5 mM $MnCl_2$ was provided directly to each 2 ml fraction. The active fractions were pooled and concentrated using centricons. The protein solution was then applied to a previously equilibrated hydroxyapatite column (8 x 1.2 cm) with 10 mM Tris-HCl buffer pH 7.5. Then the proteins were eluted by increasing the concentration of potassium phosphate from 0 to 250 mM and $MnCl_2$ was provided directly to each 2 ml fractions as before. The hydroxyl apatite fractions were checked for arginase activity. The active fractions from hydroxyl apatite were pooled, concentrated and applied to an arginine-separopore 4B affinity column (4 x 1.4 cm) previously equilibrated with 10 mM Tris-HCl buffer (pH 7.5) and arginase was eluted by increasing the concentration of NaCl (0–250 mM). $MnCl_2$ was exogenously added to each fraction.

2.5 Electrophoresis

SDS-PAGE was performed as described by Laemmli [33]. From each purification step, the samples with known protein concentration were treated with a reducing buffer for 5 min at 100° C and then subjected to 10 % polyacrylamide gel electrophoresis and stained with silver. 10 % native polyacrylamide gel (Basic-PAGE) was performed for all the samples by mixing them with a non-denaturing, non-reducing sample buffer and the gel was silver stained.

2.5.1 Biochemical characterization of partially purified horse gram arginase

In order to determine the Michaelis-Menten constant and maximum velocity, the concentrations ranging from 5 to 225 mM L-arginine were used and urea formed was estimated as described above. Values of initial velocity (Vo) were plotted against increasing concentration of L-arginine and fitted to Michaelis-Menten equation. The statistical parameters that were considered in order to determine the best-fit values were standard errors, 95 % confidence intervals and values of regression coefficients (R^2). The pH kinetics of the enzymatic activity of partially purified horse gram arginase was determined by replacing the buffer used in the respective enzyme assay with the buffers of specific pH: acetate buffer pH 5.0, phosphate buffer pH 6.0, Tris- HCl pH 7.0, 7.5, 8.0, 8.5 and 9.0. The optimum temperature for the horse gram arginase was carried out by varying the reaction temperature from 4° C to 80° C. Substrate specificity of the arginase was performed by substituting L-arginine with other substrate analogues (L-agmatine, L-argininamide, L-canavanine, L-homoarginine and D-arginine), to a final concentration of 130 mM. To determine the concentration-dependent effect of $MnCl_2$ and the effect of other divalent cations on arginase activity, the $MnCl_2$ depleted (by dialysis against $MnCl_2$ free buffer) sample was pre-incubated with indicated (0.5–200 mM) concentration of $MnCl_2$ or other cations with indicated (0.5–10 mM) concentrations at 37° C for 60 min. Enzyme modulators such as β - mercaptoethanol, DTT, EDTA and GuHCl in the concentration range of 0-100 mM, 0-100 mM, 0-10 mM and 0-2 M respectively were used in the arginase assay to check their effects on partially purified arginase. Effect of polyamines (spermine, spermidine and putrescine) was also assayed in the concentration range of 0.01 – 100 mM. Effect of various amino acids (proline, valine, ornithine, leucine, isoleucine and lysine) and substrate analogues (L-argininamide, L-canavanine and L-agmatine) was also tested in the concentration range of 0.1-25 mM. The type of inhibition and the Ki values were determined by plotting the initial velocity (V_0) as measured by amount of urea formed/mg of protein/min versus [L-arginine] in the absence and presence of various known concentration of inhibitor(s) used in the assay (substrate analogues and amino acids). The data points were then fitted to the Michaelis-Menten equation and mode of inhibition was assessed. The statistical parameters that were considered in order to determine the best-fit data points were standard errors, 95 % confidence intervals and values of regression coefficients (R^2). Graph pad prismV5.0 was used for data fitting and estimation of rate constants.

2.6 Agmatinase assay

An HPLC based detection of dansylated putrescine method was used to assess agmatinase activity as before [12]. Briefly, the reaction mixture consisted of enzyme, substrate (L-Agmatine, 1 mM or 50 mM), 50 mM Tris-HCl buffer (pH 8.5) containing 0.5 mM $MnCl_2$, in a total volume of 100 µl. The reaction mixture was incubated at 37° C for 30 min. After incubation, 100 µl of 5 % TCA was added to stop the reaction. Dansyl derivatization of the enzyme assay samples were performed as described by Marce et al [34]. The dansylated amines were separated and quantified by HPLC with Shimadzu UFLC – LC – 20AD series, amenable 5 µm C18, 120 Å, 250 × 4.6 mm LC (A8-ST5C18G120-98) column. Samples were eluted from the column with a solvent system consisting of acetonitrile: water (72:28 v/v). As a positive control for agmatinase activity, *E. coli* lysate extracted in 50 mM Tris-HCl buffer pH 7.5 containing 0.5 mM $MnCl_2$ was used. A minimum amount of 0.2 µg of dansylated putrescine can be detected by this HPLC method.

3. STATISTICAL ANALYSIS

All experiments were repeated at least three times independently. In some experiments like the effect of polyamines on arginase activity (*in vitro*), statistical significance among groups was determined by one-way analysis of variance (ANOVA). The values are presented as mean ± SD. Differences between the values were considered significant, if the P value < 0.05.

4. RESULTS AND DISCUSSION

4.1 Arginase from horse gram seedling does not loose activity upon storage

In the past, several plant arginases reported are not stable. For example, arginase from kiwifruit lost 25 % of its initial activity overnight, while 60 % of its activity was lost within 6 days [29]. Similarly, arginase from Iris bulbs [26], Jerusalem artichoke tuber [30] were also unstable. In contrast to these, arginases from ginseng [23], loblolly pine [25] and cilantro [12] are reported as stable. Apart from this, low abundance and low yield resulted in lack of interest among the scientific community on arginase. During our search for stable arginase, we found horse gram seedlings possessing stable arginase similar to cilantro [12]. Although we thoroughly characterized cilantro arginase, we were unable to molecularly characterize the subunit organisation. Therefore, we sought to characterize other stable arginase(s) from horse gram seedlings. Moreover, horse gram seeds readily germinate under laboratory condition and are easily available.

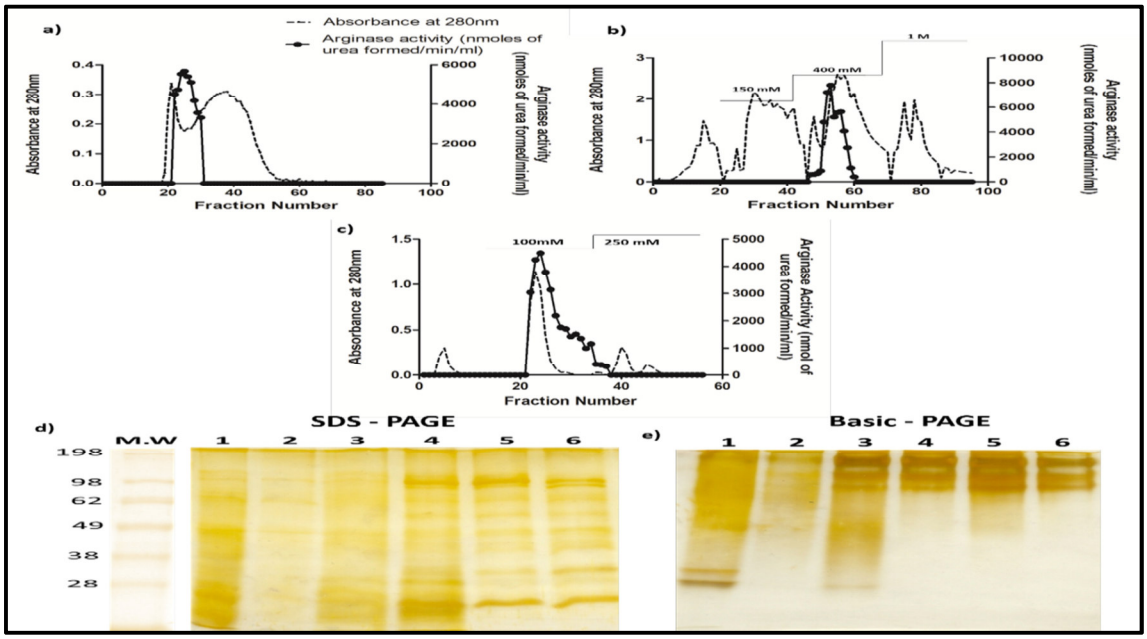
4.2 Partial purification of horse gram arginase

Results from the partial purification of horse gram arginase are summarized in table 1. The enzyme was partially purified by using various conventional chromatographic methods with a specific activity of 13752 nmoles of urea formed/mg of protein/min with 869 fold enrichment and 7.4 % recovery (table 1 and fig 1). Ammonium sulphate fractionation removed bulk of non-arginase proteins, with 4 fold purification and a specific activity of 64.47 nmoles of urea formed/mg of protein/min (table 1). Like arginase from ginseng [23], loblolly pine [25] and cilantro [12], horse gram arginase is also heat stable (stable for up to 1 hour at 70°C). This allowed the removal of a large amount of heat labile non-arginase proteins from the previous step resulting in 95-fold enrichment in activity (table 1). The heat-treated sample was concentrated using centricons and applied to sephadex G-150 to separate lower molecular weight proteins and aggregated proteins (fig 1a). The active fractions (eluting from 22-30) were pooled and further separated by DEAE-cellulose. The adsorbed arginase in DEAE-cellulose was eluted by increasing the NaCl concentration (0-1M NaCl) (fig 1b). Arginase eluted at 400 mM NaCl concentration (fractions 47-60). These active fractions were concentrated and applied to the hydroxyapatite column (fig 1c). The enzyme was eluted at 100 mM potassium phosphate (fractions 22-37) with 869 fold purification (table 1). Though we used arginine – separopore 4B affinity matrix for further purification, unfortunately we didn't achieve much purity (data not shown). Both in Arabidopsis [28] and tomato [7] two genes for arginase are reported. Although soybean is known to possess four paralogue genes for arginase [10], purification of arginase by Kang et al identified homotetramer form of arginase, with the subunit mass of 60 kD [24]. In contrast to this single gene for arginase is reported in loblolly pine [10, 35] and rice [10]. Apart from variations in the number of paralogous genes present among different plants, the subunit organization of plant arginase differs from source to source [23-27]. Though our initial aim was to characterize the subunit organisation of horse gram arginase, unfortunately, we could purify the enzyme only partially. A SDS-PAGE analysis under reducing condition and basic PAGE prolife from each purification step is shown in fig 1d and 1e. Hence, further purification of arginase from horse gram would help to reveal its subunit organization and the presence of isoforms, if any.

Table 1:Purification of arginase from horse gram seedlings

Fraction	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Purification (Fold)	Yield (%)
Crude homogenate	14040	222174	15.82	-	100 %
Ammonium sulphate precipitation 40-50 %	3105	200196	64.47	4.07	90.1 %
Heat treatment	64.25	97402.5	1515.99	95.82	43.84 %
Sephadex G-150	16.452	33686.28	2047.54	129.42	15.16 %
DEAE- Cellulose	3.487	29792.42	8543.85	540.06	13.40 %
Hydroxyapatite	1.2	16503.5	13752.9	869.33	7.4 %

The ammonium sulphate preparation and pooled fractions from DEAE-cellulose, hydroxyl apatite were assayed for arginase activity after dialysis (10 mMTris-HCl buffer (pH 7.5), 0.5 mM $MnCl_2$). The table describes the steps employed to purify arginase from horse gram seedlings.



Profiles of protein (dotted line) and arginase activity (continuous line with closed circle) from (a) Sephadex G-150, (b) DEAE-Cellulose, (c) Hydroxyapatite column chromatography. d) SDS-PAGE profile from each purification step. e) Basic-PAGE profile from each purification step. Lanes: 1) Crude homogenate (20 µg), 2) 50 % ammonium sulphate fractionation (following desalting by dialysis) (10 µg), 3) Supernatant from heat-treated homogenate (20 µg), 4) Sephadex G-150 active fraction (15 µg), 5) DEAE- cellulose active fraction (14 µg), 6) Hydroxyapatite active fraction (8 µg).

FIg 1: Purification profiles and electrophoretic analysis of partially purified horse gram arginase

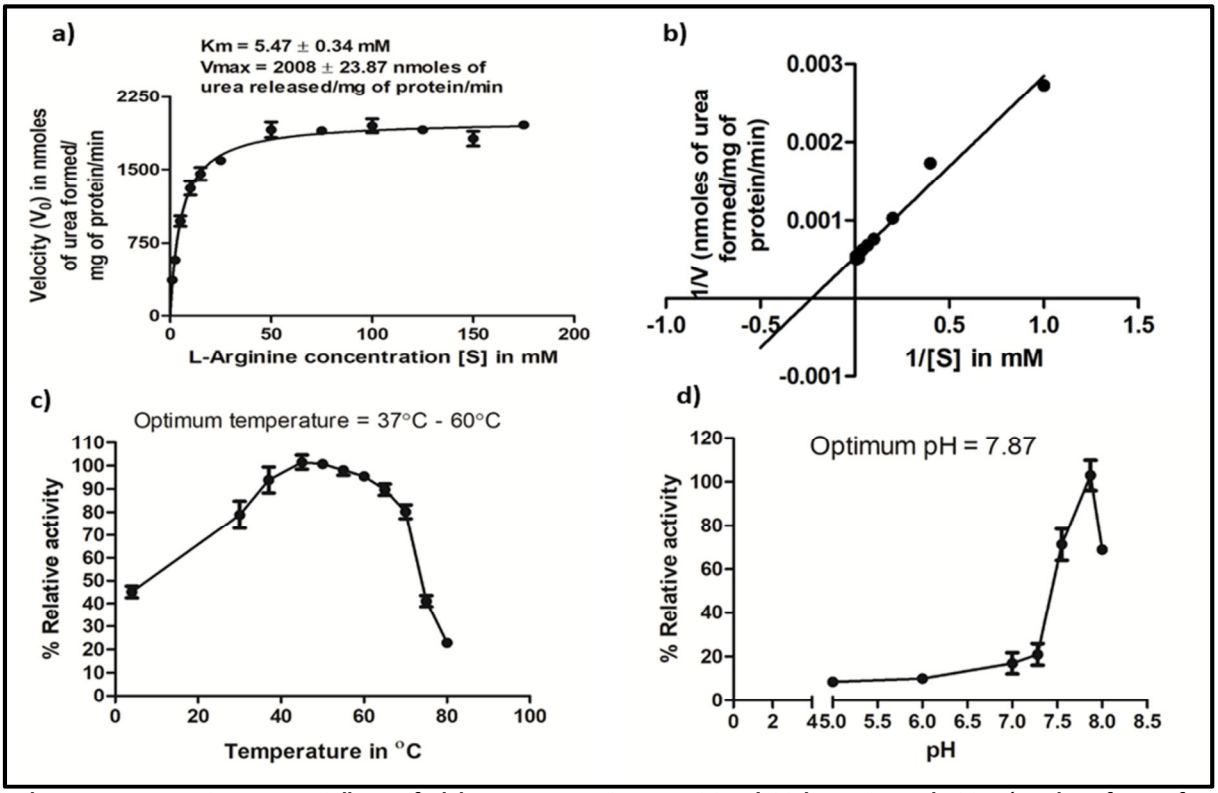
4.3 *Biochemical properties of partially purified horse gram arginase are different from other plant arginases*

4.3.1 *Determination of Km*

Though both cilantro and horse gram arginases are stable, the biochemical properties are different. The apparent Km for partially purified horse gram arginase was found to be 5.47 ± 0.37 mM (fig 2a) and it is very similar to Km reported for kiwifruit [29]. However, arginase from cilantro and other sources have higher Km values [7, 12, 23, 24, 30]. For example, arginase from Jerusalem artichoke tubers has a Km of 145 mM [30], whereas soybean [24] and ginseng [23] arginase have Km values of 83 mM and 82.7 mM respectively.

4.3.2 *Effect of temperature and pH*

The optimum temperature of the horse gram arginase lies between 37-60° C (fig 2c) and is similar to the optimum temperature reported for cilantro [12], ginseng [23] and cowpea [27] arginase. Like all other plant arginases, horse gram arginase also shows highest activity at alkaline pH (pH 7.87) (Fig 2d) [7, 12, 23, 24, 27, 29].



Effect of substrate concentration on partially purified horse gram arginase activity. Initial reaction velocities (nmoles of urea formed/mg of protein/min) were plotted against various concentrations of L-arginine and fitted to Michaelis-Menten (a) and Lineweaver-Burk (b) plot. Effect of temperature (c) and pH (d) on arginase activity was carried out at various temperature and buffers as described under methods.

Fig 2: Kinetic parameters of partially purified arginase

4.3.3 *Substrate specificity of horse gram arginase*

The sequence alignment between plant and non-plant arginase revealed that most of the plant arginases are more similar to agmatinase than to other non-plant arginases [7, 36]. Hence, the substrate specificity of horse gram arginase was tested with various arginine analogues. Most of the plant arginases are not specific for L-arginine and they show detectable activity with other arginine analogues [7, 23, 26, 27, 37, 38]. In contrast, cilantro arginase hydrolyses only L-arginine and not any other arginine analogues including L-homoarginine [12]. Intriguingly, horse gram arginase not only hydrolysed L-arginine, it also exhibited hydrolytic activity towards L-homoarginine (table 2).

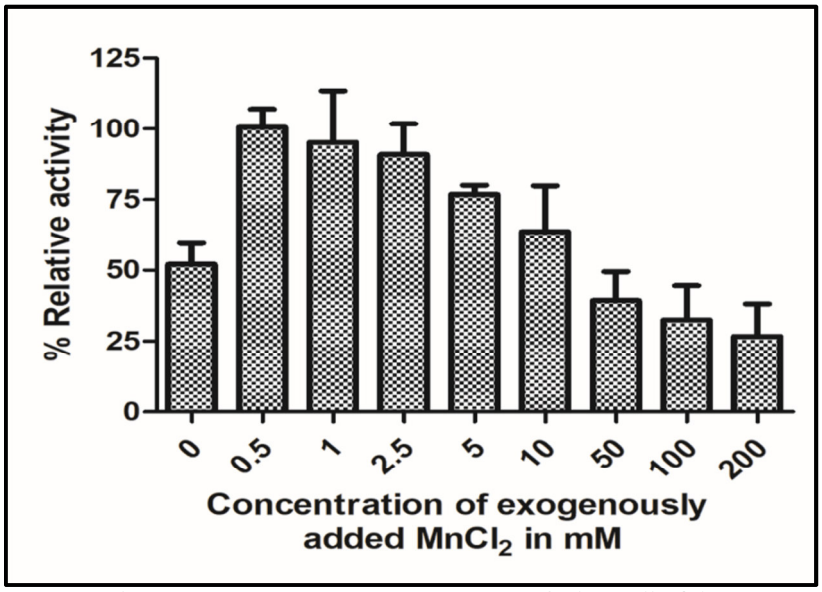
Table 2 :Substrate specificity of horse gram arginase

Substrate	Horse gram arginase Specific activity (nmoles of urea formed/mg of protein/min)
L-Arginine	1477.97 ± 48.43 (100 %)
D-Arginine	ND
L-Agmatine	ND
L-Argininamide	ND
L-Canavanine	ND
L-Homoarginine	106.55 ± 18.43 (7.2 %)

Partially purified horse gram arginase was incubated with analogues of arginine and then activity was assayed as described in the method section. N.D. stands for 'not detected'.

4.3.4 *Effect of various divalent metal ions, polyamines, amino acids, arginine analogues and enzyme modulators*

Arginase is a manganese –dependent enzyme in almost all plant arginases reported, where it acts as a cofactor/activator [1]. Partially purified horse gram arginase showed maximum activity in the presence of 0.5 mM Mn^{2+} (fig 3), while Mg^{2+} showed 10 % of the activity exhibited by Mn^{2+} (table 3). Co^{2+} and Ni^{2+} were unable to substitute for Mn^{2+}. This is in contrast to cilantro arginase where 0.5 mM Co^{2+} and Ni^{2+} partially could substitute for Mn^{2+}[12], similar to kiwifruit arginase [29].



$MnCl_2$ was removed from partially purified arginase by extensive dialysis against $MnCl_2$ free buffer (10 mM Tris HCl buffer, pH 7.5). Then the enzyme was incubated with indicated concentration of $MnCl_2$ at 37° C for 60 min and activities were assayed as described under method section. Arginase activity obtained with 0.5 mM $MnCl_2$ was considered as 100 % activity.

Fig 3: Concentration-dependent effect of $MnCl_2$ on arginase activity

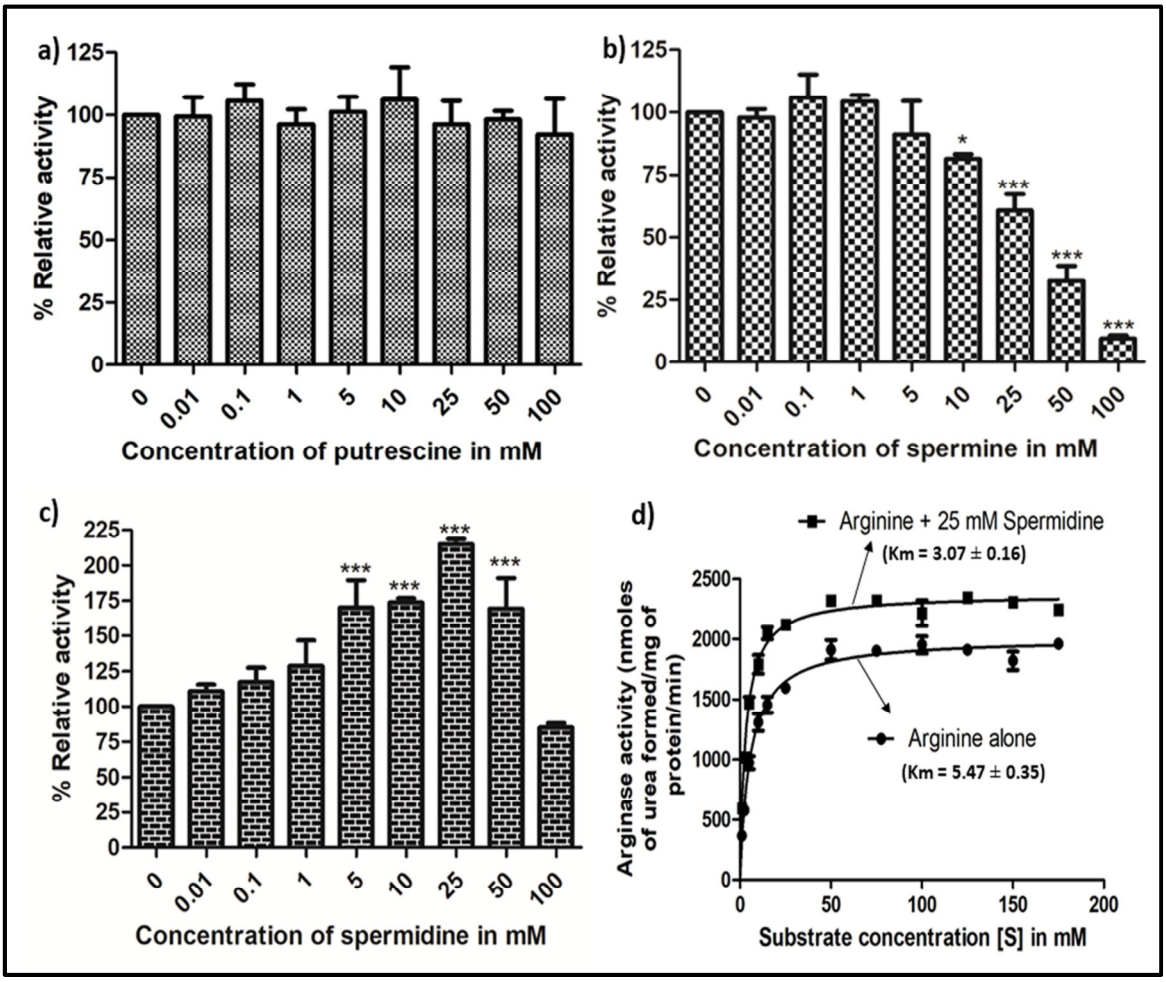
Table 3 :Effect of various metal ions on horse gram arginase activity

Metal ions	Concentration (mM)	Relative activity (%)
$MnCl_2$	0.5	100
	1	78.90 ± 15.43
	10	50.68 ± 21.04
$CaCl_2$	0.5	ND
	1	ND
	10	ND
$MgCl_2$	0.5	10.10 ± 1.23
	1	9.83 ± 2.90
	10	7.41 ± 5.28
$CdCl_2$	0.5	ND
	1	ND
	10	ND
$CoCl_2$	0.5	ND
	1	ND
	10	ND
$ZnCl_2$	0.5	ND
	1	ND
	10	ND
$NiCl_2$	0.5	ND
	1	ND
	10	ND
$CuCl_2$	0.5	ND
	1	ND
	10	ND

$MnCl_2$ depleted arginase was pre-incubated with indicated concentration of various metal ions at 37° C for 60 min, and then activities were assayed as described in the method section. Arginase activity is summarized as a percentage of control activity in the presence of 0.5 mM $MnCl_2$. N.D. stands for 'not detected

Arginine is a precursor for polyamine and various amino acid biosynthesis [1, 16]. Hence, in the next set of experiments, we tested the effect of various polyamines and amino acids on partially purified horse gram arginase. Putrescine had no effect for upto 100 mM concentration on partially purified horse gram arginase (fig 4a), while spermine progressively inhibited the activity between 10 -100 mM concentration (fig 4b). These results are very different from the effect of

polyamines we observed for cilantro arginase. Both putrescine and spermine at lower (0.01 mM) concentration enhanced the activity, while beyond 10 mM inhibited the activity of cilantro arginase [12]. On the other hand, spermidine progressively enhanced the activity of horse gram arginase between 0.01 to 50 mM and at 100 mM it inhibited the 20 % of the arginase activity (fig 4c). The effect of spermidine was very different for cilantro arginase. Between 0.01 – 1 mM concentration it inhibited the activity, while between 5 – 25 mM spermidine concentrations, it stimulated the activity of cilantro arginase. Beyond 50 mM concentration, again it inhibited the activity of cilantro arginase [12]. Since only spermidine exhibited a stimulatory effect for horse gram arginase, we calculated the Km value for L-arginine in presence and absence of spermidine. The Km value decreased for L-arginine from 5.47 ± 0.34 to 3.07 ± 0.16 in the presence of 25 mM spermidine (fig 4d). In contrast to these results, arginases from soybean [24] and ginseng [23] reported a stimulatory effect with all the polyamines at tested concentration (0.01-10 mM).



Concentration - dependent effect of Putrescine (a), Spermine (b) and Spermidine (c) on partially purified arginase activity. Arginase activity was considered as 100%, where enzyme was incubated in the absence of polyamines. d) MM-plot for reaction of arginase with arginine alone and arginine with spermidine (25 mM). Note: statistical significance was calculated for actual values (not for % relative activity). *P < 0.05; **P < 0.001; ***P < 0.0001 as compared with control without polyamines

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Fig 4: Effect of polyamines on horse gram arginase

Among the amino acids tested only L-ornithine, L-lysine, L-leucine and L-isoleucine inhibited the partially purified horse gram arginase activity (table 4), similar to cilantro arginase [12]. However, the type of inhibition exhibited by these amino acids is different except for L-lysine and it is summarised in table 4. In contrast to these results, Dabir et al reported competitive inhibition of cowpea arginase by L-proline [39], which did not show any effect on either cilantro or on horse gram arginase (table 4). We also tested the effect of various arginine analogues on the activity of partially purified horse gram arginase. Among these, L-agmatine and L-argininamide did not show any effect, while L-canavanine inhibited horse gram arginase with mixed type of inhibition (Ki value 4.83 ± 1.13) (table 4). This is in contrast to cilantro arginase, where L-argininamide inhibits the activity of the enzyme (Ki value 9.22 ± 2.11) [12]. A different arginine analogue L-agmatine selectively inhibited ginseng arginase [23].

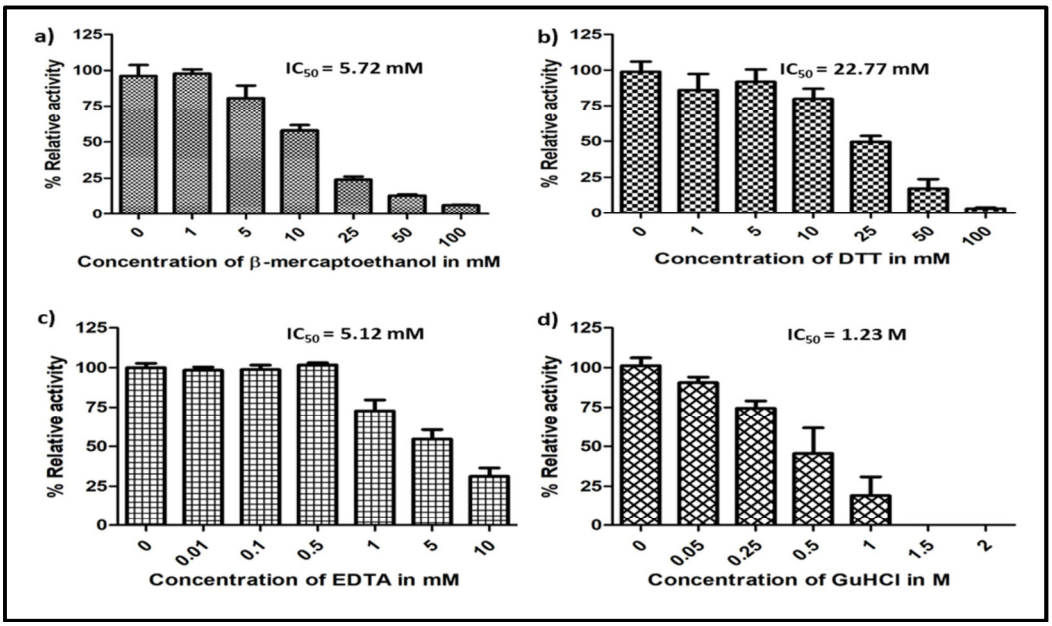
Table 4 :Inhibition studies of horse gram arginase in comparison with cilantro arginase by substrate analogues and amino acids.

Inhibition by amino acids/substrate analogues	Horse gram arginase (present study)		Cilantro arginase [12]	
	Ki (mM)	Nature of inhibition	Ki (mM)	Nature of inhibition
L-Ornithine	12.10 ± 2.63	Mixed	7.37 ± 0.82	Competitive

L-Lysine	1.03 ± 0.17	Mixed	2.43 ± 0.46	Mixed
L-Leucine	13.23 ± 2.09	Mixed	11.35 ± 0.84	Non-competitive
L-Isoleucine	9.87 ± 1.67	Mixed	17.22 ± 1.01	Non-competitive
L-Proline	No inhibition	Not applicable	No inhibition	Not applicable
L-Valine	No inhibition	Not applicable	No inhibition	Not applicable
L-Canavanine	4.83 ± 1.13	Mixed	10.95 ± 0.62	Non-competitive
L-Agmatine	No inhibition	Not applicable	No inhibition	Not applicable
L-Argininamide	No inhibition	Not applicable	9.22 ± 2.11	Mixed

The type of inhibition and the Ki values were determined by plotting the initial velocity (V_0) as measured by amount of urea formed/mg of protein/min versus [L-arginine] in the absence and presence of various known concentration of inhibitor used in the assay (substrate analogues and amino acids).

Reducing agents such as β -mercaptoethanol and DTT inhibited the partially purified horse gram arginase activity with an IC_{50} value of 5.72 mM and 22.77 mM respectively (fig 5a and 5b). Arginase is a divalent metal ion dependent enzyme, hence we also checked the effect of metal ion chelator-EDTA on arginase activity. EDTA inhibited the enzyme activity with a Ki value of 5.12 mM (fig 5c). GuHCl, the denaturant used in protein unfolding studies also inhibited the enzyme activity (fig 5d). Although the effect of above agents on cilantro arginase is similar, IC_{50} values were much lower [12]. Similarly, in tomato Chen et al showed the moderate inhibition of arginase by β-mercaptoethanol [7]. All these studies suggest that differences in the biochemical characteristics do exist among plant arginases.

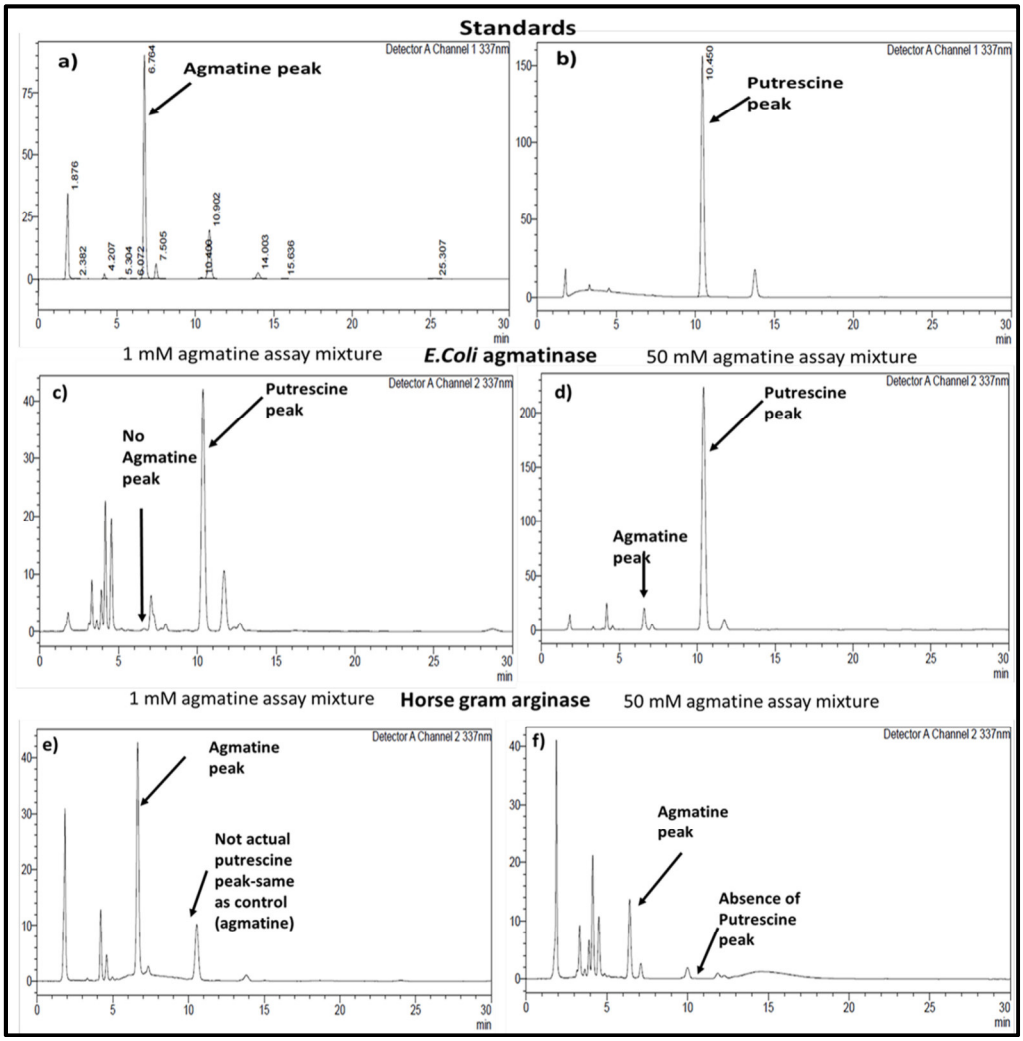


Effect of a) β-mercaptoethanol (1–100 mM), b) DTT (1–100 mM), c) EDTA (0.01–10 mM) and d) GuHCl (0.05–2 M) on arginase activity. The IC_{50} values for each inhibition was determined using graphpad prism 5: Note: In the absence of a commonly used enzyme modulator, the enzyme activity was considered as 100%.

Fig 5: Effect of enzyme modulators on horse gram arginase

4.3.5 Partially purified horse gram arginase lack agmatinase activity

As discussed before arginine is the precursor molecule for the biosynthesis of polyamines [1, 16]. In plants, polyamine biosynthesis takes place mainly via arginine decarboxylase and ornithine decarboxylase mediated pathway [1]. Recently Patel et al proposed dual functioning of plant arginase as agmatinase and its role in polyamine biosynthesis [40]. Hence, we also tested the possibility of presence or absence of agmatinase activity in horse gram arginase preparation. *E.coli* lysate was taken as positive control for these experiments. HPLC based detection of dansylated putrescine was adopted to check the agmatinase activity and we were not able to detect any agmatinase activity in partially purified horse gram arginase at both low (1 mM) and high (50 mM) agmatine concentration (fig 6e and 6f) using this technique, while *E.coli* lysate exhibited immense agmatinase activity as expected (fig 6c and 6d). These results are in accordance with our studies with cilantro arginase, which also lack agmatinase activity [12] based on this method.



A HPLC – based detection of dansylated product and substrate analysis was carried out. a) Standard dansylated agmatine (retention time 6.7 min – 20 μg) b) Standard dansylated putrescine (retention time 10.45 min – 1 μg) c) Dansylated reaction products of E.coli lysate agmatinase in presence of 1 mM agmatine sulfate and d) 50 mM agmatine sulfate, e) Dansylated reaction products of horse gram arginase in presence of 1 mM agmatine sulfate and f) 50 mM agmatine sulfate. Note: A minimum amount of 0.2 μg of dansylated putrescine can be detected by this HPLC method.

Fig 6: HPLC analysis of dansylated derivatives of agmatinase assay reaction products

5. CONCLUSION

Arginase from horse gram seedlings represents one of the stable arginases apart from cilantro among the screened plants. The enzyme is partially purified by using various conventional column chromatographic techniques such as sephadex G-150, DEAE-cellulose, and hydroxyapatite. Like cilantro arginase, horse gram arginase is also a heat stable enzyme; hence, heat treatment is used as one of the purification method. Further, the biochemical properties of partially purified enzyme is characterized and are found to be different from other plant arginases. The arginine derived polyamines and amino acids regulate the horse gram arginase *invitro*. Like all other plant arginases, horse gram arginase is also dependent on Mn^{2+} for its activity. The other divalent cation such as Mg^{2+} can partially restore the activity of horse gram arginase. EDTA and sulfhydryl reducing agents inhibit the horse gram arginase activity. In addition, GuHCl, one of the strongest denaturant used to study physicochemical unfolding of proteins also inhibited the activity of horse gram arginase. In contrast to cilantro arginase, horse gram arginase shows hydrolytic activity towards both L-arginine and L-homoarginine. Further purification would help in elucidating its subunit organisation. The studies on the effect of biotic and abiotic stress on horse gram arginase need to be examined. Such studies are likely to help in understanding the role of arginase in stress management.

6. AUTHOR CONTRIBUTION STATEMENT

Gopal Kedihithlu Marathe envisaged the original concept, designed the experiments and critically evaluated the manuscript. Shiva Siddappa performed the major experiments and wrote the manuscript. Semira Shimeles Assefa and Bettadapura Rameshgowda Nuthan performed some of the experiments.

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University Grants Commission - Special Assistance Program (for department infrastructure development, UGC-SAP, F.3-14/2012/SAP-II) India, and the Vision Group of Science & Technology (for department infrastructure development, VGST/P-15/K-FIST level 2/2010-11), Government of Karnataka, India. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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