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The kinetics of inhibition of *Vigna catjang* cotyledon and buffalo liver arginase by L-proline and branched-chain amino acids

SNEHAL DABIR[1], PANKAJ DABIR[2], & BABURAO SOMVANSHI[1]

[1]*Department of Biochemistry, Dr BA Marathwada University, Aurangabad –400 001 (MS), India, and* [2]*Department of Biochemistry and JB Tropical Disease Research Centre, Mahatma Gandhi Institute of Medical Sciences, Sevagram–442 102, Wardha, (MS), India*

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Abstract
The effect of proline, isoleucine, leucine, valine, lysine and ornithine under standard physiological conditions, on purified *Vigna catjang* cotyledon and buffalo liver arginases was studied. The results showed that *V. catjang* cotyledon arginase is inhibited by proline at a lower concentration than buffalo liver arginase and the inhibition was found to be linear competitive for both enzymes. Buffalo liver arginase was more sensitive to inhibition by branched-chain amino acids than *V. catjang* cotyledon. Leucine, lysine, ornithine and valine are competitive inhibitors while isoleucine is a mixed type of inhibitor of liver arginase. We have also studied the effect of manganese concentration which acts as a cofactor and leads to activation of arginase. The optimum Mn^{2+} concentration for *Vigna catjang* cotyledon arginase is 0.6 mM and liver arginase is 2.0 mM. The preincubation period required for liver arginase is 20 min at 55°C, the preincubation period and temperature required for activation of cotyledon arginase was found to be 8 min at 35°C. The function of cotyledon arginase in polyamine biosynthesis and a possible role of branched chain amino acids in hydrolysis of arginine in liver are discussed.

Keywords: *Arginase, branched chain L-amino acids, inhibition, kinetics, Mn^{+} ion concentration, buffalo liver,* Vigna catjang *cotyledon*

Introduction

Arginase (L-arginine amidino hydrolase, EC 3.5.3.1) catalyzes the hydrolysis of L-arginine to urea and the non-protein amino acid, L-ornithine. Urea is the principal metabolite for disposal of nitrogen in the form of a non-toxic and neutral final waste product during amino acid metabolism in mammals. L-ornithine acts as a biosynthetic precursor for proline, ornithine, glutamate and polyamines such as putrescine, spermine (eukaryotes) and spermidine (prokaryotes) [1–3]. As the urea cycle is not operative in plants, most of the studies on plant arginase have focused on its role in mobilizing arginine to provide carbon and nitrogen for expanding new organs during early seedling germination [4]. L-arginine is one of the most functionally diverse amino acids in the living cell. In addition to serving as a protein constituent, it is one of the prominent amino acids that can account for 50% of nitrogen in seed protein and up to 90% of free nitrogen in vegetative tissues [5–12]. In several plant species, including soybean, broad bean, pumpkin, *Arabidopsis thaliana* and loblolly pine, nitrogen mobilization during seedling development is correlated with large increases in arginase expression [11–13]. The coordinate action of arginase and urease is thought to recycle urea nitrogen to meet the metabolic demands of developing seedlings [11,14]. Arginase was found to exist in two forms and has a broad tissue distribution [15,1,2]. One of the forms, AI is located in the cytoplasm and is highly expressed in liver or hepatic cells. The extrahepatic AII form of

Correspondence: Dr. Snehal Dabir, Department of Microbiology, Immunology & Cell Biology Health Sciences Centre, School of Medicine, West Virginia University, Morgantown, WV, 26506, USA. E-mail: snehal_214@rediffmail.com

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arginase is found in mitochondria and has a wider tissue distribution [1,2].

In our earlier report [16], we found that arginases purified from *Vigna catjang* cotyledon and buffalo liver, differ in their optimal condition and physicochemical properties. By considering the above data and the possible functions of *V. catjang* cotyledon and buffalo liver arginases in amino acid mobilization and the urea cycle, the effects would be of special interest because of the precursor product relationship between arginine, proline and the branched chain amino acids involved in sparing free nitrogen. The present study was therefore undertaken to investigate the effect of these compounds under physiological conditions in detail.

Materials and methods

Source of enzyme

Vigna catjang cotyledon and buffalo liver arginase were purified as described by Dabir et al. [16], by employing the purification techniques of ammonium sulphate precipitation, ion exchange chromatography, gel filtration and AH-Sepharose 4B affinity chromatography.

Arginase activity assay

The arginase activity of the *V. catjang* cotyledon and hepatic tissue was determined by measuring the production of urea according to the procedure of Marsh WH et al. [17]. In brief, the reaction mixture consisting of 10 mM carbonate/bicarbonate buffer (pH 10), 2 mM $MnCl_2$, 130 mM L-arginine and enzyme solution in a total of 1 mL was incubated for 30 min at room temperature. The enzymatic reaction was terminated by adding 1mL of 10% TCA. Two mL of reaction mixture was taken for urea estimation. The control tube was run simultaneously by adding specific substrate (arginine) other than the compound tested as substrate (s) for arginase. The tubes were centrifuged, 2 mL of the clear supernatant was transferred from each tube and the enzyme activity was recorded by spectrophotometric detection of urea at A_{520} nm (UV 160A Shimatzu, Japan.) against the reagent blank by using diacetyl monoxime as described by Marsh WH et al. [17]. One unit of arginase activity was expressed as the amount of enzyme catalyzing the formation of one μmole of urea per min at 37°C. The enzyme assay and experimental conditions used for buffalo liver arginase were the same as described above for the *Vigna catjang* arginase, except for the concentrations of $MnCl_2$ (6 mM), L-arginine (25 mM) and carbonate bicarbonate buffer (50 mM, pH 9.2) used.

The amino acids (proline, isoleucine, leucine, valine, lysine and ornithine) studied for their effect on buffalo liver and *Vigna catjang* cotyledon arginase were prepared in 50 mM and 10 mM carbonate buffer respectively. The L-amino acids and all other chemicals were purchased from Sigma Aldrich, St. Louis USA.

Protein estimation

The protein content was measured by the method of Lowry et al. [18] using bovine serum albumin (Sigma, USA) as a standard.

Results

The kinetic properties of *V. catjang* cotyledon and buffalo liver arginases were studied and compared. All the enzyme assays were carried out under conditions which measured the initial velocities.

Influence of preincubation period on V. catjang *cotyledon and buffalo liver arginases*

Cotyledon arginase showed highest activity after a preincubation of 8 min at 35°C temperature. The sample with preincubation showed increased activity by approximately two-fold (Figure 1A). A 20 min

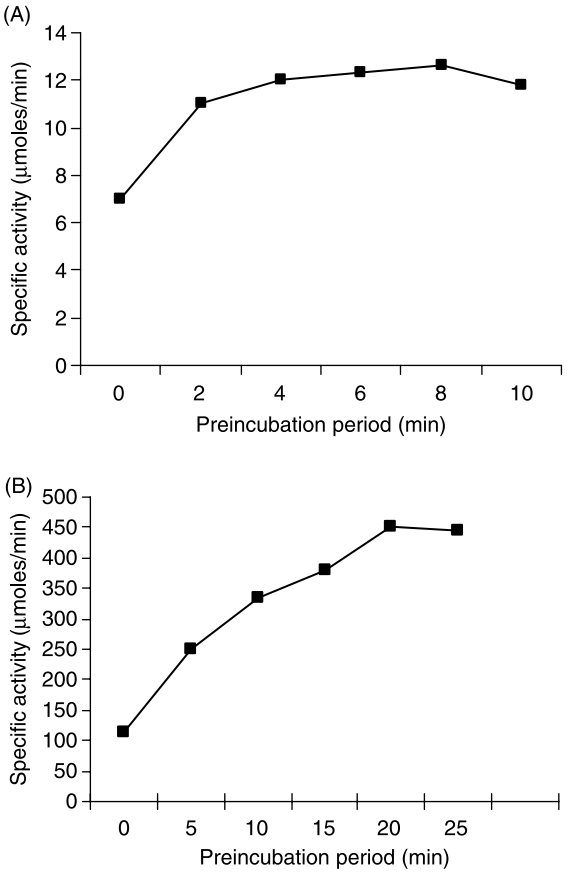

Figure 1. (A): Influence of preincubation period on *V. catjang* cotyledon arginase. (B): Influence of preincubation period on buffalo liver arginase.

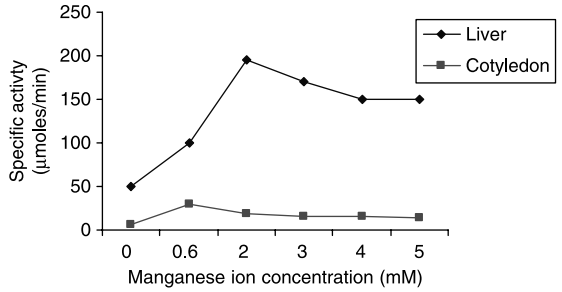

Figure 2. Effect of manganese ion concentration on cotyledon and liver arginases.

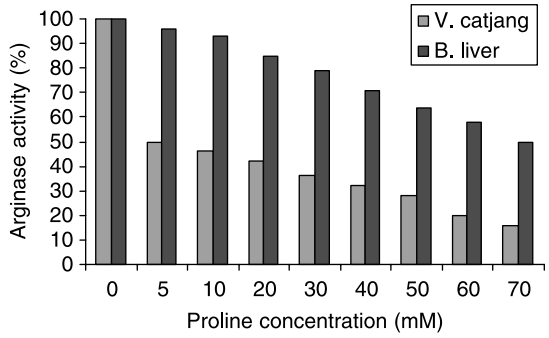

Figure 3. Effect of proline concentration on the activity of *Vigna catjang* cotyledon arginase and buffalo liver arginase.

preincubation at 55°C increased the enzyme activity approximately 4-5-fold in the liver. Therefore, for liver arginase, the optimum preincubation period was determined to be 20 min at 55°C (Figure 1B). Thus, this result shows that preincubation period and temperature had not much significance for the activation of cotyledon arginase.

Influence of manganese concentration

To determine the influence of manganese ions on the arginases activity, manganese ions at varying concentrations were added during preincubation. Preincubation at 55°C for 20 min with a 2 mM manganese ion concentration fully activated liver arginase activity and preincubation at 35°C for 8 min with 0.6 mM manganese ion concentration fully activated cotyledon arginase activity (Figure 2).

Inhibition of enzyme by proline

At 10 mM concentration of proline, *V. catjang* cotyledon arginase showed 50% inhibition whereas buffalo liver arginase required at least 70 mM proline concentration for similar inhibition (Figure 3). The inhibition was linear competitive for both the enzymes. The Ki value calculated from the data obtained with buffalo liver arginase was 5.50 mM (Figure 4).

Inhibition of arginases by branched chain amino acids

The branched chain amino acids showed stronger inhibition of the liver form of arginase than that of *V. catjang* cotyledon. At 2 mM concentration of branched chain amino acids (isoleucine, valine, leucine, lysine and ornithine) buffalo liver arginase showed approximately 50% inhibition whereas more than 70 mM of the amino acids were required for a similar inhibition of *V. catjang* cotyledon arginase (data not shown). Further we have carried out detailed kinetic studies on only buffalo liver arginase. The effect of branched-chain amino acids decreased in the order isoleucine > valine > leucine > lysine ornithine on liver arginase. The kinetics of inhibition of buffalo liver enzyme by the above branched-chain amino acids at pH 9.2 are shown in the form of Lineweaver and Burk double reciprocal plots (1/S *vs* 1/V) at 0, 2 and 4 mM inhibitor concentrations, under the same conditions as for the Km determination. From the plots, it is observed that valine, ornithine, leucine and lysine are competitive inhibitors of the liver arginase affecting only the Km value but not the maximal velocity (Figures 5A – D). The amino acid isoleucine gave non-linear reciprocal plot at various concentrations (0, 2 and 4 mM) tending towards a mixed type of inhibitor (Figure 5E). This indicates that the inhibitor is bound to a site different from the active site and the complex formed between the isoleucine and arginase was enzymatically active.

Discussion

Arginase is a metalloenzyme in which manganese acts as a cofactor, and arginase activity is manganese dependant. It is found that manganese ion stabilizes or activates arginases from different tissues as well as

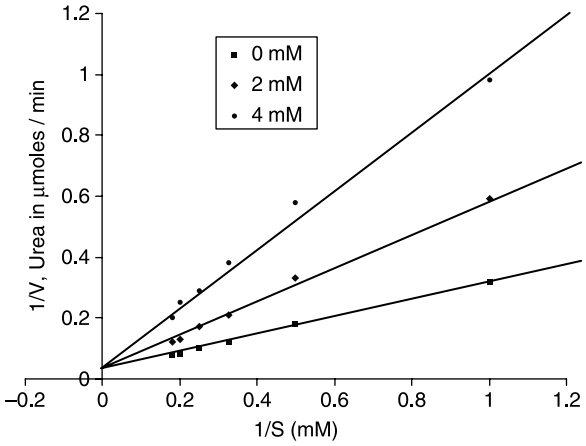

Figure 4. Lineweaver-Burk double reciprocal plot showing competitive inhibition of buffalo liver arginase by proline at 0, 2 and 4 mM concentrations.

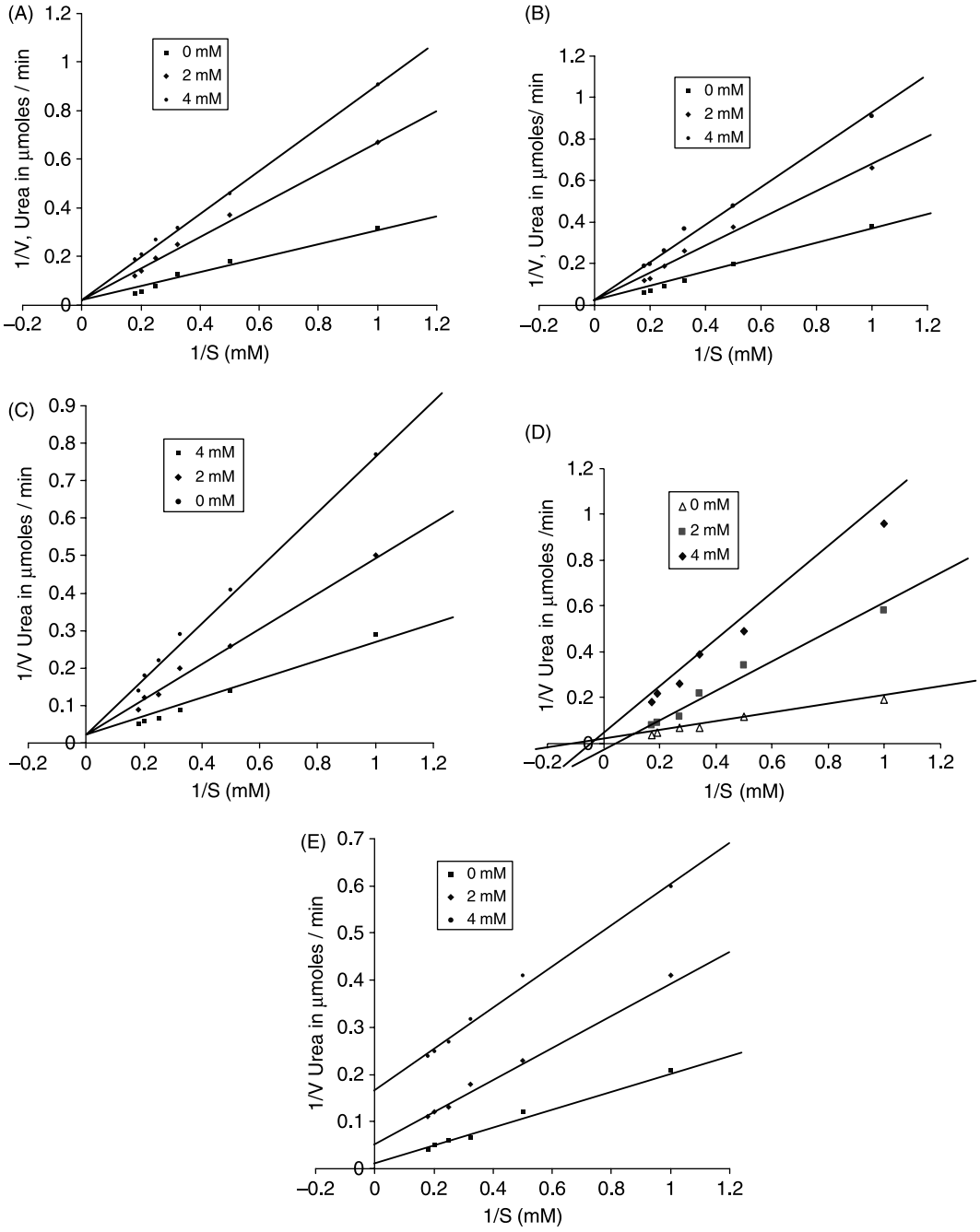

Figure 5. Lineweaver-Burk double reciprocal plot showing inhibition of buffalo liver arginase at 0, 2 and 4 mM concentrations. (**A**) ornithine, competitive. (**B**) leucine, competitive. (**C**) lysine, competitive. (**D**) valine, competitive. (**E**) isoleucine, mixed type.

plants [19–21]. In our result we have found that liver arginase requires a higher concentration of proline (70 mM) than that of *V. catjang* cotyledon (10 mM) for 50% inhibition whereas a lower concentration of branched chain amino acids are required to inhibit the liver arginase than that of the cotyledon. These observations suggest that plant arginases represent an evolutionary different group of ureohydrolase than that of non-plant arginases. In the plant arginases only that region is conserved which interacts with the guanidino moiety of substrate. However, in non-plant arginases the residues that bind the α-amino group and α-carboxyl group of arginine are conserved, whereas they are not conserved in plant arginases. Therefore arginase from plant and non-plant sources reacts differently with the same inhibitors [22].

In the present study, monocarboxylic amino acids with five or more carbon atoms such as ornithine, leucine, valine, lysine and isoleucine inhibited buffalo liver arginase. Therefore, it seems likely that the carbon chain length is critical for inhibitory amino acids to compete effectively with the substrate at the catalytic site of the enzyme molecule [23]. In this study we have found that proline, ornithine, leucine,

valine and lysine are competitive inhibitors and isoleucine is a mixed type of inhibitor of liver arginase which is in agreement with previous results [24].

The present study leads us to suggest that the differences in the inhibition properties of the arginases between the plant and animal kingdoms are due to their different role and physiological functions. The major role of arginase in mammalian species is to eliminate the waste nitrogen via the urea cycle. In contrast, the major role of arginase in plants is to coordinate the mechanism of its activity to that with urease to recycle urea-nitrogen in rapidly growing tissues [13,14]. A second significant difference between plant and non-plant arginase is their role in the synthesis of putrescine and polyamines. In animals, polyamine biosynthesis occurs primarily by the ornithine decarboxylase (ODC) pathway in which ornithine produced by arginase is converted directly to putrescine by ODC. In contrast polyamine biosynthesis in plants takes place by using arginine decarboxylase (ADC) pathway [25].

To determine the structural relationship between plant and non-plant arginases and the physiological significance of the inhibition in *Vigna catjang* cotyledon, it is necessary to elucidate the three-dimensional structure of plant arginase.

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