was dissolved in $CHCl_3$–MeOH (1:1) and analysed by GC-MS carried out under condition B: using the same column as condition A at 200° with He flow 60 ml/min. The relative retention times of the products with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, and their main fragments in the mass spectra are shown in Table 1.

*Periodate oxidation followed by Smith degradation of panaxan B.* To panaxan B (10.8 mg) in $H_2O$ (2.5 ml), 0.1 M $NaIO_4$ (2.5 ml) was added. The reaction mixture was kept at 4° in the dark. The periodate consumption was determined by a spectrophotometric method [9, 10]. After 5 days ethylene glycol was added, and the $HCO_2H$ liberation was measured by titration with 0.01 M NaOH.

The residue of the reaction mixture was treated with ethylene glycol (0.02 ml) at 4° for 1 hr and reduced with $NaBH_4$ (40 mg) at 4° for 16 hr. The soln was adjusted to pH 5 with HOAc and dialysed against $H_2O$ for 2 days. The non-dialysable fraction was concd and chromatographed over Sephadex G-25 (2.6 i.d. × 95 cm) which was eluted with $H_2O$. Fractions of 20 ml obtained from tubes 10 and 11 were combined and lyophilized yielding the product which was hydrolysed with 1 N $H_2SO_4$ containing D-mannitol as an internal standard at 100° for 6 hr. After neutralization with Dowex 2 ($OH^-$), the hydrolysate was reduced and acetylated as described above, and the resulting alditol acetate mixture was subjected to GC which was conducted under condition A. The retention times of D-glucitol hexa-acetate and D-mannitol hexa-acetate (internal standard) were 19.8 and 16.7 min.

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# AGMATINE DEIMINASE IN RICE SEEDLINGS

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**Key Word Index**—*Oryza sativa*; Gramineae; rice; agmatine deiminase.

**Abstract**—Agmatine deiminase activity in rice embryos increased gradually upto 24 hr during germination and then decreased. Gibberellic acid and kinetin inhibited the activity when added to the germination medium. The enzyme was purified 717 fold with specific activity 788.5 nkat/mg protein and yield 8.8%. The $M_r$ of the native enzyme was 18.3 × $10^4$ and the enzyme was a dimer of two identical subunits. The pH and temperature optimum of the enzyme were 6.0 and 28° respectively. The enzyme followed typical Michaelis–Menten kinetics with a $K_m$ value of $1.5 \times 10^{-2}$ M. The enzyme activity was inhibited by various divalent cations and spermidine and spermine, but putrescine showed no effect.

## INTRODUCTION

In higher plants the polyamines may be formed from arginine [1, 2] or from ornithine [3, 4]. Arginine is first decarboxylated to agmatine [5], which is then hydrolysed in two steps to putrescine. In the initial hydrolytic step agmatine is converted to *N*-carbamylputrescine with the formation of ammonia. This is effected by the enzyme agmatine deiminase (agmatine iminohydrolase; EC 3.5.3.12).

Agmatine deiminase has been reported to be present in tobacco [6, 7], maize and sunflower seedlings [8] and groundnut cotyledons [9]. The enzyme was found to be significantly more active in extracts of potassium-deficient barley leaves [10]. Despite early attempts to purify agmatine deiminase [8, 11], the enzyme was homoge-

neously purified only from corn seedlings [12]. In this paper we describe the purification and partial characterization of the enzyme from rice seedlings.

## RESULTS AND DISCUSSION

### *Agmatine iminohydrolase activity during germination of rice embryos*

In dry embryo the activity of the enzyme was 0.83 nkat/mg protein. As soon as inhibition started the enzyme activity was greatly increased, reaching 6.08 nkat/mg protein (7 fold) at 24 hr, but the activity then gradually declined (Table 1). Similar results were also obtained by Sindhu *et al.* [9] in both cotyledon and embryo of groundnut.

### *Effect of gibberellic acid and kinetin on the enzyme activity during germination*

Rice embryos were germinated for 24 hr in various concentrations of gibberellic acid ($GA_3$) and kinetin. At 1 $\mu$m concentration neither $GA_3$ nor kinetin showed appreciable effect, but above 10 $\mu$m, the enzyme activity was appreciably inhibited (Table 2). In groundnut [13] also the enzyme activity was inhibited by $GA_3$ and kinetin when added to the germination medium.

### *Purification*

With the procedure described in the Experimental, 717-fold purification of the enzyme with a specific activity of 788.5 nkat/mg protein and a yield of 8.8% was achieved (Table 3). The purity of the enzyme was judged by observing a sharp single band of protein in polyacrylamide disc gel electrophoresis. Scanning of the gel at 280 nm in a Hitachi gel scanner also showed a sharp single peak. The $M_r$ of the enzyme determined by Sephadex G-100 gel filtration was estimated to be $18.3 \times 10^4$, but SDS gel electrophoresis showed that the $M_r$ of the enzyme is about $9.5 \times 10^4$, suggesting that the native enzyme in rice is a dimer consisting of two identical subunits. A dimeric form of the enzyme was also reported by Yanagisawa and Suzuki [12] in corn seedling.

Under standard assay conditions, the activity of the enzyme was linear with protein concentration. A Lineweaver–Burk plot gave a $K_m$ value of $1.5 \times 10^{-2}$ M. The enzyme showed a pH optimum at 6.0. Half maximal activity was found at pH 5.4 and 6.6. The optimum temperature of the enzyme was 28°, but the purified enzyme stored at −20° lost 40% activity within 15 days.

Table 1. Agmatine deiminase activity during germination of rice embryos

| Hours of germination | Specific activity (nkat/mg protein) |
|---|---|
| 0 | 0.83 |
| 3 | 1.66 |
| 6 | 2.0 |
| 12 | 2.41 |
| 18 | 4.7 |
| 24 | 6.08 |
| 48 | 5.04 |

Table 2. Effect of gibberellic acid and kinetin on agmatine deiminase activity during 24 hr germination of rice embryos

| | Specific activity (nkat/mg protein) | | | | |
|---|---|---|---|---|---|
| | Concentration of hormones ($\mu$M) | | | | |
| Hormones | 0 | 1 | 10 | 50 | 100 |
| Gibberelic acid | 6.08 | 6.35 | 5.4 | 1.5 | 0.87 |
| Kinetin | 6.08 | 6.2 | 4.4 | 2.6 | 1.8 |

### *Effect of inhibitors*

The enzyme was inhibited by several divalent cations such as $Mg^{2+}$, $Mn^{2+}$, $Ca^{2+}$, and $Ba^{2+}$. Addition of 5 mM of each of the cations resulted in inhibition of the activity by 87%, 86%, 71% and 88% respectively. Putrescine showed no appreciable effect, though significant inhibition was noted with spermidine and spermine. Corn agmatine deiminase was reported to be inhibited by divalent cations though polyamine had no effect [12]. On the other hand the groundnut enzyme was inhibited by putrescine, cadaverine, spermine and spermidine [11].

## EXPERIMENTAL

*Chemicals.* Sephadex G-100, DEAE-cellulose, agmatine and polyamines were purchased from Sigma. All other chemicals used were of analytical reagent grade.

*Materials.* Rice seeds obtained from the experimental garden of Bose Institute were surface sterilized with 0.1% $HgCl_2$, washed thoroughly with $H_2O$ and then allowed to germinate in the dark at 37 ± 1° as detailed elsewhere [14].

*Enzyme activity* was routinely assayed by measuring $NH_3$ released from agmatine with Nessler's reagent following the method of ref. [8]. Assays were performed in 10 ml vials provided with a tight-fitting rubber cork having a centrally placed small vial. The reaction mixture in the vial consisted of 0.1 ml of 50 mM agmatine, 0.1 ml toluene and enzyme in a final vol. of 1 ml, and the central vial contained 0.5 ml of 0.1 M HCl. The units were sealed and normally incubated at 28° for 18 hr. At the end of this incubation period, 1 ml of saturated $K_2CO_3$ was added to the outer compartment and after standing for a further 3 hr at room temp., the ammonia which was absorbed in the central vial was measured by Nesslerization. In control experiments the boiled enzyme was used. Protein was determined by the method of ref. [15] after precipitation with TCA and solubilization in 1 M NaOH.

*Polyacrylamide gel electrophoresis* was carried out in 7.5% gel at pH 8.3 using Tris–glycine buffer according to the method of ref. [16]. Gels were stained with Coomassie Brilliant Blue and destained with 7.5% HOAc. SDS gel electrophoresis was carried out according to ref. [17].

*Enzyme purification.* A seven step purification procedure has been adopted in the present investigation. All the operations were carried out at 4°.

Step 1: Rice seedlings germinated for 72 hr were homogenized in KPi buffer (50 mM, pH 6) containing 5 mM 2-mercaptoethanol and 0.1 mM EDTA. The homogenate was filtered through cheese cloth and centrifuged at 8000 *g* for 30 min.

Step 2: The crude extract was adjusted to 7.5 mM $MnCl_2$, stirred for 30 min and the ppt was removed by centrifugation.

Step 3: The supernatant of Step 2 was subjected to $(NH_4)_2SO_4$

Table 3. Purification of agmatine deiminase from 72 hr germinated rice seedlings

| Steps | Total activity (nkat) | Specific activity (nkat/mg protein) | Purification (fold) | Yield (%) |
|---|---|---|---|---|
| Crude | 1420 | 1.1 | 1 | 100 |
| $MnCl_2$ | 9260 | 2.05 | 1.8 | 65 |
| $(NH_4)_2SO_4$ | 6320 | 5.37 | 4.9 | 44.4 |
| DEAE-cellulose | 3940 | 60 | 55 | 28 |
| $(NH_4)_2SO_4$ | 2820 | 170 | 154 | 20 |
| Sephadex G-100 | 2770 | 585 | 532 | 18 |
| DEAE-cellulose | 1250 | 788 | 717 | 8.8 |

fractionation and the protein precipitating at 30–70% satn. was dispersed in homogenization buffer and dialysed against the same buffer for 24 hr [first $(NH_4)_2SO_4$ fraction].

Step 4: The dialysed enzyme fraction (10 ml) was passed through the DEAE-cellulose column (35 × 2 cm) equilibrated with the homogenization buffer. The enzyme was eluted from the column with a pH gradient established with 400 ml of the homogenization buffer in the mixing flask and 400 ml of 0.5 M $KH_2PO_4$ containing 5 mM 2-mercaptoethanol and 0.1 mM EDTA in the reservoir.

Step 5: The active fractions from the DEAE-cellulose column were pooled, precipitated with 70% $(NH_4)_2SO_4$, dissolved in the homogenization buffer and dialysed overnight [second $(NH_4)_2SO_4$ fraction].

Step 6: The dialysed enzyme fraction (10 ml) was loaded on a Sephadex G-100 column (40 × 2 cm) equilibrated with the homogenization buffer. Active fractions were pooled and dialysed overnight.

Step 7: The enzyme fraction obtained in Step 6 was applied to a second DEAE-cellulose column (40 × 2 cm) and eluted in the similar way as in Step 4 using 200 ml of homogenization buffer and 200 ml of 0.5 M $KH_2PO_4$ containing 5 mM 2-mercaptoethanol and 0.1 mM EDTA to establish the gradient.

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