Effect of cadmium on proline accumulation and ribonuclease activity in rice seedlings: role of proline as a possible enzyme protectant

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Abstract

When seedlings of two rice (*Oryza sativa* L.) cultivars Ratna and Jaya were raised under 100 and 500 μM cadmium nitrate in the medium, a high proline content was noted in Cd^{2+} stressed seedlings compared to controls. Seedlings grown under 500 μM $Cd(NO_3)_2$ maintained increased proline level compared to non-stressed seedlings. Kinetic properties of RNase extracted from control grown and Cd^{2+} stressed seedlings showed a marked alteration in Km due to Cd^{2+} treatment. The RNase isoforms were purified from 15-d-old rice seedlings with a total purification of 22.25 fold and 74.75 % yield using conventional biochemical techniques. Three RNase isoforms, namely I, II and III were eluted from *DEAE-Sephacel* column. The isoform RNase II had Km value of 3.2 mg(RNA) cm^{-3}. The *in vitro* osmotic stress created by incorporation of PEG in the enzyme assay medium led to decreased affinity of enzyme towards its substrate with increase in Km. This loss in affinity was partially restored by the addition of 1 M proline in the assay medium, suggesting the possible protective role of proline on RNase under osmotic stress.

Additional key words: *Oryza sativa*, osmoprotectant, PEG, seedlings.

Introduction

Industrialization has led to increased introduction of several heavy metals into the soil environment. As rice is a semi-aquatic plant it suffers from increasing threat of

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Abbreviations: BSA - bovine serum albumin; Cd^{2+} - cadmium ion; Km - Michaelis constant; LB - Lineweaver-Burk; PEG - polyethylene glycol; TCA - trichloroacetic acid.

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metal stress as well as increased water deficit. On the global scale rice serves as a staple food for most of the population. Unlike other heavy metals cadmium has gained far more attention as it directly enters our food chain.

Plants growing under high levels of heavy metals tend to develop biochemical and molecular mechanisms to tolerate the effects of metal stress (for review see, *e.g.*, Bohnert *et al.* 1995). The more prominent biochemical responses known to be provoked by cadmium in higher plants include the synthesis of cysteine rich metal binding polypeptides called phytochelatins which form intracellular complexes with Cd^{2+} and help in sequestration of excess Cd^{2+} ions in plants (Sachs and Ho 1986, Robinson *et al.* 1993, Wells and Brown 1995). Accumulation of free amino acids more specially proline has been observed in various plant species subjected to heavy metal stress (Paleg and Aspinall 1981, Alia and Pardha Saradhi 1991, Bandurska 1993, Bohnert *et al.* 1995).

The activities of the enzymes like ribonuclease, peroxidase and acid phosphatase change greatly in actively dividing tissues (Wilson 1975, Dubey and Pessarakli 1995) and in tissues exposed to stressful environments (Udvardy *et al.* 1969, Dubey and Sharma 1990, Mittal and Dubey 1991). Rice seedlings raised in the presence of Cd^{2+} in the growth medium show decreased seedling vigour and suppressed RNA hydrolysis primarily due to inhibition in RNase activity (Shah and Dubey 1995). The accumulation of proline and other soluble nitrogenous compounds under environmental stresses like salinity, osmotic stress or heavy metal toxicity is regarded as one of the few important strategies adopted by plants at cellular level to prevent cellular dehydration by balancing the osmotic strength of the cytoplasm with that of the environment (Santos-Diaz and Ochoa-Alejo 1994, Dubey and Pessarakli 1995).

Nucleases are widely distributed in plants and catalyse hydrolysis of nucleic acids (Mangalekar and Sontakke 1989). RNases are the hydrolytic enzymes catalysing the degradation of RNA molecules inside the cell (Deutscher 1993). The enzyme RNase has been purified from many sources such as maize (Wilson 1968), wheat leaves (Chevrier and Sarhan 1980), pea seeds (Wilson 1975) and moth beans (Mangalekar and Sontakke 1989).

As the studies related to the role of various compatible solutes effective as osmoprotectants are a necessary step for engineering plant metabolic pathways to improve plant tolerance, the present study was undertaken to examine the level of proline and to study the kinetic behaviour of ribonucleases in Cd^{2+} grown seedlings. Attempts were made to purify RNase and to study the role of proline as an osmoprotectant for the enzyme RNase.

Materials and methods

Plants: Seedlings of rice (*Oryza sativa* L.) cvs. Ratna and Jaya were raised in sand in plastic pots with Hoagland nutrient solution which served as control and nutrient solution supplemented with 100 and 500 μM $Cd(NO_3)_2$ which served as treatment solutions. Seedlings were maintained in a growth chamber at 28 ± 1 °C, 80 % relative humidity and 12-h light/dark cycle (irradiance of 40 - 50 μmol m^{-2} s^{-1}) as described

earlier (Shah and Dubey 1995). Pots were maintained at field saturation capacity and irrigation was done when required. Seedlings were uprooted at 5-d-intervals and experiments were performed in triplicate.

Amino acid estimation: About 200 mg dried root or shoot samples were homogenized in 5 cm^3 of 80 % ethanol followed by heating at 70 °C for 1 min and centrifugation at 22 000 *g* for 10 min. Extraction was repeated thrice, contents were mixed and alcohol was evaporated at 80 °C. Contents were dissolved in water and volume was made upto 10 cm^3. Amino acids were estimated according to the method of Rosen (1959). To 0.5 cm^3 of sample 1 cm^3 of 0.1 M acetate cyanide buffer (pH 5.35) and 0.5 cm^3 of 3 % ninhydrin solution were added. The mixture was boiled on a water bath for 15 min at 100 °C. Volume was made upto 5.0 cm^3 with distilled water. Leucine was used as standard. Values were expressed in mg(amino acids) g^{-1}(dry mass of the samples).

Proline estimation: Proline was estimated in roots and shoots according to the method of Bates *et al.* (1973). Fresh samples (500 mg) were homogenized in 10 cm^3 of 3 % aqueous sulfosalicylic acid and centrifuged at 22 000 *g* for 5 min. To 2 cm^3 of the supernatant 2 cm^3 of acid ninhydrin was added. Further, 2 cm^3 of glacial acetic acid was added and the contents were boiled for 1 h at 100 °C in a water bath. The mixture was further extracted with 10 cm^3 of toluene by mixing the two thoroughly in a test tube with vigorous stirring. Absorption of chromophore was read at 515 nm in an *Elico CL24* spectrophotometer (Hyderabad, India). L-Proline (*Sigma*) was used for the preparation of the standard curve. The amount of proline in the samples was calculated in mg(proline) g^{-1}(d.m.).

Determination of Km of ribonucleases extracted from control and Cd^{2+} stressed seedlings: RNase was extracted from the roots of 15-d-old rice seedlings in 100 mM sodium acetate buffer (pH 5.4). Enzyme extracts were dialyzed in cellophane membrane dialysis tubings in cold (4 °C) for 12 h with 3 - 4 changes of buffer. Ribonuclease (EC.1.4.23) activity was assayed in the enzyme preparations according to the method of Wilson (1967) using increasing concentrations of RNA (*Sigma*) as substrate. RNase activity was determined in each case and Michaelis constant (Km) values were calculated by constructing Lineweaver-Burk plots.

Purification of ribonuclease: Fresh root samples (10 g) from 15-d-old control rice seedlings were washed with double-distilled water and homogenized in 22.0 cm^3 of 100 mM sodium acetate buffer (pH 5.4) containing 0.1 M KCl. The homogenate was centrifuged at 22 000 *g* for 10 min. All the operations unless otherwise stated were performed at 4 °C. The clear supernatant obtained served as crude extract which was subjected to heat treatment at 60 °C for 15 min and immediately cooled in ice. After centrifugation at 22 000 *g* for 5 min, the precipitate was discarded (as it had no enzyme activity) and to the supernatant chilled acetone was added slowly at -10 °C in a cryostat with shaking, so as to achieve 80 % acetone concentration in the extract. The precipitate was collected by centrifugation and dissolved in 10 cm^3 of 0.1 M sodium acetate buffer (pH 5.4). The enzyme solution was subjected to 25 to 60 %

ammonium sulfate precipitation maintaining pH at 5.4. The resulting precipitate was dissolved in 5 cm^3 of 0.1 M sodium acetate buffer, pH 5.4 and was extensively dialysed against the same buffer to remove excess salt. The RNase activity and protein was measured at each purification step.

The dialyzed enzyme was loaded onto a *DEAE-Sephacel* column (1.5 × 14 cm) equilibrated in 100 mM sodium acetate buffer, pH 5.4. The column was washed with the same buffer (three times the void volume). The eluate was assayed for enzyme activity and protein. Stepwise elution of bound enzyme was carried out using increasing strengths of sodium acetate buffer, pH 5.4 (0.2, 0.3, 0.4 and 0.5 M). Three cm^3 aliquots were collected. Protein was measured at 280 nm on a UV-VIS *Shimadzu* spectrophotometer (Kyoto, Japan) in each aliquot. The aliquots possessing RNase activity were individually assayed for deoxyribonuclease and 5' nucleotidase activities according to the methods of Wilson (1967) and Leon *et al.* (1955), respectively.

Determination of osmoprotective role of proline: To study the effect of osmotic stress on RNase activity, the *in vitro* system of Paleg *et al.* (1984) was followed. To 0.5 cm^3 of purified enzyme (RNase II) polyethylene glycol (PEG-6000) was slowly added to achieve 40 % PEG concentration in a total volume of 2 cm^3. In another set of experiments, to 0.5 cm^3 of purified enzyme, was added 1 M proline in addition to 40 % of PEG. Both the reaction mixtures were kept at 0 °C for 4 h. Thereafter, RNase activity was assayed in both the sets and Michaelis constants (Km) for RNase with respect to RNA as substrate were determined by constructing Lineweaver-Burk plots. In all enzyme preparations protein was estimated by the method of Lowry *et al.* (1951) using BSA (*Sigma*) as standard.

Results and discussion

Cadmium and free amino acids: In seedlings of both rice cvs. Ratna and Jaya a steady increase in the level of free amino acids was observed with an increase in Cd^{2+} concentration in the growth medium (Fig. 1). Under 500 μM Cd^{2+} the content of free amino acids increased about 40 % in roots and 60 % in shoots after 15-d treatment as compared to controls. Considerable increase in the level of free amino acids has been observed in different parts of plants growing under stressful conditions (Stewart and Larher 1980, Reddy and Vora 1983, Dubey and Rani 1989, Alia and Saradhi 1991, Alfocea *et al.* 1994). Accumulation of amino acids as observed in our experiments in Cd^{2+} stressed plants is a possible consequence of their increased synthesis and decreased utilisation (Dubey and Pessarakli 1995).

Proline accumulation in response to cadmium: Proline accumulation in response to environmental stresses has been reported by various workers, who suggested a possible role of proline in osmotic adjustment, however direct evidence for such a function is still lacking (Kishor *et al.* 1995). The control plants grown without $Cd(NO_3)_2$ for 20 d showed an average proline content of 0.40 mg g^{-1}(d.m.) in roots

and 0.50 mg g^{-1}(d.m.) in shoots, whereas those that were grown at 100 μM and 500 μM $Cd(NO_3)_2$ showed an increased proline content (Fig. 1). At 20-d treatment with 100 μM Cd^{2+} 0.5 and 0.8 mg(proline) g^{-1}(d.m.) in roots and shoots, respectively, was noted in cv. Ratna and 0.8 and 0.7 mg(proline) g^{-1}(d.m.) in roots and shoots in cv. Jaya was observed. Under 500 μM Cd^{2+} the roots and shoots of cv. Ratna contained 1.2 and 1.0 mg(proline) g^{-1}(d.m.) and those of cv. Jaya 1.6 and 1.1 mg (proline) g^{-1}(d.m.).

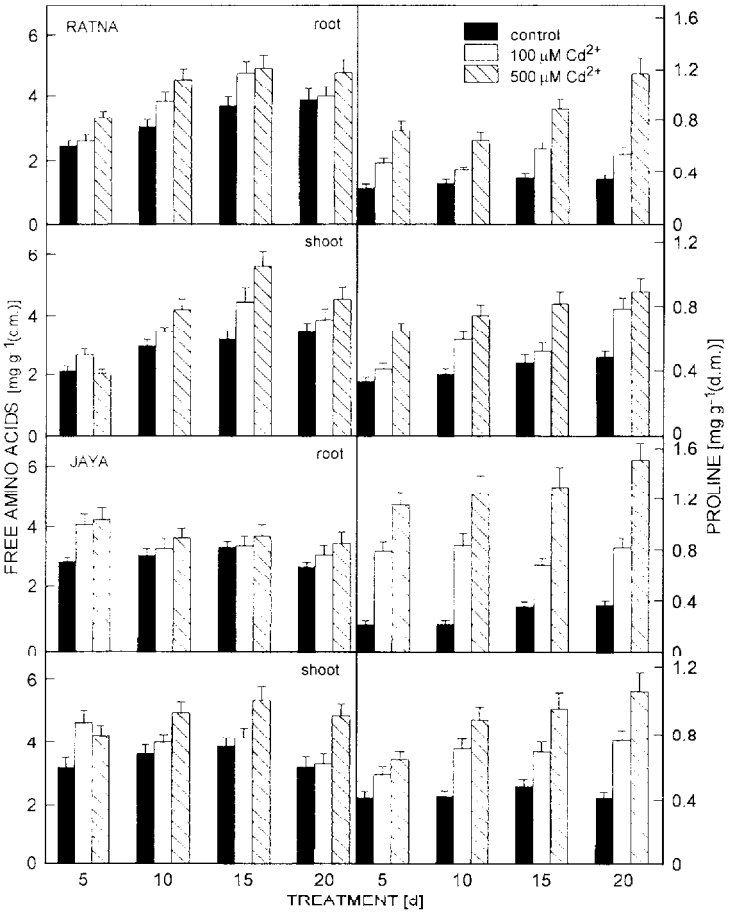

Fig. 1. Effect of increasing concentrations of $Cd(NO_3)_2$ in the growth medium on content of amino acids and proline in rice cvs. Ratna and Jaya after 5, 10, 15 and 20 d of treatment. Means based on three independent determinations; *bars* indicate standard deviations.

High proline level in Cd^{2+} stressed seedlings appears to be primarily due to its increased *de novo* synthesis under stressful condition (Rhodes *et al.* 1986, Joshi *et al.* 1992, Madan *et al.* 1995). As proline is a compatible solute it may have a role in providing protection to the enzymes and biopolymers at the cellular level against heavy metal damage. Similar to our findings Alia and Saradhi (1991) showed Cd^{2+} to be the strongest inducer of proline accumulation in *Triticum aestivum* plants. An

increase in amino acid content *L. esculentum* with salinity was mainly due to increase in proline (Alfocea *et al.* 1994). It is regarded that proline in addition to its role as solute that protects macromolecules against denaturation (Schobert and Tschesche 1978), also serves as a sink for energy to regulate redox potentials and as a hydroxyl radical scavenger (Smirnoff and Cumbes 1989, Alia and Saradhi 1991).

Comparison of RNase kinetics - with and without Cd^{2+}: Earlier we have reported that under *in vitro* conditions $Cd(NO_3)_2$ upto 100 μM in the reaction medium stimulated RNase activity whereas concentration beyond this caused a gradual decrease in enzyme activity (Shah and Dubey 1995). Here we report that the RNase extracted from 15-d-old control grown plants had a Km of 3.40 mg(RNA) cm^{-3}, whereas a significant shift in Km value was observed in dialyzed enzyme extract from Cd^{2+}-treated seedlings (Fig. 2). Enzymes extracted from seedlings grown under 100 μM and 500 μM $Cd(NO_3)_2$ showed Km values of 3.0 and 4.5 mg(RNA) cm^{-3}, respectively, as evident from the LB plot (Fig. 2). This suggested that in seedlings grown at moderate Cd^{2+} concentration (100 μM) the affinity of RNase towards its substrate increased slightly whereas a significant decrease in enzyme affinity was observed in seedlings grown at higher Cd^{2+} (500 μM) concentration.

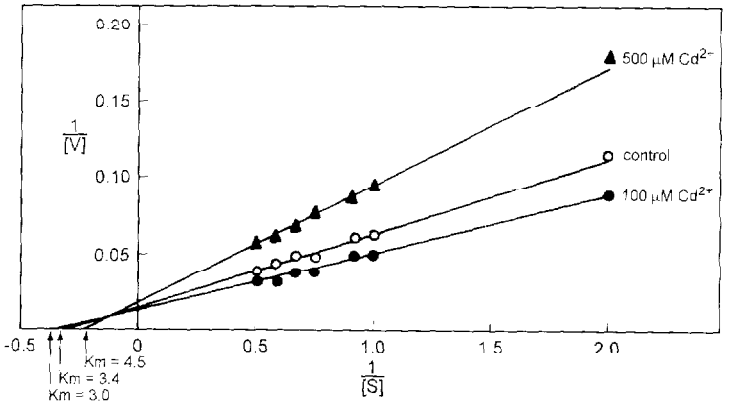

Fig. 2. Lineweaver-Burk plots for determination of Km values of RNase preparations obtained from roots of 15-d-old control, as well as 100 μM and 500 μM $Cd(NO_3)_2$ treated rice seedlings. Km values obtained were 3.0, 3.4 and 4.5 mg(RNA) cm^{-3} for control, 100 μM and 500 μM $Cd(NO_3)_2$ grown seedlings, respectively.

Inhibition in the activities of many enzymes with Cd^{2+} and other heavy metals has been attributed to the inactivation of their proteins by the interaction of heavy metals with -SH groups (Kumar and Banerjee 1992, Somashekaraiah *et al.* 1992). Our earlier studies suggested that rice seedlings raised under 500 μM Cd^{2+} had decreased RNase activity with decreased number of its isoforms compared to control plants (Shah and Dubey 1995). An increased Km value of RNase due to high Cd^{2+} *in situ* as observed in our experiments further suggests possible alteration in conformation or inactivation of RNase due to high level of Cd^{2+}.

RNase isoforms in root tissues of rice: Three RNase isoforms were partially purified using successive steps of heat treatment (65 °C for 15 min), acetone treatment, ammonium sulfate precipitation and *DEAE-Sephacel* column chromatography. Using these techniques the RNase isoforms were purified to a total of 22.25 fold with 74.75 % yield (Table 1). The elution profile from *DEAE Sephacel* column is shown in Fig. 3. When 0.753 mg protein with 820 enzyme units was loaded on the column, three different protein peaks showing RNase activity were observed. These peaks were termed RNase I, II and III, representing the three RNase isoforms. Maximum RNase activity was associated with RNase II, followed by RNase III and RNase I. The pH optima for RNase II in our case was found to be 5.4 and Km 3.2 mg cm^{-3}.

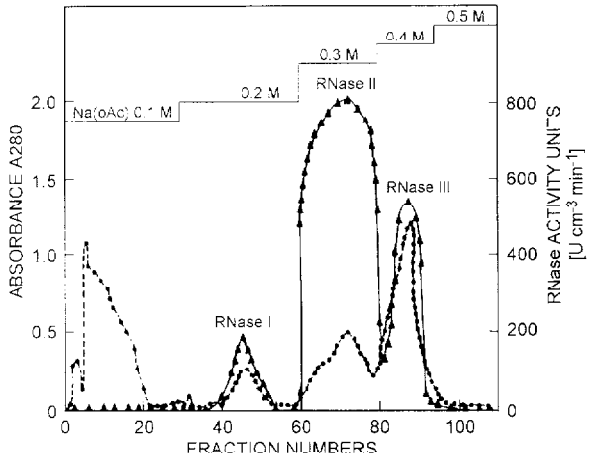

Fig. 3. Elution profile of RNase and its isoforms from *DEAE-Sephacel* column chromatography.

Table 1. Purification of ribonuclease from roots of 15-d-old seedlings of rice cv. Jaya.

Purification steps		Enzyme volume [cm^3]	Total protein [mg]	Total activity [U]	Specific activity [U mg^{-1}(prot.)]	Purification [fold]	Yield [%]
I crude extract		22.0	6.880	1070	155.52	1.000	100.00
II heat treatment (60 °C, 15 min)		20.0	4.370	1010	231.12	1.486	94.39
III acetone treatment (80 %, -10°C)		10.0	1.990	1000	526.30	3.380	93.46
IV amm. sulphate predipitation (25-60 %)		5.0	0.994	900	905.43	5.820	84.11
V dialysis (O.1 M sod. acetate buffer, pH 5.4)		6.0	0.735	820	1115.65	7.170	76.63
VI DEAE sephacel column chromatography	RNase I	4.0	0.086	45	523.25	3.364	4.20
	RNase II	10.0	0.325	624	1920.00	12.340	58.31
	RNase III	5.0	0.152	131	861.84	5.541	12.24

Various classes of RNase are present in plant tissues (Wilson 1975). Our experiments revealed 3 peaks with RNase activities and all of these were devoid of any DNase or 5'-nucleotidase activity. Plant RNases have been reported to be heat stable. The RNase from barley (Prentics and Heisel 1985), mung bean (Walter and Loring 1966) and moth bean (Mangalekar and Sontakke 1989) showed variable stability towards heat, depending upon the relative purity of the preparation. Our results suggest that as RNase from rice could withstand a heat treatment of 60 °C for 15 min, it is fairly heat stable, although some loss in enzyme activity was apparent during the purification process.

Proline as an osmoprotectant: Different authors have assigned various roles to the stress induced proline. Accumulating proline has been suggested to act as a compatible cytosolute (Stewart and Lee 1974), an osmoprotectant for cytosolic enzymes and various cellular structures (Csonka 1989) and also as a sink for energy to regulate redox potentials (Alia and Saradhi 1991). We have developed an *in vitro* experimental system to study the role of proline in controlling the activity of RNase under osmotic stress. In the presence of 40 % PEG in the medium RNase activity was partially lost with an increase in Km from 3.20 to 5.71 mg cm^{-3} (Fig. 4). This

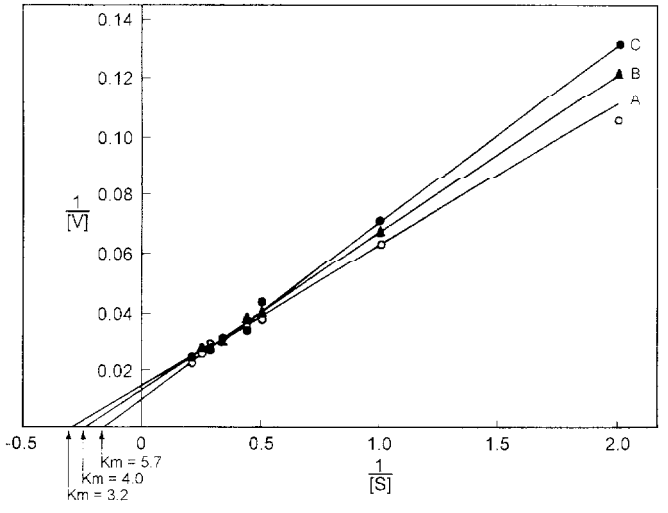

Fig. 4. Lineweaver-Burk plots showing Km values of purified Rnase II preparation in presence of enzyme alone (*A*), enzyme + 40 % PEG + 1 M proline (*B*) and enzyme + 40 % PEG (*C*) in the medium. Km values obtained were 3.20, 4.00 and 5.71 mg(RNA) cm^{-3}, respectively, for enzyme alone, enzyme + PEG + proline and enzyme + PEG.

suggested decreased affinity of RNase towards its substrate under osmotic stress similar to that under Cd^{2+} stress. With the incorporation of 1 M proline in the medium partial restoration of this decreased activity was noted with decrease in Km from 5.71 to 4.0 mg cm^{-3} which suggested some protective role of proline for RNase under osmotic stress. The results of the present study thus confirm the role of proline in reducing the precipitation of ribonuclease and other proteins caused by PEG (Paleg

et al. 1984, Bandurska 1993). Similar to our results Miranda-Ham and Loyola Vargas (1987) also observed a possible protective role of proline for the nitrogen assimilatory enzymes glutamine synthetase and glutamate dehydrogenase in maize plants under water stress.

It can be concluded that cadmium toxicity in rice seedlings lead to accumulation of free proline and loss in substrate affinity of the enzyme RNase. Furthermore, proline accumulation under stressful conditions appears to provide considerable protection to the enzyme RNase against the denaturing effect of osmotic stress.

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