Journal of Environmental Sciences Vol. 7, No. 1, pp. 24—30, 1995 ISSN 1001—0742

Effect of aluminum on NAD kinase activity in chloroplast fraction from leaves of rice seedlings

Liu Jinqi, Liu Houtian*

Chinese Research Academy of Environmental Sciences, Beijing 100012, China

Wang Youai, Wu Shuping

Department of Biology, Hebei Normal University, Shijiazhuang 050016, China

Abstract—The effect of aluminum on NAD kinase *in vivo* and *in vitro* was studied. The concentration of aluminum and exposure time are the main factors determining aluminum toxicity. Al can accumulate in the chloroplast of rice seedlings and affect calmodulin-dependent NAD kinase activity, however, calmodulin-independent NAD kinase was insensitive to Al application. Calmodulin can reverse the inhibition of Al at low Al concentrations (0.001mmol/L-0.5mmol/L), but the relaxing ability decreases at higher Al(2mmol/L). It is suggested that, aside from impairing calmodulin, Al may also affect calmodulin-dependent NAD kinase activity by changing the enzyme structure.

Keywords: aluminum; calmodulin; NAD kinase; chloroplast fraction; rice seedlings.

1 Introduction

Aluminum leached from acid soil has come to threaten the structure and function of the ecosystem (Foy, 1974; Ulrich, 1983). Aluminum toxicity is one of the major limiting factors of rice production. Excess Al in plants interferes with cell division and damages certain enzyme activities involved in some physiological processes. NAD kinase catalyses the only known biochemical reaction of NADP synthesis in plants (McGuinness, 1985). This enzyme plays an important role in many physiological processes of plants, e.g. photosynthesis. It is therefore necessary to study the effect of environmental streesses on NAD kinase. Maciejewska (Maciejewska, 1990) studied the relationship between NAD kinase and cold tolerance of plants. The response of NAD kinase in the root tips of some cereal crops to the application of Al was studied (Slaski, 1989; 1990). Nevertheless, Al effect on the NAD kinase of rice and its possible mechanism are still unknown.

The present work is to study the relationship between the activity of NAD kinase in the chloroplast fraction of rice seedlings and aluminum injury. It is evident that Al markedly decreases both the gross photosynthesis and photosynthetic rate of many plants. The aim of this research

* To whom correspondence should be addressed

is to provide some available data for better understanding one of the possible of pathway of Al toxicity on photosynthesis and some other catabolic processes in rice.

2 Materials and methods

2.1 Plan material

Seeds of rice (*Oryza sativa* L.), an aluminum tolerant species, were sterilized with 0.1% $HgCl_2$ for 10 min, rinsed thoroughly with running water and germinated at 25±4℃ in vermiculite for a week. The seedlings were transferred to nutrient solution after the first week.

2.2 Treatment of Al to rice seedlings

When the first leaves of rice seedling appeared, the seedlings transferred to the same nutrient solution with Al added in the form of $AlCl_3 \cdot 6H_2O$(0, 0.001, 0.01, 0.1, 0.5, 1.0, 2.0, 5.0 mmol/L) for 96h. Immediately after Al treatment NAD kinase activity in the chloroplast was assayed.

2.3 Aluminum treatment to NAD kinase *in vitro*

Aluminum was added as $AlCl_3 \cdot 6H_2O$ to the incubation mixture of NAD kinase with concentration of 0, 0.001, 0.01, 0.1, 0.5, 1.0, 2.0 mmol/L, respectively. An incubation mixture without aluminum was used as the control.

2.4 Analysis aluminum content in chloroplast fraction

After Al treatment for one week, with all concentration: 0, 0.001, 0.01, 0.1, 0.5, 1.0, 2.0 mmol/L, chloroplast of rice seedlings was isolated according to the modified method of Hincha (Hincha, 1986). Al content was assayed by a flameless atomic absorption spectrophotometer.

2.5 Preparation of the NAD kinase extract

NAD kinase was extracted according to the modified method of Anderson (Anderson, 1978). The leaves of rice seedlings were chilled, homogenized, filtered and centrifuged orderly. The supernatant was used as the enzyme preparation. All the procedures were performed at 0—4℃.

2.6 NAD kinase assay

The NAD kinase activity was estimated by the modified method of Muto (Muto, 1977). The incubation medium contained: 3mmol/L ATP, 10mmol/L $MgCl_2$, 1.0 mmol/L $CaCl_2$, 100 mmol/L Tris-HCl (pH 7.8), 3.0 mmol/L NAD and 0.1 ml NDA Kinase preparation in a final volume of 0.5 ml. The reaction was run for 30 min at 37℃. At the end of incubation, the mixture was chilled in ice and 100 μl of 1.0 mol/L HCl was added. The acidified sample was neutralized with 100μl 1.0 mol/L NaOH and finally clarified by centrifugation. The clear supernatant obtained was used for the determination of NADP. Simultaneously another 0.1 ml NAD kinase preparation was inactivated before it was added to the incubation medium, using as the control.

The assay system of NADP contained: 0.2mmol/L glucose-6-p. 20 μl/ml of phenazine methosulfate, 30 μl/ml of 2,6-dichlorophenol indophenol. 0.25 mol/L Tris-HCl and 0.1ml of the supernatant (NADP), in a final volume of 1.5ml. After 3 min of preincubation, the reaction was started by adding 0.1 unit of G-6-P dehydrogenase. The rate of decreases in absorbance at

600 nm was recorded by use of SHIMADZU UV -265 spectrophotometer. The enzyme activity was expressed as nmol $NADP^+$ formed (mg protein)$^{-1}$. h^{-1}.

Calmodulin - independent NAD kinase was assayed in the presence of 0. 1 mmol/L trifluoperazine. Calmondulin - dependent NAD kinase activity was obtained by subtracting the calmondulin - independent NAD kinase from the total activity of the enzyme (Allan, 1985).

2. 7 Extraction and purification of calmodulin

Cabbage (*Bressica capitata* L.) was used to extract calmodulin according to the method of Biro (Biro, 1984). 1000g of cabbage was homogenized in three volumes per weight of buffer (50 mmol/L Tris, 2mmol/L EGTA, 0. 15mmol/L NaCl, 0. 25 mmol/L PMSF, 20mmol/L $NaHSO_3$, 10mmol/L dithiothreitol, pH 8. 0). The homogenate was filtered, and then centrifuged at 5500g for 30 min. 30% TCA was added to the supernatant to final TCA concentration of 3%. After stirring at 4℃ for 30 min, the mixture was centrifuged again at 5500 g for 30 min. The pellet was resuspended in 200ml of cooled buffer (20mmol/L Tris, 1mmol/L EDTA, 20mmol/L $NaHSO_3$, 0. 15 mmol/L PMSF, 10mmol/L dithiothreitol, pH8). After being heated on a steam bath to 90—95℃ for 3min, the mixture was placed on ice and rapidly cooled to room temperature. The resulting thick mixture was centrifuged at 18000g for 1h. The supernatant was adjusted to Ca^{2+} concentration of 5 mmol/L, and the homogenate was centrifuged at 18000 g for 20 min. The supernatant were combined and applied to a phenyl - sepharose 4B column equilibrated with buffer A (50mmol/L Tris, 0. 1 mmol/L $CaCl_2$, pH 7. 5) and buffer B (50mmol/L Tris, 0. 1 mmol/L $CaCl_2$, 0. 5 mmol/L NaCl, pH 7. 5), the calmodulin was eluted with buffer C(50 mmol/L Tris, 0. 5mmol/L NaCl, pH 7. 5), the calmodlin was eluted with buffer C(50 mmol/L Tris, 5 mmol/L EGTA, pH 8. 0). Fractions were collected and those containing calmodulin were combined, the pooled calmodulin - containing fractions were dialyzed against water. Calmodulin prepared by this procedure was free of polypeptide contamination as determined by SDS polyacrylamide gel electrophoresis.

2. 8 Reversal of aluminum inhibition on the activity of NAD kinase by calmodulin

Different concentration of calmodulin were added to the incubation mixture treated by aluminum and the restoration of the enzyme activity was estimated. The amount of calmodulin was 6, 4, 2, 0. 8, 0. 4, 0. 05 and 0. 01μg, respectively.

2. 9 Protein determination

Protein content was determined according to Bradford (Bradford, 1976).

3 Results

Aluminum accumulation in the chloroplasts of rice seedlings is shown in Table 1. The quantity of Al accumulation increased with the increase of Al concentration. When intact seedlings were exposed to Al and their NAD kinase was assayed, the total NAD kinase activity was increased with increasing aluminum concentration. At 1 mmol/L of Al concentration in the medium, the total NAD kinase activity reached its maximum. However, this increase was only associated with increased calmodulin - independent NAD kinase activity, the calmodulin - dependent NAD kinase activity was decreased from 75 to 23 percentage of the total enzyme activity (Fig. 1).

Table 1 Al accumulation in the chloroplast of rice seedlings after Al treatment for one week*

Aluminum concentration, mmol/L	Aluminum accumulation, μmol/mg. chl.
0	7.9
0.001	9.5
0.01	10.3
0.1	12.8
0.5	18.2
1.0	47.6
2.0	59.5

* Values are averages of three replications

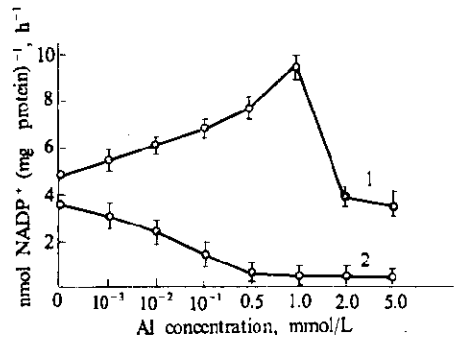

Fig. 1 Effect of Al on NAD kinase activity *in vivo* Al treatment time: 96h1. total enzyme activity; 2. calmodulin - dependent NAD kinase activity; error bars represent the SD.

The effect of increased concentration of aluminum ions on NAD kinase activity in vitro is shown in Fig. 2. Total NAD kinase activity gradually decreased at increasing concentration of Al in the medium. However, the extent of aluminum effect on calmodulin - dependent and independent NAD kinase activity was different. The decrease of total enzyme activity was associated with the remarkably decreased calmodulin - dependent NAD kinase activity, whereas the activity of calmodulin - independent NAD kinase activity remained almost unchanged. Furthermore, the activity of these two types of NAD kinase changed with the increasing concentration of aluminum. In control samples (not exposed to aluminum), the ratio of calmodulin - dependent NAD kinase activity to the total enzyme activity was 76%, however, with an enhancement of Al concentration up to 2 mmol/L, the share of calmodulin - dependent NAD kinase activity in the total enzyme activity decreased to 21%, while the ratio of calmodulin - independent NAD kinase activity increased remarkably.

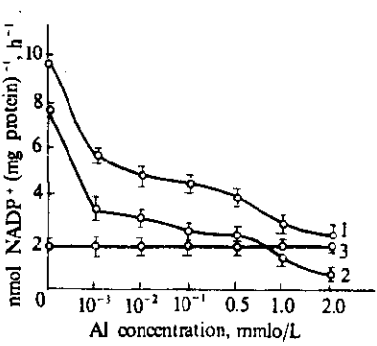

Fig. 2 Effect of Al on NAD kinase activity *in vitro*
1. Total NAD kinase activity;
2. calmodulin - dependent NAD kinase activity;
3. calmodulin - independent NAD kinase activity.
Al treatment time: 10 min. Error bars indicate SD

The pattern of the time effect of aluminum on NAD kinase activity in vitro was observed. The prolongation of the treatment time of aluminum was accompanied by the decrease of total enzyme activity, but the calmodulin - independent NAD kinase activity remained unchanged (Fig. 3). The share of calmodulin - independent NAD kinase activity in total enzyme activity distinctly increased with the prolongation of treatment time of aluminum. The total enzyme activity was almost constituted by the calmodulin

-independent NAD kinase activity at 20 min treatment of aluminum (Fig. 3). Both the structure and function of calmodulin are affected by aluminum (Haug, 1984; 1985). In the present experiment, the restoration ability of calmodulin to the inhibition of aluminum on NAD kinase activity was investigated. Under low Al stress (0.1 mmol/L), the restoration of enzyme activity was obvious with the increased of calmodulinconcentration, and the enzyme activity could reach to the control level at a calmodulin concentration of 2 μg. However, when the aluminum concentration was increased to 2 mmol/L, the concentration of 6 μg calmodulin was seemingly too low for the restoration of enzyme activity(Fig. 4).

4 Discussion

Some researchers have studied the transportation and accumulation of Al in rice seedlings. It is reported that most of aluminum exists in the roots, but there was still some in leaves (Foy, 1978; Hao, 1989). The present work further detected Al content in the chloroplast of rice seedling and showed Al accumulation in the chloroplast after application of Al. A study indicated that upon application of Al to rice, the reaction between chloropyll a and b declined, accompanied by a marked decrease in gross photosynthesis and photosynthetic rate (Sarkunan, 1984). It is expected that accumulation of Al in the chloroplasts of rice might be one of the causes for Al affecting many physiological activities of the chloroplast, including some enzyme activities.

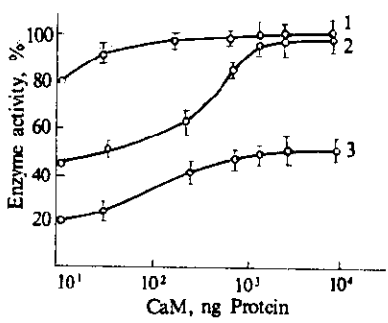

Fig. 3 Time effect of Al on the activity of NAD kinase
Al concentration: 0.5mmol/L
1. total enzyme activity;
2. calmodulin-dependent NAD kinase activity;
3. calmodulin-independent NAD kinase activity.
Error bars represent the *SD*

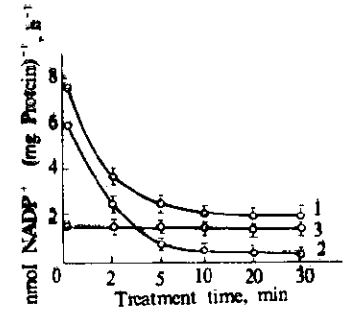

Fig. 4 Reversal of extrinsic calmodulin to the inhibition of Al on NAD kinase activity
Al concentration:
1. control (without Al); 2. 0.1mmol/L;
3. 2.0mmol/L. Al treatment time: 30min.
Calmodulin
treatment time: 30min. Error bars indicate the SD.
Al and calmodulin are added simultaneously

In the present study, NAD kinase activity *in vivo* increased in certain range of Al concentrations, accompanied by the enhancement of calmodulin-independent NAD kinase activity and the gradual decrease of calmodulin-dependent NAD kinase activity. This might be ascribed to the induction of calmodulin-independent NAD kinase synthesis *in vivo* of rice seedlings under Al stress. It is reasonable, however, that the induction of synthesis could not be detected in vitro

experiments. But the study *in vitro* still showed the strong tolerance of calmodulin - independent NAD kinase in response to Al stress. These results suggested that calmodulin - independent NAD kinase may play a role in Al tolerance. Under some environmental stresses, plants can modify glycolytic and pentose - phosphate pathways by regulation the pyridine nucleotide pool (Allan, 1985). This regulation can affect the production of organic acid or mucilage, which protects against aluminum toxicity (Suhayda, 1984; 1985). Catalyzing the only known biochemical reaction of NADP synthesis, NAD kinase plays a regulating role to the pyridine nucleotide pool and thereby may indirectly regulate the production of organic acid or mucilage. It is expected that, under Al stress, the high activity of calmodulin - independent NAD kinase might be helpful to ameliorate Al toxicity.

The mechanism of Al toxicity to plants is still very far from being understood, but it has been suggested that calmodulin is a main target of aluminum action (Haug, 1984; Liu, 1990).

Calmodulin is a multifunctional, Ca^{2+} dependent, regulatory protein. Aluminum can bind stoichiometrically to calmodulin and induce changes in the functioning of this protein due to the increase of its hydrophobic surface, the loss of calmodulin - helix content and changes of its association with the activation of target enzymes (Haug, 1984; 1985). It has been found that in our experiment that in a certain range of aluminum concentrations, extrinsic calmodulin can reverse or even completely resist the inhibition of aluminum on calmodulin - dependent NAD kinase activity, supporting that aluminum can affect calmodulin - dependent NAD kinase activity *via* modification of calmodulin structure. It has also been noticed, however, that the reversal of calmodulin to aluminum toxicity remarkably decreased at higher concentrations of Al (2 mmol/L), suggesting that at higher concentrations, Al affect the structure of the enzyme in addition to impairing calmodulin.

Acknowledgements—The authors are thankful to Professor D. Y. Sun for his helpful assistance in this study.

References

Allan E, Trewavas A. Planta, 1985;165:493

Anderson JM, Cormier MJ. Biophys Res Commun, 1978;84:595

Barber SA. Plant root and its environment (Ed. by Carson EW). Virginia:Charlottesville University Press. 1974:525

Biro RL, Dun D, Serlin BS, Terry ME, Dalta N, Roux SJ. Plant Physiol,1984;75,382

Bradford MM. Biochem, 1976;72:248

Foy CD. Plant root and its environment (Ed. by Carson EW). Virginia:Charolottesville University Press. 1974:601

Foy CD, Fleming AL. Crop tolerance to suboptimal land conditions(Ed. by Jung GA). Wisconsin:ASA Spec Pub Am Soc of Agron Madison. 1978;(32)301

Hao Luning, Liu Houtian. Acta Botanical Sinica, 1989;31:847

Haug A. CRC Crit Rev Plant Sci, 1984;1:345

Haug A, Caldwell CR. Frontiers of membrane research in agriculture (Ed. by St John JB, Berlin E, Jackson PC). Beltsville symposium 9. Totowa:Rowman & Allanheld. 1985:359

Henry EW, Miller JE, Dung LJ, Johnson MT, Yeekpeh JK. J Pl Nutr, 1981;4:353

Hincha DK. Biochim Biophys Acta, 1986;861:152

Liu Houtian, Hao Luning. Acta Botanica Sinica, 1990; 32:528

Maciejewska U. Physiol Plantarum, 1990; 80:133

McGuinness ET, Butler JR. Int J Biochem, 1985;17:1

Muto S, Miyachi S, Plant physiol, 1977;59:55

Sarkunan V, Biddappa CC, Mayak SK. Curr Sci, 1984;53:822

Slaski JJ, J Plant Physiol, 1989;133:696

Slaski JJ. J Plant Physiol, 1990;136:40

Suhayda CG, Haug A. Biochem Biophys Res Commun, 1984; 119:376

Suhayda CG, Haug A. Can J Biochem Cell Biol; 1985;63:1167

Ulrich B, Parbrath J. Effects of accumulation air pollutants in forests ecosystems (Ed. by Reided Publ Comp), 1983;331

(Received March 8, 1993)