Short Communication

Nitrate assimilatory enzymes of *Spirulina (Arthospira) platensis* are more thermotolerant than those of rice

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ABSTRACT

In order to assess the potential of *Spirulina (Arthospira) platensis* as a source of abundant, thermostable nitrate assimilatory enzymes, the specific activities and thermal tolerance of nitrate reductase (NR), nitrite reductase (NiR) and glutamine synthetase (GS) were compared with those of rice in crude extracts *in vitro*. The results show that *Spirulina* enzymes have relatively higher thermotolerance. When the extracts were pre-exposed to 80 °C for 1 hr, *Spirulina* enzymes retained higher activities by 3.4, 1.7 and 3.7 fold, respectively than corresponding enzymes in rice. This property was not due to salts and other small proteins/molecules, as their removal by gel filtration (G-25) did not affect their thermotolerance. **[Physiol. Mol. Biol. Plants 2009; 15(3) : 277-280]** *E-mail : raghuram98@hotmail.com*

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INTRODUCTION

Nitrate is the most common form of nitrogen nutrient available for the growth of cyanobacteria and plants. It is taken up through nitrate transporters and sequentially reduced into nitrite and ammonium ions by nitrate reductase (NR) and nitrite reductase (NiR), and further assimilated into amino acids through the glutamine synthetase (GS) and glutamate synthase (GOGAT) cycle. The detrimental environmental effects of excess nitrate/nitrite and other forms of reactive nitrogen generated from unutilized fertilizers and industrial effluents have been highlighted recently (Abrol *et al.,* 2007; Galloway *et al.,* 2008). Purified nitrate assimilatory enzymes are used in environmental testing (Patton *et al.,* 2002; Campbell *et al.,* 2006), nitrate decontamination (Mellor *et al.,* 1992), meat processing (Gotterup *et al.,* 2007), biosensors (Chen *et al.,* 2008) and other applications (Angeby *et al.,* 2002), while whole organisms are useful for bioremediation (Hu *et al.,* 2000). However, the poor stability and thermosensitivity of these enzymes is a major limitation in their widespread use.

Spirulina (Arthospira) platensis is a cyanobacterium commercially well known for its high protein content and other neutraceutical properties, and is also becoming increasingly popular in environmental biotechnology (Vonshak, 1997; Eriksen, 2008). For example, *Spirulina* was used for decontamination of nitrate, phosphate etc. from swine waste (Kim *et al.,* 2000). However, the nitrate assimilating enzymes and genes of *Spirulina* have not been characterized to design better-informed strategies for *in situ* and *ex situ* applications. Our earlier studies have shown that NR in *Spirulina* is substrate-inducible (Jha *et al.,* 2007), a fact well known in higher plants but not in non-nitrogen-fixing, nitrate-dependent cyanobacteria. We also showed that NR, NiR and GS in *Spirulina* have higher specific activities and stabilities at room temperature than those from rice (Ali *et al.,* 2008). The present study was aimed at examining the thermotolerance of NR, NiR and GS *in vitro* in *Spirulina*, compared to those of rice, since higher plants are traditional sources for these enzymes.

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MATERIALS AND METHODS

Growth conditions

Axenic culture of *Spirulina platensis* (*Arthospira platensis*) strain PCC 7345 was obtained from the Pasteur Culture Collection (Institüt Pasteur, Paris). It was grown photoautotrophically till exponential phase on a shaker (125 rpm) at 27 ± 2 °C under white light (4

Klux) for 8 days, on a synthetic medium containing BG-11 and ASN-III media (1:1 v/v) (www.pasteur.fr/recherche/banques/PCC/). Crude extracts were prepared as described previously (Jha *et al.,* 2007) with minor modifications: the cell pellet to buffer ratio was 1: 2 (w/v) and the suspension was sonicated for 7 min (5 sec on and 10 sec off) at 35 % amplitude. The crude extract was transferred into a pre–cooled microfuge tube and kept on ice for immediate use or frozen at –20 °C for later use. *Spirulina* crude extracts were used either directly, or after gel filtration using Sephadex G-25 (Amersham, USA). The matrix was swollen using Tris-HCl (50 mM, pH 7.0) and a column of 5 ml bed volume was packed in a 10 ml syringe using the extraction buffer. The column was kept at 4 °C, loaded with 2 ml of crude extract and fractions of 0.5 ml each were collected using the same extraction buffer for elution. The fractions that contained enzyme activity were pooled and used.

Rice seeds (*Oryza sativa* var. Panvel 1) were obtained as described earlier (Ali *et al*., 2008), surface-sterilized, imbibed in distilled water and grown on washed wet cotton in plastic trays at 28 °C and 80 % humidity under white light (1 Klux, 12 hour diurnal cycle) for 9 days. Excised leaves were floated on 40 mM KNO_3 for 6 hrs, frozen in liquid N_2 and stored at –70 °C till use. Crude extracts were prepared as described earlier (Ali *et al.,* 2008).

Enzyme assays

All the enzyme assays were set up in triplicates as described below and protein was estimated by Bradford's method. Protein concentration in the crude extracts of both organisms was maintained at ~5 mg/ml for effective comparison. Means from the three independent experiments (with internal duplicates) were plotted, along with standard errors.

Nitrate reductase specific activity was measured as described earlier for *Spirulina* (Jha *et al.,* 2007) and rice (Ali *et al.,* (2008). It was defined as nmoles of nitrite produced per mg protein per min.

Nitrite reductase activity in both the organisms was assayed as described by Ali *et al.* (2008). The reaction mixture contained 75 mM potassium phosphate buffer (pH – 7.5), 0.5 mM KNO_2, 2 mM methyl viologen, 20 mM $Na_2S_2O_4$ freshly prepared in 0.3 M sodium bicarbonate and 50 µl of enzyme extract. The specific activity of NiR was defined as nmoles of nitrite consumed per mg protein per min.

Glutamine synthetase was assayed as phosphate released from ATP utilization as described earlier (Ali *et al.,* 2008). The crude extract was desalted using a Sephadex G-25 column before assay. The reaction mixture consisted of sodium ATP, (7.5 mM, pH 7.0), sodium glutamate, (100 mM, pH 7.0), $MgCl_2$, (50 mM), NH_4Cl, (50 mM), imidazole buffer (50 mM, pH 7.0) and 10 ml of desalted extract in a total reaction volume of 0.8 ml. The blanks contained all assay components except ATP. The reaction was incubated for 15 min at 25 °C and stopped by adding 50 ml of 2.02 M ammonium molybdate reagent and 150 ml of 6 mM ANSA (1- amino-2-naphthol-4-sulfonic acid). The contents were vigorously mixed and the color developed was measured spectrophotometrically (A_{680}) after 15 minutes. GS specific activity was defined as µg of phosphate released per mg protein per min.

RESULTS AND DISCUSSION

In order to evaluate thermotolerance, crude extracts from *Spirulina* cultures and rice leaves were aliquoted into different microfuge tubes and pre-incubated at different temperatures (4, 25, 40, 60 and 80 °C) for different durations (5, 15, 30, 45 and 60 min) before assaying them separately for NR, NiR and GS at 25 °C as described above. The data presented in Figure 1 clearly indicate that the kinetics of thermal inactivation of each of the enzymes was significantly slower in *Spirulina* (top panel) than in rice (bottom panel), especially after 30 minutes of preincubation at 60 and 80 °C.

Thermotolerance of nitrate reductase in *Spirulina*

NR had maximal specific activity at 4 and 25 °C, and remained stable for at least an hour in both organisms. Using a different strain of *Spirulina*, Ali *et al.* (2008) reported that at 25 °C, NR activity had a half life of 22 hrs in *Spirulina*, as compared to 5 hrs in rice *in vitro,* but thermotolerance at higher temperatures was not tested. Indeed, our analysis of NR from two more strains of *Spirulina* showed that while there may be strain-specific differences in specific activities *vis-à-vis* rice, higher stability is a common feature across strains (data not shown). At higher temperatures, *Spirulina* NR revealed higher thermotolerance than that of rice (Fig. 1A). For example, at 60 °C and 80 °C, the specific activity of rice NR declined to negligible levels within 5 min indicating total inactivation, whereas in *Spirulina*, even after 30 min, NR retained full activity at 40 °C, over half the activity at 60 °C, and 28 % of the initial activity at 80 °C. In view of the fact that earlier attempts to enhance

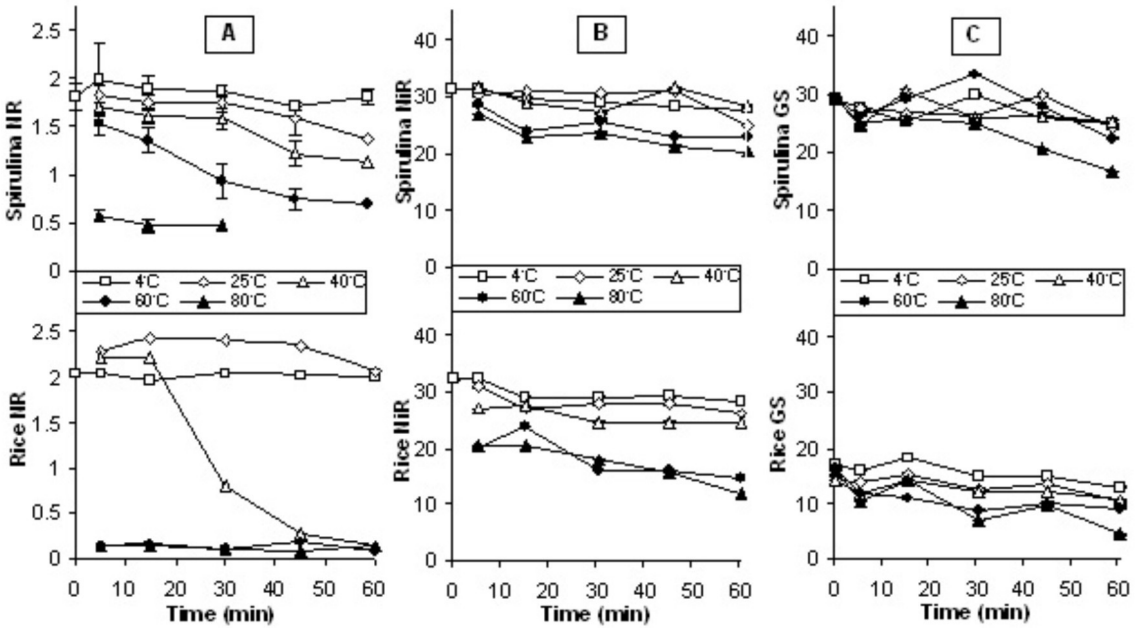

Fig. 1. Thermal tolerance of NR, NiR and GS specific activity in *Spirulina* (top) and rice (bottom). Crude extracts from *Spirulina* cultures and rice leaves were pre-exposed to a range of temperatures (4, 25, 40, 60 & 80 °C) and NR, NiR and GS activities were assayed after different time intervals (5, 15, 30, 45, 60 min) as described in Materials and Methods. The mean specific activities from three independent experiments are plotted with standard error bars.

the stability of higher plant NR by genetic manipulation met with limited success (Campbell *et al.,* 2006), our finding that *Spirulina* could be a natural source for stable and thermotolerant NR bears significance.

Thermotolerance of nitrite reductase in *Spirulina*

NiR activity remained stable for at least an hour in both organisms at 4, 25 and 40 °C. At higher temperatures, *Spirulina* NiR revealed higher thermotolerance than that of rice, though to a lesser degree than in NR (Fig. 1B). Its specific activity in rice declined to 45 % and 37 % of the initial activity in 1 hr at 60 and 80 °C respectively, whereas in *Spirulina*, NiR retained over 73 % and 64 % of its initial activity under similar conditions. Ali *et al* (2008) did not observe significant differences in the half lives of NiR at RT between *Spirulina* and rice (13 and 12 hrs respectively), whereas our present data at higher temperatures clearly bring out that thermotolerance is an advantage of NiR in *Spirulina* as compared to that of rice.

Thermotolerance of glutamine synthetase in *Spirulina*

The specific activity of GS, in general, was higher in *Spirulina* than in rice, indicating the difference in steady state abundance of the enzyme, corroborating earlier results from a different strain (Ali *et al*., 2008). At 4, 25 and 40 °C, the enzyme remained nearly stable for at least an hour in both organisms. At higher temperatures, *Spirulina* GS revealed higher thermotolerance than that of rice (Fig. 1C). Its specific activity in rice declined to 55.62 % and 28.1 % of the initial activity in 1 hr at 60 and 80 °C respectively, whereas in *Spirulina*, GS retained over 76.7 % and 56.8 % of its initial activity under similar conditions. We note that at 60 °C, the specific activity of *Spirulina* GS increased marginally by about 10 % in 30 min, but the significance of this, if any, is not clear. However, it does not affect the main result that the *Spirulina* enzyme (GS) is more thermotolerant than that of rice.

Thermotolerance following gel filtration

In order to verify the possible role of salts, small proteins and any other small molecules in conferring thermotolerance to the *Spirulina* enzymes, its crude extracts were subjected to Sephadex G-25 gel filtration at 4 °C and again tested for thermal tolerance of NR and NiR at 80 °C for 30 min and 1 hr. The data shown in Fig. 2 clearly indicates that gel-filtered extracts retained their original thermotolerance levels for both the enzymes. GS was always assayed in gel-filtered extracts, as crude

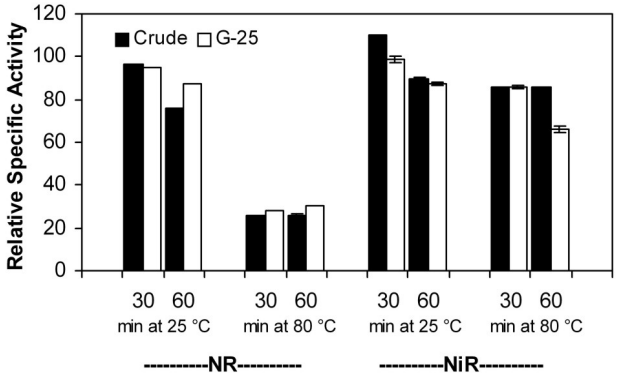

Fig. 2. Thermal tolerance of NR and NiR before and after Sephadex G-25 gel filtration of *Spirulina* extracts. The enzymes were assayed in crude or G-25-purified extracts pre-incubated at 25 °C or 80 °C for 30 and 60 min as described in Materials and Methods. For the calculation of relative specific activities, the absolute specific activity at 4 °C was taken as 100 % for both fresh and purified extracts. The actual activities were 1.80 and 2.44 IU for NR and 30.24 and 31.19 for NiR before and after fractionation, respectively. The means of replicates were plotted along with SD bars.

extracts were not suitable for direct use in the assay. Therefore, it is very likely that thermotolerance is a property inherent to these enzymes in *Spirulina*, though it can only be confirmed after their complete characterization by cloning or purification, which is currently underway.

In conclusion, NR, NiR and GS of *Spirulina (Arthospira) platensis* revealed relatively higher thermotolerance than those of rice, especially at above-ambient temperatures. Even at 80 °C for 1 hr, these enzymes were less affected in *Spirulina* than in rice, retaining 3.4, 1.7 and 3.7 fold higher activities respectively. The thermal tolerance of all three nitrate assimilatory enzymes renders *Spirulina* an attractive natural source of sturdy enzymes for various applications. This is also the first report of a single source for all three thermotolerant nitrate assimilatory enzymes.

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