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Effects of proline and betaine on heat inactivation of ribulose-1,5-bisphosphate carboxylase/oxygenase in crude extracts of rice seedlings

M.L. DIONISIO-SESE*, M. SHONO, and S. TOBITA**

Okinawa Subtropical Station, Japan International Research Center for Agricultural Sciences (JIRCAS), Ishigaki, Okinawa 907, Japan

Abstract

Activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) in heated crude extracts from seedlings of the rice cultivars Hitomebore and IR28 was investigated in the presence of proline and betaine. Both solutes retarded the inactivation of the enzyme extracted from the leaves of both cultivars at temperature-stress from 35 to 45 °C. At 50 °C, however, betaine was effective in both cultivars. Stabilization of RuBPCO activity was independent of the added solute from 1 to 2 M concentration.

Additional key words: cultivar differences; *Oryza sativa* L.

Introduction

The accumulation of compatible solutes, such as proline and betaine, is well recorded in many plant species subjected to water stress (Singh *et al.* 1972, Hanson and Nelsen 1978, Velitchkova and Fedina 1998), salt stress (Storey and Wyn Jones 1975, 1977, Wyn Jones and Storey 1978), cold stress (Naidu *et al.* 1991), and heat stress (Chu *et al.* 1974, Kuo *et al.* 1986). Apart from their major role, however, as cytoplasmic osmotica, these compounds may have other possible adaptive functions in plants under these stresses. *In vitro* studies have shown that proline and/or betaine stabilize biomembranes (Jolivet *et al.* 1982), the oxygen-evolving photosystem 2 complex (Papageorgiou and Murata 1995), and activities of the enzymes NAD- and NADP-dependent malate dehydrogenase (Pollard and Wyn Jones 1979, Nikolopoulos and

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*Corresponding author. Present address: Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines at Los Baños, College, Laguna 4031, Philippines; fax: +63 49 536 2517, e-mail: mlds@mudspring.uplb.edu.ph

**Present address: Environmental Resources Division, JIRCAS, Tsukuba, Ibaraki 305-8686, Japan.

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Manetas 1991), pyruvate kinase (Pollard and Wyn Jones 1979), and phosphoenolpyruvate carboxylase (Shomer-Ilan and Waisel 1986, Nikolopoulos and Manetas 1991).

Heat can be one of the environmental stresses, which adversely affect plant metabolic processes through alteration of enzyme structure and disruption of its function. Enzymes from higher plants, which have been studied so far with regard to heat stability in the presence of compatible solutes, were either cytoplasmic or mitochondrial enzymes (Paleg *et al.* 1981, Nash *et al.* 1982, Laurie and Stewart 1990, Nikolopoulos and Manetas 1991, Shomer-Ilan *et al.* 1991). Report on the effect of compatible solutes on heat stability of chloroplastic enzymes, notable of which is RuBPCO, seems to be lacking. An *in vivo* study, however, has shown that high temperature stress for prolonged periods inhibits RuBPCO activity in rice plants, especially in the heat-sensitive cultivar (Bose and Ghosh 1995). This work is a preliminary investigation on the possible protective effect of proline and betaine on the heat-induced inactivation of extracted RuBPCO from rice seedlings.

Materials and methods

Two rice (*Oryza sativa* L.) cultivars, Hitomebore and IR28, were grown in hydroponic solution (modified Yoshida nutrient solution) for one month from emergence in the glasshouse under natural irradiation and day/night temperature of 28 °C. RuBPCO was extracted from 500 mg leaf tissues by grinding the leaves to a powder in liquid nitrogen after which 5 cm^3 extraction buffer containing 100 mM HEPES-NaOH (pH 7.8 at 4 °C), 5 mM dithiothreitol, 15 mM $MgCl_2$, and 1 mM EDTA was added. The resulting supernatant, after centrifugation at 30 000×*g* for 30 min at 4 °C was designated as the crude extract, in which RuBPCO activity was stable for at least 5 h at 4 °C.

RuBPCO activity in the extract was measured using a coupled-spectrophotometric assay described by Lan and Mott (1991). For heat inactivation experiments, 100 mm^3 of the crude extract was incubated in water bath for 15 to 30 min at the indicated temperature with the desired concentration of compatible solutes. All experiments were performed twice and the spread of values is shown as error bars representing standard errors of the means.

Results and discussion

Our *in vitro* study supports the thermolability of the rice RuBPCO; complete inactivation was observed in both Hitomebore and IR28 cultivars after 15 min exposure to temperature as high as 50 °C (Fig. 1, *left*). The enzyme from Hitomebore, however, was more heat stable at lower temperatures than that from IR28; at 35–45 °C, the Hitomebore enzyme was only 10–40 % inactivated whereas 40–80 % of the activity was lost in IR28. The differences between the two cultivars could well be due to difference in the RuBPCO proteins or alternatively, differences in the other constituents of the extracts, such as proteases and phenolics. Although the measurements

fail to differentiate between direct heat inactivation of RuBPCO and temperature-dependent inactivation due to other components (such as proteases) of the leaf extract used as the source of RuBPCO, the possibility of intraspecific variation of rice RuBPCO in response to heat stress still remains. In a previous study, Bose and Ghosh (1995) showed that at 45 °C, the specific activity and holoenzyme level of RuBPCO were more stable in the heat-tolerant cultivar (N22) than in the heat-sensitive one (IR8). Nevertheless, at 50 °C both rice cultivars exhibited a pronounced decline in RuBPCO activity and holoenzyme concentration.

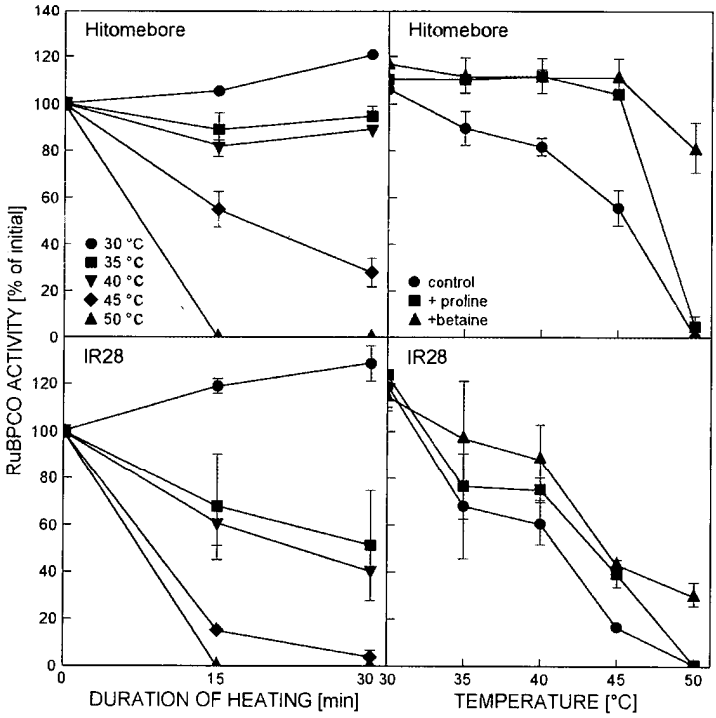

Fig. 1. Heat inactivation of RuBPCO extracted from rice leaves at various temperatures. *Left*: incubation at 30 (●), 35 (■), 40 (▼), 45 (◆), and 50 °C (▲). *Right*: Incubation for 15 min in the absence (●) or presence of 1 M proline (■) or betaine (▲). Activities expressed as a % of the initial activity at time zero.

Both 1 M proline and betaine completely protected RuBPCO from Hitomebore against 15 min heat inactivation when incubated at 35 to 45 °C (Fig. 1, *right*). However, at 50 °C addition of proline had no effect in protecting the enzyme from heat-induced loss of activity while betaine addition resulted in about 80 % retention of RuBPCO activity. Unlike in Hitomebore, addition of compatible solutes did not completely protect the IR28 enzyme against heat inactivation. Nevertheless, although inactivation of both the proline- and betaine-treated samples did occur at 35-45 °C, they were always slower than the control. Thus, the inclusion of proline and betaine in IR28 samples did not prevent heat inactivation, but they did retard it. The stabili-

zation of enzyme activity was independent of solute concentration from 1 to 2 M, whereas proline or betaine addition below 1 M caused an increasing protection against heat inactivation of the enzyme (Fig. 2).

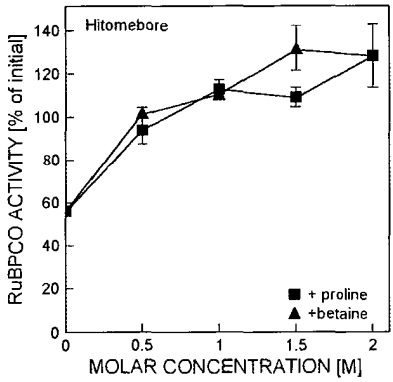

Fig. 2. Protection from heat inactivation of RuBPCO from rice leaves by varying concentrations of proline (■) and betaine (▲) after heating at 45 °C for 15 min. Activity is expressed as a % of the initial activity at time zero.

In stressed plants, compatible solutes accumulate in the cytosol, where they prevent the stress-related inactivation of metabolic processes (Greenway and Munns 1980). This cytoplasmic localization of solutes may not be strict since membrane alterations accompanying stresses may also facilitate changes in its distribution within cell compartments. For example, in the halophyte *Mesembryanthemum crystallinum*, chloroplasts isolated in conditions of high external salt revealed accumulation of osmotic solutes (Demmig and Winter 1986). The osmoprotectant glycinebetaine in particular was reported to be synthesized in the cytoplasm and in the chloroplasts of halophilic plants belonging to family *Chenopodiaceae* (Hanson *et al.* 1985). In the chloroplast it is synthesized from the oxidation of choline by specific enzymes, and can be found in this organelle at concentrations up to 1 M when leaves grow in saline environments (Hanson *et al.* 1985). Thus under high temperature, the solutes in concentration around 1 M may also accumulate in the chloroplast and protect chloroplastic enzymes against heat-induced destabilization.

Surprisingly, in both Hitomebore and IR28 proline was completely ineffective while betaine caused a small retention of enzyme activity at 50 °C. Thus, betaine unlike proline offered protection against heat inactivation of RuBPCO throughout the whole range of temperatures studied. Similarly, betaine was more effective than proline with regard to the heat-induced destabilization of chickpea glutamine synthetase (Laurie and Stewart 1990), and nitrate reductase and NADH-dependent malate dehydrogenase from *Salsola soda* (Nikolopoulos and Manetas 1991). Betaine is also the most effective enzyme-stabilizing agent in cases of destabilization by electrolytes like NaCl (Pollard and Wyn Jones 1979). It also protects the photosynthetic oxygen-evolving complex from heat-induced and salt-induced inactivation (Papageorgiou and Murata 1995). The co-evolution of enzyme structure and compatible solute systems,

that is the protective effect of a solute, may be restricted to enzymes of the species which accumulate the particular solute (Manetas *et al.* 1986, Nikolopoulos and Manetas 1991). Yet this is not the case with the observed betaine-over-proline protection of heat-inactivated rice RuBPCO (for a similar glycinebetaine-over-proline preference in alleviating stomatal and non-stomatal inhibition of photosynthesis in wheat see Rajasekaran *et al.* 1997). In contrast to plant families such as the *Chenopodiaceae* in which betaine occurs universally as a consistent chemical character, the *Gramineae* show numerous discontinuities (Storey *et al.* 1977). In particular, rice does not appear to have any betaine-accumulating capability (Rathinasabapathi *et al.* 1993) and proline is the compound observed to increase in response to salt stress (Roy *et al.* 1992). With the aim of enhancing the drought- and salt-tolerance of rice plants, the enzyme involved in betaine biosynthetic pathway is presently introduced by genetic engineering (Hayashi *et al.* 1997). The resulting salt-tolerant transgenic rice plants may also be resistant to heat stress.

There seems little clear indication of how proline and betaine exert their protective effects on thermal denaturation of enzymes. In the case of RuBPCO, the quaternary structure of the enzyme is probably essential for its catalytic activity. Thus, the stabilization of RuBPCO activity in the presence of compatible solutes is likely due to conservation of the quaternary structure of the enzyme, such as with phosphoenolpyruvate carboxylase (Stamatakis *et al.* 1988). The present consensus concerning the stabilization of enzymes by compatible solutes is explained in terms of the minimization of protein-water interactions as proposed by Arakawa and Timasheff (1985). The compatible solutes through a preferential exclusion from the protein domain may act as protein aggregating agents; the enzymes are confined into a small fraction of the total volume and the organic cosolutes increase subunit interaction in preventing protein unfolding thereby maintaining protein configuration. This 'exclusion volume' theory is put forward as an encompassing explanation for the protective function of compatible solutes. However, the effects of compatible solutes are not always identical, *e.g.*, the greater effectiveness of betaine over proline in the stabilization of rice RuBPCO at 50 °C as well as other cases (Laurie and Stewart 1990, Nikolopoulos and Manetas 1991). Any explanation of the solute-induced enzyme protection response would therefore have to take into account also the evident molecular disparity between proline and betaine.

To the best of our knowledge, this is the first report regarding the *in vitro* protection of RuBPCO of a higher plant against heat stress by compatible solutes such as proline and betaine. Since measurements were done on crude enzyme extracts, it would be interesting to investigate further the structural differences between RuBPCO from the two rice cultivars, which may result in the differences in inactivation and protection.

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