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Citrulline functions as an efficient hydroxyl radical scavenger: Implication for the drought-tolerance of wild watermelon plant

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Introduction

Wild watermelon plants inhabit in the Kalahari desert, Botsuwana, and exhibit exceeding tolerance to water deficits. The plants hold the photosynthetic apparatus intact during prolonged drought in the strong light, suggesting that there should be some mechanisms to tolerate oxidative stress arisen from the excess light energy in the leaves (Kawasaki *et al*, 2000; Miyake and Yokota, 2000). Drought stress induces an ArgE-related protein in the leaves, results in the massive accumulation of free amino acids citrulline and, to a lesser extent, arginine (Kawasaki *et al*, 2000). However, possible protective roles of citrulline in drought tolerance of wild watermelon plants have remained to be investigated.

It has been reported that side chains of arginine residues in a polypeptide are sensitive to oxidation by hydroxyl radicals (Amici *et al*, 1989), implying that citrulline, structurally analogous to arginine, may also exhibit high reactivity toward hydroxyl radicals. Excellent performance of wild watermelon plants against drought/oxidative stresses, together with a proposed hypothesis that compatible solutes act as radical scavengers (Smirnoff and Cumbes, 1989; Shen *et al*, 1997), prompted us to evaluate the role of a new compatible solute, citrulline, as a free radical scavenger.

Materials and methods

Materials. Wild watermelon (*Citrullus lanatus* sp.; Tottori International Watermelon Seed Bank # 101117-1) was grown as described previously (Kawasaki *et al*, 2000). Purified lactate dehydrogenase (LDH) from pig heart were purchased from Oriental Yeast (Tokyo). All other reagents were obtained from Nakalai Tesque (Kyoto)

Determination of the rate constant for the reaction between citrulline and hydroxyl radicals. The reaction mixture contained 40 mM potassium phosphate buffer (pH 7.4), 0.26 mM ascorbate, 0.15 mM EDTA-Na-Fe(III), 0.6 mM H_2O_2, 2 mM salicylate and various concentrations of compounds in question in a final volume of 400 µL. The reaction mixtures were incubated at 25 ˚C for 90 min, and then resultant products 2,3-dihydroxy-benzoic acid (DHBA) were quantified as described (Smirnoff and Cumbes, 1989). The second-order rate constants of the reactions between various compounds and hydroxyl radicals were calculated according to the kinetic competition model for ROS scavengers as described (Mitsuta *et al*, 1990). The inhibitory dose-fifty (ID_{50}) of each compounds was calculated by fitting the experimental data to the equation: $I/Io = ID_{50}/([S] + ID_{50})$, where I and Io were the concentrations of DHBA formed in the presence and absence of the compound, respectively, and [S] was the concentration of the compound. The second-order rate constants of the compounds were calculated from the following equation: $k_2 = k_1 \times ([SAL]/ID_{50})$, where k_1 and

k_2 were the second-order rate constants for the reactions of hydroxyl radicals with salicylate and the compound, respectively, and [SAL] was the initial concentration of salicylate. A constant for salicylate, 1.2 X 10^{10} M^{-1} s^{-1} (Maskos *et al*, 1988), was used for calculations of the constants for the competitors.

Enzyme assays. Leaves of wild watermelon were frozen by liquid nitrogen, and ground to fine powder in a mortar and pestle. The extraction buffer that consisted of 50 mM Tris-HCl buffer (pH 7.5), 5 mM DTT, 15 % glycerol was added and the resulting homogenate was centrifuged at 10,000 *g* for 10 min. The supernatent was then used for MDH assays. Malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) activities were assayed by measuring the oxidation of NADH spectrometrically at 25 ˚C as described previously (Ochoa, 1955; Kornberg, 1955).

Results

Determination of the rate constant for the reaction between citrulline and hydroxyl radicals.

The reactivity of citrulline with the hydroxyl radicals was examined *in vitro*, by allowing it to compete with salicylate for the radicals (Smirnoff and Cumbes, 1989; Halliwell and Gutteridge, 1984). In this assay, salicylate reacts with hydroxyl radical and yields its hydroxyl derivative 2,3-dihydroxy-benzoic acid (DHBA), which can be quantified spectrometrically. A compound in question added to the reaction mixture traps hydroxyl radicals and reduces the amount of DHBA in concentration-dependent manner. The data obtained were used to estimate the second-order rate constant for the reaction between hydroxyl radicals and citrulline, and compared with those of other representative compatible solutes from plants; *i.e.*, mannitol, proline and glycine betaine (Table I). The rate constant for mannitol was calculated as 2.1×10^9 M^{-1} s^{-1}, which was in a good agreement with the value published previously (Buxton *et al*, 1988). The rate constant for citrulline was estimated to be 3.9×10^9 M^{-1} s^{-1}. This value was the highest among the four solutes investigated in this study, and notably, exceeded that of mannitol, which was well-known as an efficient radical scavenger. The rate for citrulline was also 7- and 48-times higher than those of proline and glycine betaine, respectively.

Citrulline is Compatible with Metabolic Enzymes

In order to address whether the accumulated citrulline interfere with cellular metabolisms, the effects of this compound on the activities of two representative cytosolic enzymes, MDH and LDH, were investigated *in vitro.* The increasing concentrations of citrulline did not exert any inhibitory effect on the MDH activity (Fig. 1A). Rather, citrulline slightly stimulated the MDH activity; 109 % and 103 % of the control activity in the presence of 200 mM and 600 mM citrulline, respectively. As for LDH, citrulline reduced the activity only marginally (Fig. 1B).

In contrast, these enzymes were strongly inhibited by high concentrations of arginine chloride (Fig. 1). The MDH and LDH activities in the presence of 600 mM arginine chloride dropped to 10 % and 54 % of the control values, respectively. To examine whether these inhibitory effects are attributable solely to chloride ion used as the counter ion in this assay, the effects of KCl were also examined. The MDH and LDH activities in the presence of 600 mM KCl resulted in 21 % and 64 % of the control values, respectively, suggesting that the arginine ion is more inhibitory for the metabolic enzymes than K^+.

Table I
The second-order rate constants for the reactions of various compatible solutes with hydroxyl radicals

Compounds	ID_{50} (mM)	Rate constant ($M^{-1}s^{-1}$)
Citrulline	6.6 ± 1.2	(3.9 ± 0.82) x 10^9
Mannitol	13 ± 3.0	(2.1 ± 0.58) x 10^9
Proline	48 ± 9.0	(5.4 ± 0.94) x 10^8
Glycinebetaine	500 ± 280	(8.2 ± 0.31) x 10^7

ID_{50} represents concentration of respective compounds which reduces hydroxylation of 2 mM salicylate by 50 %. Data represent the mean ± SE (n=3).

Discussion

The present study demonstrated that citrulline, a novel compatible solute in drought-tolerant wild watermelon plants, fulfils properties as an efficient protective compound. The reaction rate constant between hydroxyl radicals and citrulline was shown to be 3.9×10^9 M^{-1} s^{-1}, showing that citrulline was one of the most potent radical-scavengers among compatible solutes (Smirnoff and Cumbes, 1989). The accumulated citrulline in watermelon leaves could significantly increase antioxidative potential of the cells and contribute to the tolerance of wild watermelon plants to oxidative stress brought about by drought. Considering the estimated concentration of citrulline is 200 mM in chloroplasts of watermelon leaves (Kawasaki *et al*, 2000), the half life of hydroxyl radicals generated in chloroplasts would be 0.9 ns ($t_{1/2} = \ln 2/(k \times [c])$, where k is the reaction constant and [c] is the concentration of citrulline). This value is significantly smaller than those estimated for ascorbate and glutathione in chloroplasts; $t_{1/2}$ values of these compounds are 1.9 and 17.5 ns, respectively, considering the reported concentrations and rate constants of these reductants (Noctor and Foyer, 1998; Ambar and Neta, 1967). Therefore, the function of citrulline as a hydroxyl radical-scavenger may be more important than those of the classical antioxidants.

During drought, levels of citrulline and arginine increase up to 49 and 11 % of total free amino acids in the watermelon leaves, respectively (Kawasaki *et al*, 2000). Our results revealed that citrulline of up to 600 mM in concentration had no inhibitory effects on the two representative metabolic enzymes, suggesting that the accumulated citrulline does not perturb cellular metabolism *in vivo*. Being an amphiphilic compound at the physiological ranges of pH, citrulline fulfills physicochemical properties as a compatible solute (Yancey *et al*, 1982).

In contrast, our results showed that the metabolic enzymes were markedly inhibited in the presence of arginine, possibly due to its net positive charge at physiological pH. Therefore, it

is reasonable to assume that wild watermelon plant has evolved developed regulatory mechanisms for accumulating citrulline preferentially rather than arginine, to avoid inhibitory effect of arginine.

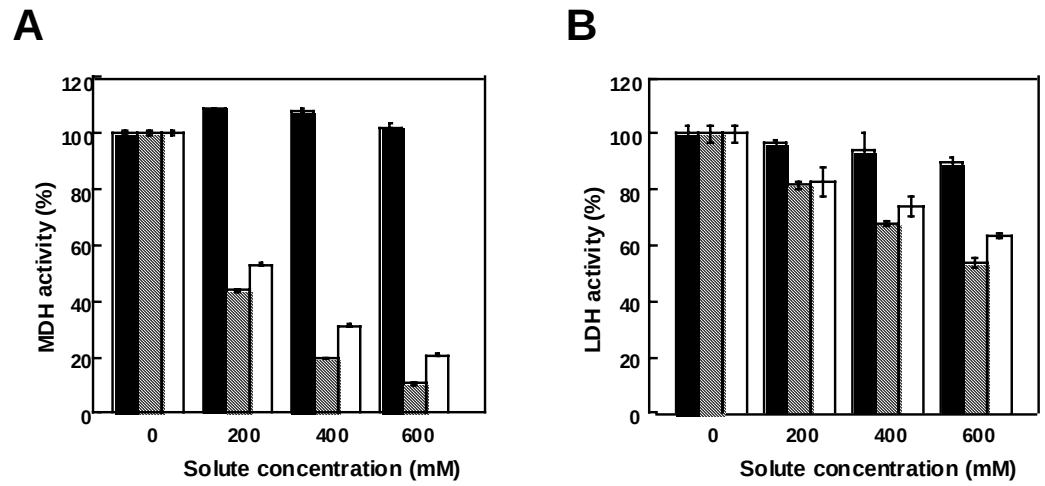

Fig. 1. Effects of increasing concentrations of citrulline (filled bars), arginine-Cl (grey bars) and KCl (open bars) on the enzymatic activities of MDH (A) and LDH (B). Values refer to percentage of the control activities and represent the mean ±SE (n=3).

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