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Citrulline, a novel compatible solute in drought-tolerant wild watermelon leaves, is an efficient hydroxyl radical scavenger

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Abstract Drought-tolerant wild watermelon accumulates high levels of citrulline in the leaves in response to drought conditions. In this work, the hydroxyl radical-scavenging activity of citrulline was investigated in vitro. The second-order rate constant for the reaction between citrulline and hydroxyl radicals was found to be 3.9×10^9 M^{-1} s^{-1}, demonstrating that citrulline is one of the most efficient scavengers among compatible solutes examined so far. Moreover, citrulline effectively protected DNA and an enzyme from oxidative injuries. Liquid chromatography-mass spectrometry analysis revealed that at least four major products were formed by the reaction between citrulline and hydroxyl radicals. Activities of metabolic enzymes were not inhibited by up to 600 mM citrulline, indicating that citrulline does not interfere with cellular metabolism. We reasoned, from these results, that citrulline contributes to oxidative stress tolerance under drought conditions as a novel hydroxyl radical scavenger.

Key words: Citrulline; Hydroxyl radical scavenger; Drought tolerance; Wild watermelon (*Citrullus lanatus* L.)

1. Introduction

Drought is one of the major abiotic stresses affecting plant productivity [1]. Under drought conditions, generation and proliferation of reactive oxygen species (ROS) increases and results in oxidative damage to plant cells [2,3]. To detoxify these radicals, plant cells contain multiple defense systems composed of scavenging enzymes and antioxidative metabolites [4,5]. Progression of severe drought stress, however, overrides these defense mechanisms and causes many of the ROS-mediated lethal reactions [2,3].

Wild watermelon plants inhabit the Kalahari desert, Botswana, and exhibit exceedingly high drought tolerance. The plants keep the photosynthetic apparatus intact during prolonged drought in strong light, suggesting that there are mechanisms present which make the plant tolerant to oxidative stress arising from excessive light energy falling on the leaves [6,7]. Drought stress induces an ArgE-related polypeptide and causes massive accumulation of the free amino acid citrulline in the leaves [6]. It would thus be useful to determine the physiological function of the accumulated citrulline under conditions where the plants are subjected to severe drought.

It has been reported that side chains of arginine residues in a polypeptide are particularly sensitive to oxidation by hydroxyl radicals [8], implying that citrulline, which is structurally analogous to arginine, may also exhibit high reactivity toward hydroxyl radicals. The excellent performance of wild watermelon plants against drought/oxidative stresses, together with a proposed hypothesis that compatible solutes act as radical scavengers [9,10], prompted us to evaluate the role of citrulline as a hydroxyl radical scavenger.

2. Materials and methods

2.1. Materials

Wild watermelon was grown as described previously [6]. Purified lactate dehydrogenase (LDH) and pyruvate kinase (PK) from pig heart were purchased from Oriental Yeast (Tokyo, Japan). Plasmid DNA pBluescriptII™ was obtained from Stratagene (La Jolla, CA, USA). All other reagents were obtained from Nakalai Tesque (Kyoto, Japan).

2.2. Determination of the rate constant

The hydroxyl radical-scavenging activity of a compound was analyzed by the competitive trapping assay as described previously [9]. The second-order rate constant for the reaction between the compound and hydroxyl radicals was calculated according to the kinetic competition model for ROS scavengers as reported [11]. The constant for salicylate, 1.2×10^{10} M^{-1} s^{-1} [12], was used for calculation of the constant for the competitor.

2.3. DNA and enzyme assays

The DNA-nicking assay was performed as described [13] using supercoiled pBluescriptII™ DNA. Crude malate dehydrogenase (MDH) was prepared from leaves of wild watermelon as described [14]. MDH and LDH activities were assayed as described previously [14,15].

To analyze the effect of citrulline on the inactivation of PK by hydroxyl radicals, the reaction mixture contained 100 mM Tris–HCl buffer (pH 7.4), 1 U of the PK enzyme, 0.2 mM ascorbate, 0.15 mM EDTA-Na-Fe(III), 0.6 mM H_2O_2 and various concentrations of citrulline in a final volume of 250 μl. The inactivation reaction was started by adding H_2O_2 at 25°C, and aliquots (10 μl) were withdrawn after appropriate time intervals and used for assays of the remaining activity. The PK activity was measured as described previously [16].

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Abbreviations: ROS, reactive oxygen species; DHBA, 2,3-dihydroxybenzoic acid; ID_{50}, the concentration of a compound required to inhibit hydroxyl radical-catalyzed hydroxylation of salicylate by 50% of the maximum yield observed in the absence of the compound; MDH, malate dehydrogenase; LDH, lactate dehydrogenase; PK, pyruvate kinase; LC/MS, liquid chromatography-mass spectrometry

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2.4. Liquid chromatography-mass spectrometry (LC/MS)

The reaction mixture containing 160 mM citrulline, 88 mM H_2O_2 and 0.1 mM EDTA-Na-Fe(III) in a total volume of 100 μl was incubated for 6 h at 25°C. The mixture was applied to a 2.1 mm×250 mm Inertsil ODS-2 column (GL Sciences, Tokyo, Japan) installed in an HPLC system HP1100 (Hewlett-Packard, Palo Alto, CA, USA) and eluted at a flow rate of 0.2 ml min^{-1} with a mixture of methanol:trifluoroacetic acid:water (5:0.1:95, v/v) for 10 min, and then by a gradient of 5–40% (v/v) methanol over 15 min. The absorbance of the eluents was monitored at 210 nm. The eluted compounds were analyzed by time-of-flight mass spectrometry (TOF-MS) using the LCT™ mass spectral analyzer (Jasco Int., Tokyo, Japan) in the atmospheric chemical ionization mode with a cone potential of 30 V. Electrospray mass spectrometry was performed at a voltage of 3.0 kV. Mass spectra were acquired by scanning the *m/z* range of 50–1000.

3. Results and discussion

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

Hydroxyl radicals are extremely toxic ROS which damage virtually all cellular macromolecules [17]. In order to know whether or not the citrulline accumulated in wild watermelon leaves effectively decomposes the hydroxyl radicals, the reactivity of citrulline with the radicals was examined in vitro, by allowing it to compete with salicylate for the radicals [9]. In this assay, salicylate reacts with hydroxyl radicals and yields its hydroxyl derivative 2,3-dihydroxy-benzoic acid (DHBA), which can be quantified spectrometrically. The compound in question added to the reaction mixture traps hydroxyl radicals and reduces the amount of DHBA in a concentration-dependent manner. Fig. 1 illustrates the change in the amount of DHBA formed versus the concentrations of citrulline and other representative compatible solutes reported from plants, i.e. proline, mannitol and glycine betaine. Of the four solutes used in the experiments, citrulline was the most effective scavenger of hydroxyl radicals. Mannitol and proline were less effective than citrulline at any concentration examined. Glycine betaine was virtually ineffective.

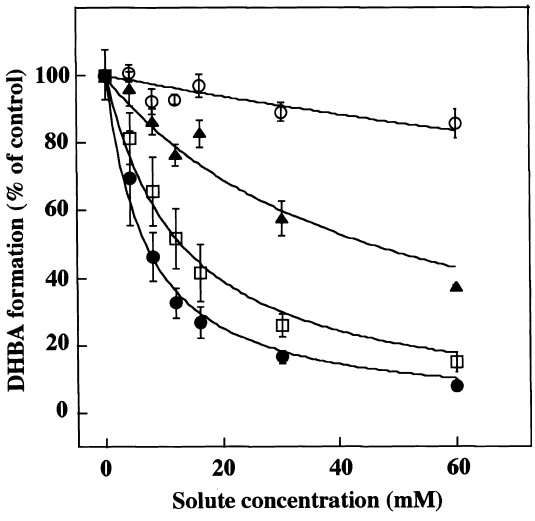

Fig. 1. Hydroxyl radical-scavenging activities of citrulline and various compatible solutes. The competitive inhibition of radical-induced DHBA formation by adding increasing concentrations of citrulline (●), mannitol (□), proline (▲) and glycine betaine (○) was analyzed. Values refer to percentages of the control activity and represent the mean ± S.E.M. ($n = 3$). The lines represent the regression curves fitted to the plotted points.

Table 1
Second-order rate constants for the reactions between hydroxyl radicals and various compounds

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

Data represent the mean ± S.E.M. ($n = 3$).

The data points in Fig. 1 were used to estimate the concentration which reduces hydroxylation of 20 mM salicylate by 50% (ID_{50}) of these compounds, and the second-order rate constant for the reaction between hydroxyl radicals and each compound was calculated (Table 1). The rate constant for mannitol, 2.1×10^9 M^{-1} s^{-1}, was in good agreement with the value reported previously [18]. The rate constant for citrulline was estimated to be 3.9×10^9 M^{-1} s^{-1}, indicating that citrulline is one of the most potent scavengers among compatible solutes examined so far [9] and notably exceeded that of mannitol, which is well-known as an efficient radical scavenger [9,10]. Thus, citrulline accumulated in watermelon leaves may increase the antioxidative potential of the cells and, in principle, protect cells from oxidative stresses. Further experiments are needed to examine whether citrulline could plays this role in vivo under conditions of drought.

The subcellular distribution of the citrulline accumulated in watermelon leaves is currently unknown. The DRIP-1 protein involved in citrulline biosynthesis in drought-stressed watermelon leaves has been suggested to be localized in the cytosol [6]. However, ornithine carbamoyltransferase, responsible directly for citrulline synthesis, is localized in chloroplasts in other plants [19,20]. If one assumes that the occurrence of citrulline is confined to chloroplasts, which occupy 9.5% of mesophyll cell volume [21], the observed content of 24 μmol (g fresh weight)$^{-1}$ in the watermelon leaves [6] could correspond to as much as 300 mM in concentration. Alternatively, if citrulline is distributed through the cytosol and nucleus in addition to chloroplasts by an amino acid transporter located in the chloroplastic envelope [22], the level of citrulline is estimated to be around 200 mM in concentration.

Considering the estimated concentration of citrulline (200 mM) in watermelon leaves, the half-life of hydroxyl radicals generated in vivo would be 0.9 ns ($t_{1/2} = \ln 2/(k \times [C])$, where k is the rate constant for the reaction between citrulline and hydroxyl radicals, 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 for these reductants [4,23]. Therefore, the function of citrulline as a hydroxyl radical scavenger may be more important than that of the classical antioxidants.

3.2. Protection of DNA and an enzyme from hydroxyl radicals

We next examined whether or not citrulline effectively protects DNA from ROS attacks. ROS-induced DNA damages are mediated by the interaction of hydroxyl radicals with C-4′ of deoxyribose, leading to cleavage of the phosphodiester backbone [24]. Hydroxyl radicals caused cleavage of one strand of the supercoiled plasmid DNA and yielded the nicked form (Fig. 2). Addition of citrulline at concentrations

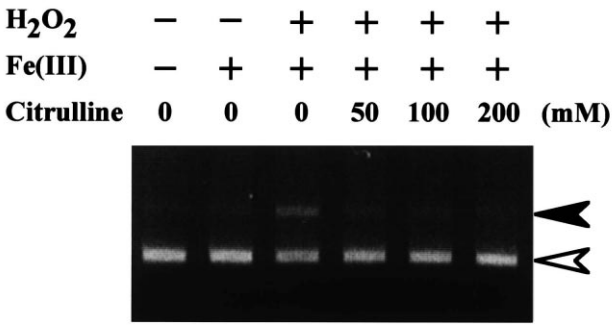

Fig. 2. Protection of DNA from oxidative damages by citrulline. pBluescriptII™ plasmid DNA was incubated in the ROS-generating system for 2 h in either the presence or absence of citrulline, resolved on an agarose gel and visualized by ethidium bromide staining. The final concentrations of citrulline are indicated on the top. Supercoiled and nicked DNAs are marked by open and closed arrowheads, respectively.

of 50–200 mM strongly reduced the formation of nicked DNA, demonstrating that citrulline effectively protected DNA from ROS attacks.

Subsequently we examined whether or not citrulline is capable of protecting metabolic enzymes from oxidative injuries, using PK as a model enzyme. PK has previously been reported as one of the enzymes which are readily inactivated by ROS [25]. PK was incubated with hydroxyl radicals generated by the Fenton reagent in the presence of various concentrations of citrulline. As shown in Fig. 3, citrulline at 200 or 400 mM clearly protected PK from oxidative damage.

These results strengthen the idea that the citrulline accumulated in watermelon leaves is an effective defense against oxidative injuries during drought stress. Under oxidative stress conditions, various cellular components are easily damaged by ROS, leading to extensive breakdown of cellular organization and homeostasis [2,3,5,26]. Citrulline accumulated in watermelon leaves may keep these vulnerable components active and maintain the cellular potential to survive during prolonged drought.

3.3. Analysis of the products formed by the reaction between citrulline and hydroxyl radicals

The compounds generated by the reaction between citrulline and hydroxyl radicals were analyzed by reversed-phase HPLC with UV detection and on-line MS. Incubation of 160 mM citrulline for 6 h with hydroxyl radicals produced from 88 mM H_2O_2 resulted in a 15% reduction of the amount of citrulline, which was eluted at 3.1 min (peak 1 in Fig. 4). The identity of peak 1 as citrulline was confirmed by its MS signal $m/z = 176$, which corresponds to the protonated parental ion for citrulline (data not shown). H_2O_2, which eluted at 2.7 min, was completely consumed during the reaction (data not shown). At least four major products with distinct retention times were resolved by HPLC analysis (Fig. 4, peaks 2–5). The MS pattern of peak 2 showed major signals at $m/z = 286$, 243 and 221 (data not shown), thus a compound with a molecular mass of 285 was suggested. The mass spectrum of peak 3 could not be obtained. The MS profile of peak

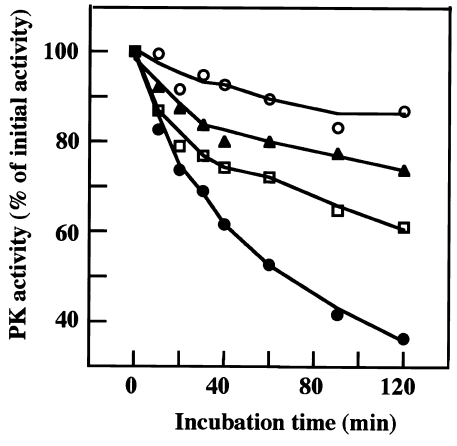

Fig. 3. Effect of citrulline on the inactivation of PK by hydroxyl radicals. Purified PK enzyme was incubated at 25°C with (●, □, ▲) or without (○) the Fenton reagent for indicated times in the presence of citrulline at 200 mM (□) and 400 mM (▲), respectively, or in its absence (●, ○). The PK activity is expressed as percentage of the initial activity.

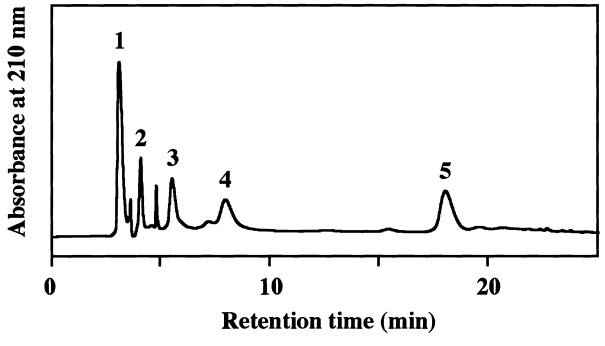

Fig. 4. Analysis of the products formed by the reaction between citrulline and hydroxyl radicals. Citrulline was incubated with the Fenton reagent and the resultant compounds were resolved by reverse-phase HPLC. The eluted compounds were monitored by UV absorbance at 210 nm and analyzed by MS. The MS profiles of major products are described in the text.

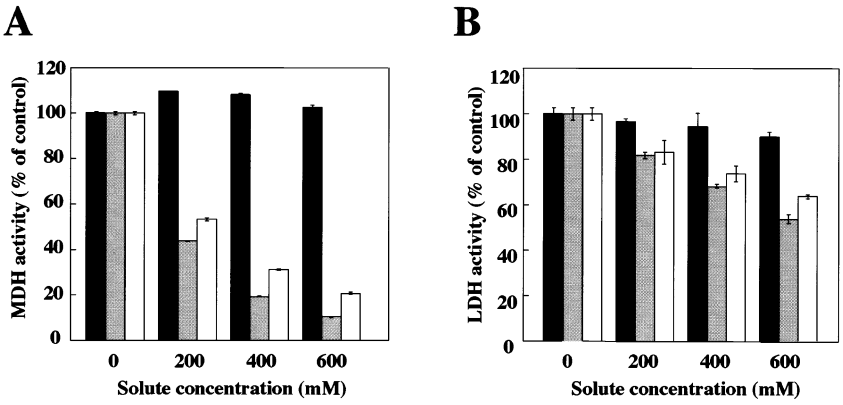

Fig. 5. Effects of increasing concentrations of citrulline (filled bars), arginine chloride (gray bars) and KCl (open bars) on the enzymatic activities of MDH (A) and LDH (B). Values refer to percentage of activity in the absence of any of these compounds and represent the mean ± S.E.M. ($n = 3$).

4 exhibited major signals at $m/z = 489$, 377 and 265 (data not shown), indicating the oligomeric nature of the compound with 112 mass increment. The MS pattern of peak 5 exhibited a complicated profile with major signals at $m/z = 409$, 366 and 151 (data not shown).

These results confirmed that citrulline was indeed capable of scavenging hydroxyl radicals. Although the precise chemical nature of the products formed in the reaction remains to be established definitely, the MS data suggest that the majority of the products are likely to be larger in molecular weight than citrulline. Stadtman and Berlett [27] have reported that the oxidation of leucine by the Fenton reagent leads to the formation of the corresponding α-ketoacid, oxime, aldehyde and carboxylic acid with a carbon skeleton one less than leucine. The proposed reaction scheme involves the initial abstraction of the hydrogen atom from the α-carbon of the amino acid to form a carbon-centered radical as an intermediate [28]. An increase in the length of a side chain, however, is accompanied by an increasing complexity of the reaction products comprising variously cross-linked derivatives, as exemplified by the oxidation of norvaline [29]. The LC/MS results suggest that citrulline, due to its long side chain with multiple sites for attacks by hydroxyl radicals, may be decomposed to several radical derivatives, which give rise to a variety of secondary products with various molecular weights by condensation and/or polymerization reactions.

3.4. Citrulline is compatible with metabolic enzymes

During drought, levels of citrulline and arginine increase up to 49 and 11% of total free amino acids in the watermelon leaves, respectively [6]. In order to assess whether or not the accumulated amino acids interfere with cellular metabolism, the effects of these compounds on the activities of two representative metabolic enzymes, MDH and LDH, were investigated in vitro (Fig. 5). Increasing concentrations of citrulline did not exert any inhibitory effect on the MDH activity (Fig. 5A). As for LDH, citrulline reduced the activity only marginally (Fig. 5B). These results suggest that citrulline does not perturb cellular metabolism in vivo. Being an amphiphilic compound at the physiological range of pH, citrulline exhibits the physicochemical properties of a compatible solute [30].

In contrast, these metabolic enzymes were strongly inhibited by high concentrations of arginine chloride (Fig. 5). The MDH and LDH activities in the presence of 600 mM arginine chloride dropped to 10% and 54% of the control values, respectively. The negative effects of arginine chloride were more severe than those of KCl, indicating that the arginine ion itself is inhibitory for these enzymes. These results raise the possibility that arginine, if accumulated at high concentrations in the cells, may impose detrimental effects on cellular activities. It is therefore reasonable to assume that the wild watermelon plant has evolved regulatory mechanisms for accumulating citrulline preferentially rather than arginine. The enzymological mechanism for the massive accumulation of citrulline is under investigation in this laboratory.

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