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Effects of Oxygen Stress on ^{1}H–NMR Relaxation Time (T_1) of *Vigna radiata* Seedling

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Effects of the oxygen exposure on plants were investigated in intact hypocotyl tissues of etiolated seedlings from mung bean (*Vigna radiata* L.). We report here that spin–lattice NMR relaxation time (T_1) of water proton of intact tissues, which can be measured with a 180° – τ – 90° pulse sequence at 20 MHz, is responsible to the oxygen exposure. When the tissues placed in the NMR tube were exposed to 95% oxygen for 5 s, a rapid decrease in the T_1 values and a subsequent relaxation process were observed. The value was fully recovered to the initial one during a 40 min incubation. Heat–denatured tissues did not show such recovery response, suggesting that the T_1 recovery requires heat–sensitive components contained in the tissues. To examine a possibility that scavenging activities of oxygen radicals is involved in the mechanism for the T_1 response, effects of supplemental scavengers were tested. We have found that the shortening of T_1 induced by oxygen exposure to membrane fractions was strongly suppressed by the radical scavenger superoxide dismutase (SOD), Tiron and ascorbate. These results suggest that activities of O_2^- production in the tissues are involved in the mechanism for abrupt shortening of T_1.

Key words: free radical – mung bean (*Vigna radiata*) – NMR relaxation time (T_1) – paramagnetic substance – superoxide

Abbreviations: BSA, bovine serum albumin; NMR, nuclear magnetic resonance; SOD, superoxide dismutase; T_1, spin–lattice relaxation time; T_2, spin–spin relaxation time; Tiron, 4,5–dihydroxy–1,3–benzene disulfonic acid.

INTRODUCTION

Relaxation time (T_1 or T_2) of water proton magnetic resonance in biological systems provides important clinical information by virtue of the fact that relaxation mechanism against various stresses depends on the intrinsic state of water in cells (Kaku and Iwaya–Inoue, 1990 references therein). Despite plants require molecular oxygen for

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survival, oxygen can become a causative factor for stress and is toxic to plants, because it can be readily reduced to reactive oxygen species such as super oxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH) that can oxidize and damage cellular components (Halliwell, 1982; Hendry and Crawford, 1994). Oxygen radicals can react very rapidly with DNA, causing severe cellular damages (Pinhero *et al.*, 1997). O_2 toxicity to bacteria is also in part due to the activities of O_2^- formation in the tissues (Gregory and Fridovich, 1973b).

Spin-lattice relaxation time (T_1) is strongly influenced by paramagnetic substances, such as paramagnetic ions (Stout *et al.*, 1977; Ratkovi'c, 1987; Iwaya-Inoue *et al.*, 1993), molecular oxygen (Lanir and Gilboa, 1981) and stable free radicals (Brasch, 1983) which possess large magnetic moments due to unpaired electrons in their orbital, and thereby exert a relaxing effect on neighboring hydrogen nuclei. Despite many studies on superoxide (VanToai and Bolles, 1991 references therein) which are also paramagnetic, they contain no information regarding the relationship between the production of superoxide and the water proton relaxation time. The present paper will discuss that the T_1 can reflect activities of O_2^- formation in the plant tissues.

MATERIALS AND METHODS

Plant material

Seeds of mung bean (*Vigna radiata* (L.) Wilczek) kindly provided by Taishin Jitsugyo Co. Ltd., were used as materials. Seeds were immersed into sodium hypochloride (200 ppm) for one hour and were successively immersed in 1 mM $CaSO_4$ solution at 25 °C in the dark for about 15 h. Just after the seeds initiated germination, they were seeded in cotton gauze unfolded on vinyl wire placed in a plastic pot (10 cm in diameter, 7.5 cm in depth). These pots were placed into a plastic vat filled with 1 mM $CaSO_4$ solution at 25 °C in the dark. Hypocotyls were excised on the 4 th day after germination and used for the experiments.

Measurements of proton T_1 relaxation times

The T_1 values of mung bean seeds were measured using a $180^\circ - \tau - 90^\circ$ pulse sequence (Farrar and Becker, 1971) at 20 MHz with a Bruker Minispec PC 20 pulsed NMR spectrometer as described previously (Kaku and Iwaya-Inoue, 1988; Iwaya-Inoue *et al.*, 1989; 1993). The hypocotyl (about 4 cm in length) of three intact seedlings of mung bean was cut into two pieces and then six pieces were packed into a 7.5 mm diameter NMR tube. The probe temperature (20 °C) was controlled by a thermostat (Lauda Kryo-SK65) connected to the sample chamber of the spectrometer.

Oxygen exposure

Effects of the oxygen exposure on T_1 for intact hypocotyl tissues of etiolated seedlings from mung bean were compared between tissues denatured at 120 °C for 15 min and control. Furthermore, the hypocotyl segments were homogenized at 0 °C in a 30 mM Hepes-tris solution (pH 7.5). When the tissues in the NMR tube were exposed to 95% oxygen or nitrogen for 5 s, T_1 values for both control and heat-denatured tissues were immediately determined. In the case of fluid samples, oxygen or nitrogen was added to

the fluids by bubbling gas in an NMR tube for 5 s, and T_1 values for respective samples were also immediately determined.

Effects on T_1 of addition of radical scavengers

Centrifuged at 10,000×g for 15 min at 4 °C, the homogenate prepared in the same way described above was separated into soluble and insoluble fractions. The soluble fraction obtained was filtered through a cellulose-acetate filter (0.45 μm, Advantec DISMIC-25). Prior to the exposure of oxygen, a mixture solution of 0.2 mg/ml SOD, 1 mM sodium ascorbate and 1 mM Tiron was added to each soluble or insoluble fraction obtained from fresh control and heat-denatured tissues. A BSA solution at the same concentration was used as a control for SOD. Effects on T_1 of the addition of radical scavengers to the soluble fractions, insoluble fractions and non-biological solutions were determined by using the samples mentioned above.

RESULTS AND DISCUSSION

Effects of oxygen exposure on spin-lattice relaxation time (T_1) for intact tissues

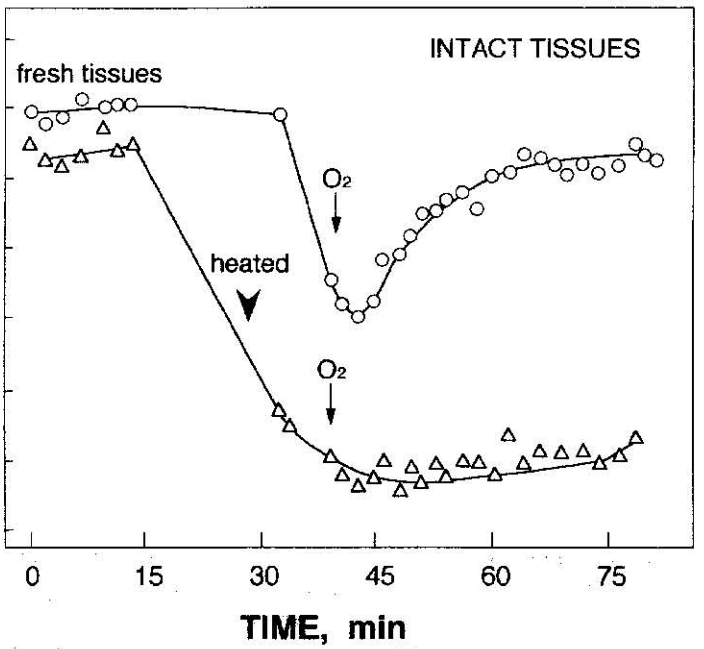

Fig. 1. The changes of T_1 for intact hypocotyl tissues of etiolated *Vigna radiata* seedlings affected by heat denaturing treatment and oxygen exposure. Fresh tissues (○) were exposed to (95% v/v) oxygen for 5 s at the point indicated by arrow. Tissues were suffered from heat-denatured treatment (arrow head) at 120 °C for 15 min (△) and then exposed to oxygen for 5 s (arrow). The probe temperature during all measurements was 20 °C.

The effects of the oxygen exposure on spin-lattice relaxation time (T_1) for intact hypocotyl tissues of etiolated seedlings from mung bean were compared between tissues denatured at 120 °C for 15 min and control (Fig. 1). The T_1 of the hypocotyl of intact seedlings of mung bean was measured using $180^\circ - \tau - 90^\circ$ pulse sequence by pulse NMR spectrometer as described previously (Kaku and Iwaya-Inoue, 1988; Iwaya-Inoue *et al.*, 1989; 1993). After the denaturing heat treatment, T_1 values for intact tissues decreased from 1.1 s to 0.8 s. A similar tendency has also been observed in frog lens tissues exposed to heat stress by microwave (Neville *et al.*, 1974). Even 40 °C exposure to mung bean also induced a time-dependent decrease in T_1; the decrease in T_1 values during the denaturing treatment seemed to depend on increases in protein content, macromolecular rearrangements and conformational changes that affect water-protein interaction (Iwaya-Inoue *et al.*, 1993). When the tissues in the NMR tube were exposed to 95% oxygen for 5 s, T_1 values for both control and heat-denatured tissues immediately decreased. The local magnetic field of molecular oxygen (present as $^3O_2$) will participate in decreasing T_1 of water proton due to the paramagnetic property of unpaired electron in their orbital: the magnetic susceptibility of oxygen molecule is 1.35×10^{-6} m^3/kg, while that of hydrogen molecule is -0.2×10^{-8} m^3/kg (Reitz and Milford, 1960 references therein). The subsequent relaxation behaviors were entirely different; the T_1 values for fresh control gradually increased to the initial values during 40 min, while those for tissues suffering from denaturing treatment remained low during the same period. This evidence suggests that some substances in fresh tissues protect against the toxicity caused by the exposure of oxygen, while not in heat-denatured tissues.

Effects of oxygen exposure on T_1 for tissue homogenate

Fig. 2 shows time courses of T_1 for fresh tissue homogenate and heat-denatured tissue homogenate caused by the addition of oxygen. T_1 values for the homogenate of the hypocotyl were significantly smaller than those of water and a buffer solution. When oxygen was dissolved to the fluids by bubbling oxygen in an NMR tube for 5 s, T_1 values for each solution immediately decreased and remained at low levels for 40 min until the dissolved oxygen was displaced by 95% nitrogen. As soon as the addition of nitrogen, T_1 values of the four solutions were almost restored to the initial levels or slightly higher levels because nitrogen is diamagnetic (Reitz and Milford, 1960). Apparently the relaxation behaviors are quite different from intact hypocotyl tissues and homogenate even obtained from fresh tissues, which have different intactness in cellular compartmentalization (Figs. 1 and 2).

The increased concentration of O_2 lead to increased rates of O_2^- production in yeast cells (Gregory *et al.*, 1974), and O_2^- is a common product of the biological reduction of oxygen in many vascular plants (Bridges and Salin, 1981). The induction of SOD is a response to the O_2 exposure (Gregory and Fridovich, 1973a) and SOD catalizes the following reaction: $2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$ (McCord and Fridovich, 1969). In transgenic maize (*Zea mays* L.) with the highest FeSOD activities plants enhanced tolerance toward oxidative stress and increased growth rates (Van Breusegem *et al.*, 1999). Therefore, the abrupt decrease in T_1 caused by the addition of oxygen probably depends either on the oxygen concentration itself or the activities of O_2^- production in fresh tissues, and hypocotyl tissues are protected from toxic oxygen by the induction of SOD. Unlike the

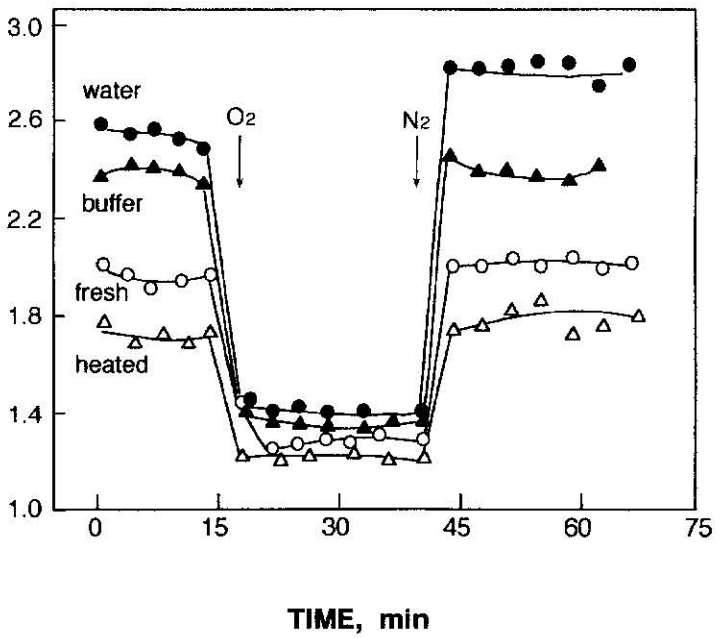

Fig. 2. Effects of oxygen and nitrogen exposure on T_1 for a homogenate from mung bean hypocotyl tissues. Symbols are homogenate from fresh tissues (○), that from heat-denatured tissues (△), non-biological solutions such as water (●) and a 30 mM Hepes-tris (pH 7.5) buffer solution (▲). 95% oxygen was bubbled into fluids for 5 s, subsequently nitrogen for 5 s at the points indicated by arrows, respectively. The probe temperature during measurements was 20 °C.

paramagnetic inorganic substances such as Mn^{2+} and Fe^{2+}, an unpaired electron in a free radical occupies the outermost molecular orbital and readily available for electron pairing with reducing agents (Brasch, 1983).

Effects on T_1 of radical scavengers to the soluble fractions and insoluble fractions

Negatively charged O_2^- cannot move across inside cells, but it is rapidly converted to membrane-diffusible H_2O_2 by SOD, ascorbate and thiols (Cheeseman and Slater, 1993). Effects on T_1 of addition of radical scavengers to the soluble fractions, insoluble fractions and non-biological solutions were shown in Fig. 3. The addition of radical scavengers (0.2 mg/ml SOD, 1 mM sodium ascorbate and 1 mM Tiron) prior to the exposure of oxygen, was only effective to the membrane fractions obtained from fresh control tissues (Fig. 3B). BSA at the same concentration, which was used as a control for SOD, gave no effect on the T_1 for the insoluble fractions even prepared from fresh tissues. It has been widely investigated that superoxide is produced in organelles such as chloroplasts (Asada, 1984), mitochondria (Boveris, 1984) and glyoxisomes (Sandalio *et al.*, 1988), while SOD

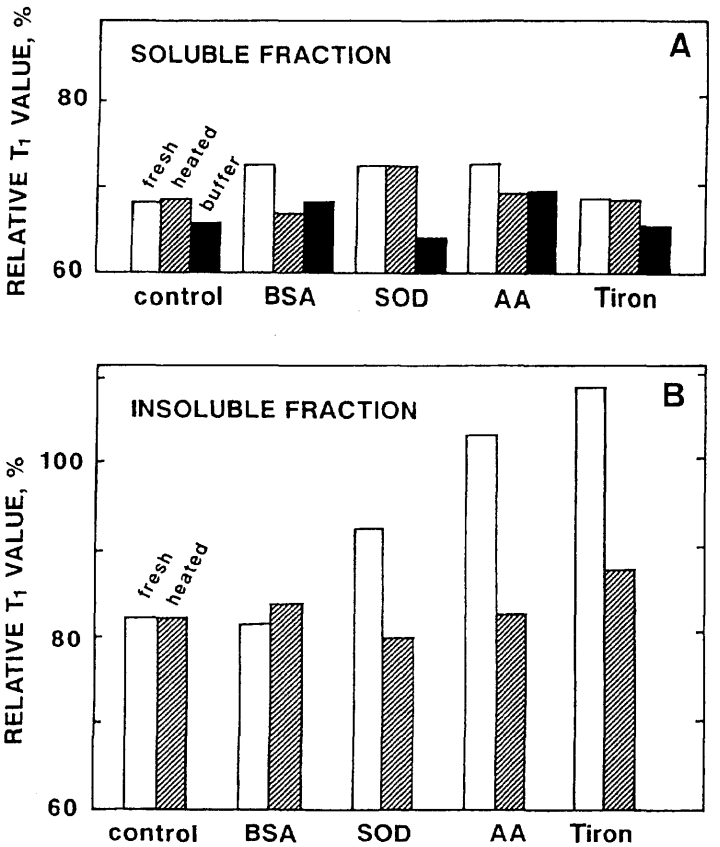

Fig. 3. Effects of radical scavengers on T_1 for soluble and insoluble fractions from fresh and heat-denatured tissues. Concentrations of BSA and SOD were 0.2 mg/ml, sodium ascorbate and Tiron were 1 mM, respectively. A: Soluble fraction from fresh tissues (open bars), heat-denatured tissue (oblique bars) and buffer (closed bars). B: Insoluble fractions from fresh control (open bars) and heat-denatured tissue (oblique bars). Quoted values of T_1 were expressed as relative T_1 values of samples after the oxygen treatment to the initial ones. T_1 values were the means of 7 to 10 replicates for each sample. The probe temperature during measurements was 20 °C.

is in water-soluble fractions in plant cells (Giannopolitis and Ries, 1977). Since the water-soluble fractions in mung bean hypocotyl contain most of the matrix proteins (Iwaya-Inoue *et al.*, 1993), O_2^- was produced in the membrane fractions but not in the soluble fractions. T_1 is not restored in heat-denatured tissues since the enzyme is known to be inhibited by 120 °C treatment for 15 min and SOD is not induced (Nakano, 1988). These results support our idea that the T_1 value is useful to monitor the O_2^- formation in intact tissues under stress conditions.

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