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BRIEF COMMUNICATION

Inhibitory effects of flavonoids on alternative respiration of plant mitochondria

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Abstract

Inhibitory effects of flavonoids on plant alternative respiration were investigated using isolated mitochondria of *Vigna radiata* seedlings. The antioxidant flavonoids quercetin and myricetin effectively inhibited alternative respiration. We suggest that radical scavenging activity is involved in the inhibitory mechanism.

Additional key words: alternative oxidase, quinone, reactive oxygen species.

Plant mitochondria possess two electron transport pathways, *i.e.* the cytochrome pathway and the alternative pathway (Wagner and Krab 1995). The cytochrome pathway, in which cytochrome oxidase (COX; EC 1.9.3.1) functions as a terminal oxidase, is almost identical to the respiratory electron transport system in animal mitochondria. The alternative pathway, in which alternative oxidase (AOX) acts as a terminal oxidase, is only found in plants, green algae, fungi, and protozoa (Affourtit *et al.* 2002). It has been known that AOX is inhibited by hydroxamic acids or alkyl gallates (Schonbaum *et al.* 1971, Siedow and Bickett 1981). In particular, the alkyl gallate *n*-propyl gallate (nPG) has been extensively used as an effective inhibitor for AOX. In biochemical studies, nPG is known not only as an AOX inhibitor but also as an effective commercially available antioxidant. Phytophenolics including flavonoids are potent antioxidants widely distributed in plants (Yamasaki *et al.* 1997, Sakihama *et al.* 2002). The antioxidant activity of nPG is fundamentally ascribed to polyphenolic structure similar to that of antioxidant phytophenolics (Fig. 1). We hypothesized that phytophenolics also display inhibitory actions on AOX activity due to a structural similarity to nPG and antioxidant character. The aim of this study was to show inhibitory effects of flavonoids on AOX activity.

Three flavonoids (myricetin, quercetin and kaempferol) and nPG were used to inhibit AOX activity (Fig. 1). Plant mitochondria were isolated from hypocotyls of mung bean *Vigna radiata*. Preparation of mitochondria was similar to the method previously described (Yamasaki *et al.* 2001). Hypocotyls were ground in a medium containing 0.3 M mannitol, 10 mM KH_2PO_4, 1 mM EDTA, 5 g dm^{-3} polyvinylpyrrolidone (PVP)-30, 4 mM cysteine, and 0.25 mM potassium pyrophosphate (pH 7.6) and the homogenate was centrifuged at 1 900 *g* for 10 min. The supernatant was centrifuged at 12 000 *g* for 20 min to spin down mitochondria and the pellet was then suspended in a medium containing 0.3 M mannitol, 10 mM KH_2PO_4 (pH 7.2). The suspension was centrifuged at 1 500 *g* for 5 min and the supernatant was centrifuged again at 12 000 *g* for 20 min. The resulting pellet of mitochondria was used for experiments. All procedures were conducted below 4 °C. Respiration activity of isolated mitochondria was measured by the rate of oxygen consumption with a Clark-type oxygen electrode at 25 °C. Antioxidant activity of AOX inhibitors and flavonoids was determined with a 1,1-diphenyl-2-picrylhydradil (DPPH) radical-generating system (Yokozawa *et al.* 1998). The concentration for 50 % inhibition of radical formation (IC_{50}) was calculated for comparisons.

Table 1 shows the antioxidant activity of phenolics determined by the DPPH method. Degradation of DPPH

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Abbreviations: AOX - alternative oxidase; COX - cytochrome oxidase; nPG - *n*-propyl gallate.
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radical was monitored with absorbance decrease at 517 nm. The concentration required for 50 % scavenging of 60 μM DPPH radical (IC_{50}) are represented in μM. It is apparent that number of OH group on aromatic ring determines antioxidant activities, namely, in order of effectiveness, trihydroxy (gallate), dihydroxy (catechol) and monohydroxy (phenol) structures in effective order.

Myricetin and quercetin exhibited strong inhibitory effects on the alternative pathway almost equivalent to that of the AOX inhibitor nPG (Fig. 2). In contrast, kaempferol did not show any inhibitory effect on the activity of the alternative respiration (Fig. 2). It should be noted that there was no substantial difference between those flavonoids in inhibitory effects on respiration when both COX and AOX were operational (Fig. 2).

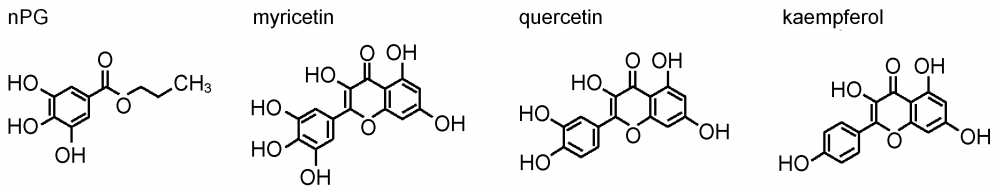

Fig. 1. Structures of nPG and flavonoids.

Table 1. Antioxidant activity of phenolics determined by the DPPH method. Values are means ± SD; *n* = 3.

Compounds	IC_{50} values [μM]
nPG	3.38 ± 0.32
Myricetin	4.92 ± 0.63
Quercetin	7.03 ± 0.27
Kaempferol	11.53 ± 0.99

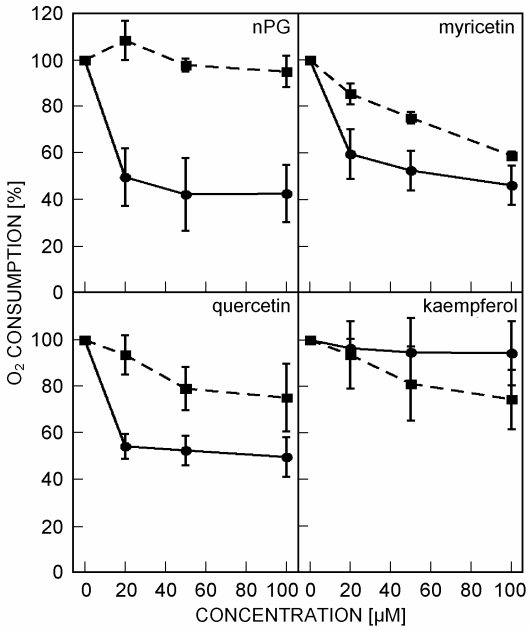

Fig. 2. Effects of nPG and flavonoids on respiration activity of isolated mitochondria. Rates of O_2 consumption (COX + AOX - *squares*) were measured with a reaction medium containing 250 μg(protein) cm^{-3} mitochondria determined by the Bradford method, 0.5 mM NADH, 0.5 mM ADP, 1 mM pyruvate, 0.3 M mannitol, 10 mM KH_2PO_4, 5 mM $MgCl_2$, and 10 mM KCl (pH 7.2). AOX activity (*circles*) was monitored in the presence of 2 μM antimycin A, a specific inhibitor for the cytochrome pathway. Values are means ± SD; *n* = 3.

These results have demonstrated that antioxidant flavonoids are capable of inhibiting the alternative respiration. IC_{50} value (μM) of the AOX inhibitor salicylhydroxamic acids (SHAM), that also bears phenol structure, was estimated to be 192 ± 48, a value more than 50 times higher than nPG. Consistently, nanomolar concentrations of nPG are needed for 50 % inhibition of AOX, whereas SHAM needs much higher concentrations (μM) to obtain the same effect (Berthold 1998). The *O*-3'-4'-dihydroxy (catechol) moiety, which both myricetin and quercetin include, is important for strong antioxidant activity (Rice-Evans *et al.* 1997). nPG also includes a dihydroxy moiety and indeed exerts strong antioxidant activity (Table 1). Recently, *Arabidopsis thaliana* AOX mutants that are SHAM-insensitive but nPG sensitive were isolated (Berthold 1998). This report suggests that the inhibitory mechanism of nPG would be different from that of SHAM. Together with the present results demonstrating that both nPG and flavonoids exert strong antioxidant activity (Table 1), one could speculate that antioxidant activity is involved in the inhibition mechanism.

According to a recently proposed model for the structure of AOX, the enzyme contains a functionally important tyrosine residue neighbouring a diiron center (Andersson and Nordlund 1999, Affourtit *et al.* 2002). The substitution of the tyrosine residue results in the inactivation of AOX (Albury *et al.* 2002). Since tyrosil radical plays an important role in the reaction of the diiron center, such as in the R2 subunit of ribonucleotide reductase (RNR R2) (Graslund 2002), radical chemistry of tyrosine is likely involved in the reactions of AOX. Ubisemiquinone ($^{\cdot}QH^{-}$), a radical form of ubiquinone (Q), is produced as an intermediate during the reduction of Q. Ubisemiquinone is thought to act as a prooxidant which potentially produces ROS in mitochondria through

reduction of O_2 (Turrens *et al.* 1985). It has been demonstrated that AOX lowers ROS generation while COX is inhibited (Maxwell *et al.* 1999). Thus, it is reasonable to presume that the ubisemiquinone radical, in addition to ubiquinol (QH_2), is also a substrate for AOX. We consider that AOX can be inhibited by antioxidative AOX inhibitors, such as nPG and flavonoids, because of their radical scavenging activity to remove the substrate ubisemiquinone radical. In this context, AOX would function as "enzymatic antioxidant" for the respiratory chain in plant mitochondria, a mechanism to avoid oxidative damage under stress conditions (Yamasaki *et al.* 2001).

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