Singlet Oxygen Quenching by Phenylamides and their Parent Compounds

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This paper demonstrates for the first time that plant metabolites of the phenylamide type, conjugates of putrescine with hydroxycinnamic acids (*p*-coumaric, caffeic and ferulic), possess $^1O_2$ quenching properties. Data were obtained confirming that their acidic parent compounds were also able to quench $^1O_2$, as did polyamines (putrescine, spermidine and spermine), and that this ability depends on the number of amino groups. Potentiation of the $^1O_2$ quenching ability of the conjugates relative to both parent components was established. The importance of polyamines and phenylamides in the plant non-enzymatic antioxidant defence at sites of intensive $^1O_2$ generation, such as the photosynthetic centers, was suggested.

Key words: Antioxidants, Hydroxycinnamic Polyamine Conjugates, Singlet Oxygen

Introduction

In the diversified network of reactive oxygen species (ROS) in aerobes, $^1O_2$ is of considerable physiological significance because of its relatively long life in aqueous media, ability to cross the cell membrane and high reactivity and damage to biomolecules. This is particularly valid for plants, given that $^1O_2$ is generated mainly by spin reversal of oxygen in photosensitization reactions in chloroplasts, and is particularly injurious to the photosynthetic apparatus (Knox and Dodge, 1984; Hideg *et al*., 1998). In the $^1O_2$ molecule, containing two electrons with antiparallel spins in the outer orbitals, the spin restrictions are abolished, hence its oxidizing power and energetic state are highly increased as compared to ground state oxygen, $^3O_2$. Quenching of $^1O_2$ can be performed by a physical mechanism of energy transfer to conjugated double bond-containing compounds and a chemical mechanism of oxidation of H-donating groups. As $^1O_2$ does not undergo enzymatic processing, its quenching uniquely by non-enzymatic tools is of key importance for the regulation of its toxicity effects, protection of cellular structures and functioning of the photosynthetic machinery (Khan *et al*., 1992; Halliwell and Gutteridge, 1999; Edreva, 2005). Significant $^1O_2$ quenching by chloroplastic components, carotenoids and tocopherols, has been reported (Edge *et al*., 1997; Cantrell *et al*., 2003; Kim *et al*., 2006).

Aliphatic polyamines (PA), ubiquitously distributed and essential for cell functioning metabolites, are involved in the regulation of growth and development processes of plants (Kakkar and Sawhney, 2002) and in the responses to both biotic and abiotic stress stimuli (Facchini *et al*., 2002; Walters, 2003; Kuehn and Phillips, 2005). The role of chloroplast-associated polyamines in the functioning of the photosynthetic apparatus has been shown (Kotzabasis, 1996; Logothetis *et al*., 2004). The antioxidant and ROS scavenging ability of PA is suggested as a rationale of their homeostatic and protective functions displayed against stress-induced oxidative damage (Khan *et al*., 1992; Edreva, 1996; Bouchereau *et al*., 1999, and references therein). In model systems PA have been found to act as potent antioxidants and scavengers of superoxide and hydroxyl radicals (Drolet *et al*., 1986; Das and Misra, 2004) and as inhibitors of lipid peroxidation (Tadolini, 1989; Belle *et al*., 2004). However, Bors *et al*. (1989) showed relatively low reaction rate

Abbreviations: PA, polyamines; Put, putrescine; ROS, reactive oxygen species; Spd, spermidine; Spm, spermine.

constants of PA with hydroxyl, superoxide and hydroperoxyl radicals, whilst demonstrating a high radical scavenging activity of phenylamides (hydroxycinnamic acid conjugates of PA) – a property conveyed by their acidic moiety. The important antioxidant and antiradical capacity of the hydroxycinnamic acids and their PA conjugates was confirmed by subsequent experiments (Chen and Ho, 1997; Grace and Logan, 2000; Son and Lewis, 2002), with phenylamides being assigned a relevance to disease resistance and environmental stress tolerance in plants (Facchini *et al*., 2002; Walters, 2003; Langebartels *et al*., 1991; Edreva *et al*. 2007). These findings promoted again the question addressed by Bors *et al*. (1989) as to whether PA may block cell injury by a direct scavenging of ROS, or whether their effect is mediated by conjugation with hydroxycinnamic acids.

While various radical ROS have largely been studied in ROS scavenging tests with PA and phenylamides (Das and Misra, 2004), singlet oxygen ($^1O_2$), which is a non-radical ROS, has received relatively little attention. Das and Misra (2004) evaluated the $^1O_2$ quenching effect of putrescine (Put), cadaverine (Cad), spermidine (Spd) and spermine (Spm) and found a relationship between the quenching ability and the number of amino groups in the tested compounds. Hydroxycinnamic acids were also reported to quench $^1O_2$ (Foley *et al*., 1999). However, to our knowledge, no information on the $^1O_2$ quenching ability of phenylamide conjugates is available.

The aim of the present study was to determine whether phenylamide compounds, namely putrescine conjugates with hydroxycinnamic acids, are able to quench $^1O_2$ *in vitro*, and to confirm the $^1O_2$ quenching ability of PA and hydroxycinnamic acids. The model system involves PA varying in the number of amino groups (diamine putrescine, triamine spermidine and tetraamine spermine), mono-/di-hydroxy and methoxy, hydroxy-substituted cinnamic acids (*p*-coumaric, caffeic and ferulic, respectively), and their conjugates with putrescine. We provide evidence indicating that the putrescine conjugates, as well as PA and hydroxycinnamic acids, are effective scavengers of $^1O_2$.

Materials and Methods

Experimental procedure

Rose Bengal (RB, 4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodofluorescein) was used as a photosensitizer that produces $^1O_2$ upon absorbing light. RB belongs to the water-soluble xanthene dye group and acts as a photodynamic sensitizer by a mechanism involving energy transfer from the excited sensitizer to dioxygen resulting in $^1O_2$ formation (Knox and Dodge, 1984). RB (10 μM) was irradiated with 500 μmol m^{-2} s^{-1} PFD (photon flux density) in the range 400–700 nm in the presence of 5 mM potassium phosphate buffer (pH 7.0) in a Hansatech oxygen electrode chamber (Hansatech, Burwell, Cambs, UK) maintained at 25 °C. Time of irradiation was 5 min. Then the solution of $^1O_2$ quenchers was added to the reaction mixture and illuminated for 10 min. After adding of imidazole (10 mM) the oxygen consumption was recorded. In the above-described procedure illumination of RB generates $^1O_2$. The level of $^1O_2$ was measured by the rate of its consumption for the oxidation of imidazole, added to the reaction mixture, with high consumption corresponding to a high $^1O_2$ level, and *vice versa*. If $^1O_2$ quenchers were previously applied, then the level of $^1O_2$ will be lowered, which results in a decreased rate of its consumption by imidazole. Thus, high $^1O_2$ quenching ability will correspond to low oxygen consumption. The difference between 100% (maximal oxygen consumption without quenchers) and the per cent of oxygen consumption in the presence of quenchers is a measure of the $^1O_2$ quenching ability of the tested compounds. All PA were applied at near-physiological concentrations (0.5, 1.0, 2.0, 3.0 and 4.0 mM). As for putrescine these concentrations were not effective, further experiments with it were performed by applying higher doses of 10 to 40 mM. Put conjugates (*p*-coumaroylputrescine, caffeoylputrescine and feruloylputrescine) and free hydroxycinnamic acids (*p*-coumaric, caffeic and ferulic) were applied at concentrations of 0.5, 1.0 and 2.0 mM.

Reagents

Putrescine, spermidine, spermine, *p*-coumaric, caffeic and ferulic acids, and Rose Bengal were purchased from Sigma. Hydroxycinnamoyl putrescines were a generous gift from Prof. Jeffrey K. Atkinson of Brock University, Ontario, Canada. The protocol of synthesis and the chemical characteristics of these substances have been described in detail (Fixon-Owoo *et al*., 2003).

Statistics

All data shown are the average values from at least five measurements ±S.E. One-way ANOVA

and pos-hoc Tukey's tests were applied to identify the significant differences between the different concentrations of compounds used.

Results

The results obtained showed that all PA tested were able to quench $^1O_2$ (Fig. 1). The highest rate was registered for Spm, ranging from 77.3% (0.5 mM) to 86.5% (2.0 mM) and reaching a plateau at concentrations higher than 2.0 mM. The quenching by Spd was much lower (12.7% at 0.5 mM and 35.6% at 2.0 mM), with a saturation effect being observed at concentrations higher than 2.0 mM.

In the concentration range applied in Spd and Spm tests (0.5 mM–4.0 mM) no $^1O_2$ quenching by Put was detected (data not shown). A slight quenching (9.7%) was hardly observed at concentrations 20 times higher than those for Spd and Spm, *i.e.* 10 mM, the effect being not significantly changed at 20 mM, 30 mM and 40 mM (Fig. 1).

Fig. 2 represents the $^1O_2$ quenching by Put conjugates – *p*-coumaroylputrescine, caffeoylputrescine and feruloylputrescine, as well as by the corresponding acidic parent compounds. The results obtained showed that these conjugates exerted marked $^1O_2$ quenching, which was generally similar for all conjugates, being somewhat lower for *p*-coumaroylputrescine. Substantial effect (86.5% and 82.9% for caffeoyl- and feruloylputrescine, respectively) was observed at their lowest concentration (0.5 mM), and maximal quenching, of above 90%, was attained at 2.0 mM for both feruloylputrescine and caffeoylputrescine (Fig. 2A).

The parent free hydroxycinnamic acids also acted as $^1O_2$ quenchers. The effect was less pronounced for *p*-coumaric acid (16.1% inhibition at 0.5 mM and 49.4% at 2.0 mM) than for ferulic acid (62.9% and 87%) and caffeic acid (55.4% and 81%) at 0.5 mM and 2.0 mM concentrations, respectively (Fig. 2B). Noticeably, the effect of the acids was lower relative to their conjugates with Put, the difference being particularly evident when comparing *p*-coumaroylputrescine with *p*-coumaric acid.

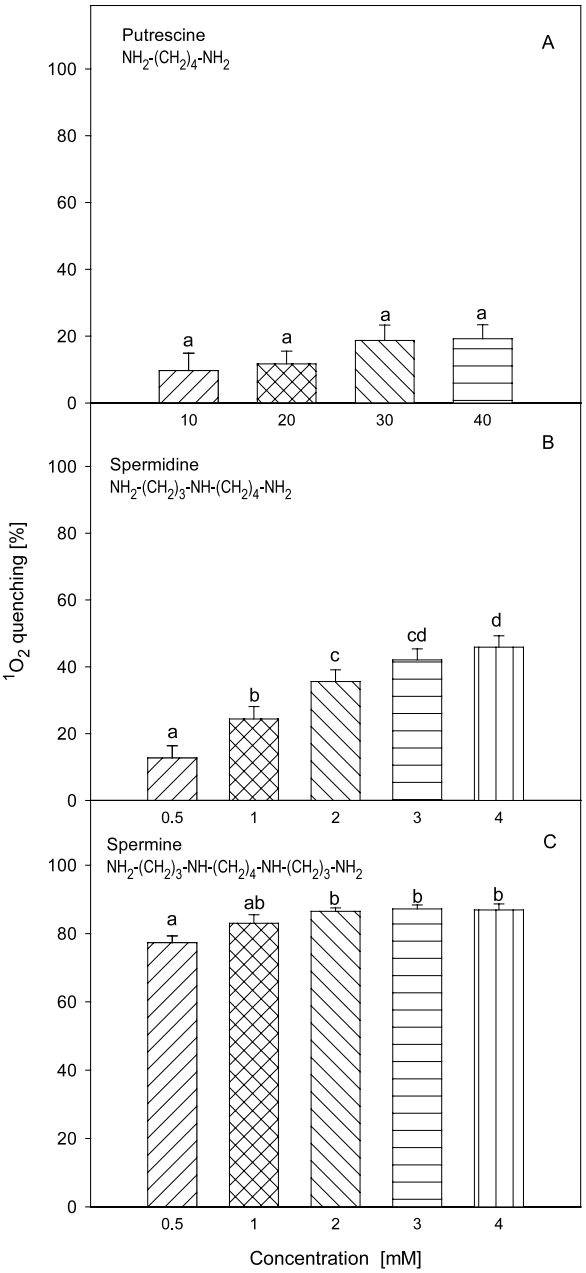

Fig. 1. $^1O_2$ quenching (%) by different concentrations of (A) putrescine, (B) spermidine and (C) spermine. The values with the same lower case letters in the group of every compound are not significantly different with $P < 0.05$.

Discussion

Our results clearly showed that free PA, a source of H-donating amino groups, quench $^1O_2$, with the antioxidant effect being dependent on the number of amine functionalities (Fig. 1). The triamine Spd and the tetraamine Spm successfully quenched $^1O_2$ at near-physiological concentrations, whereas the diamine Put became effective only at higher concentrations. The data are in accordance with those of Das and Misra (2004) ob-

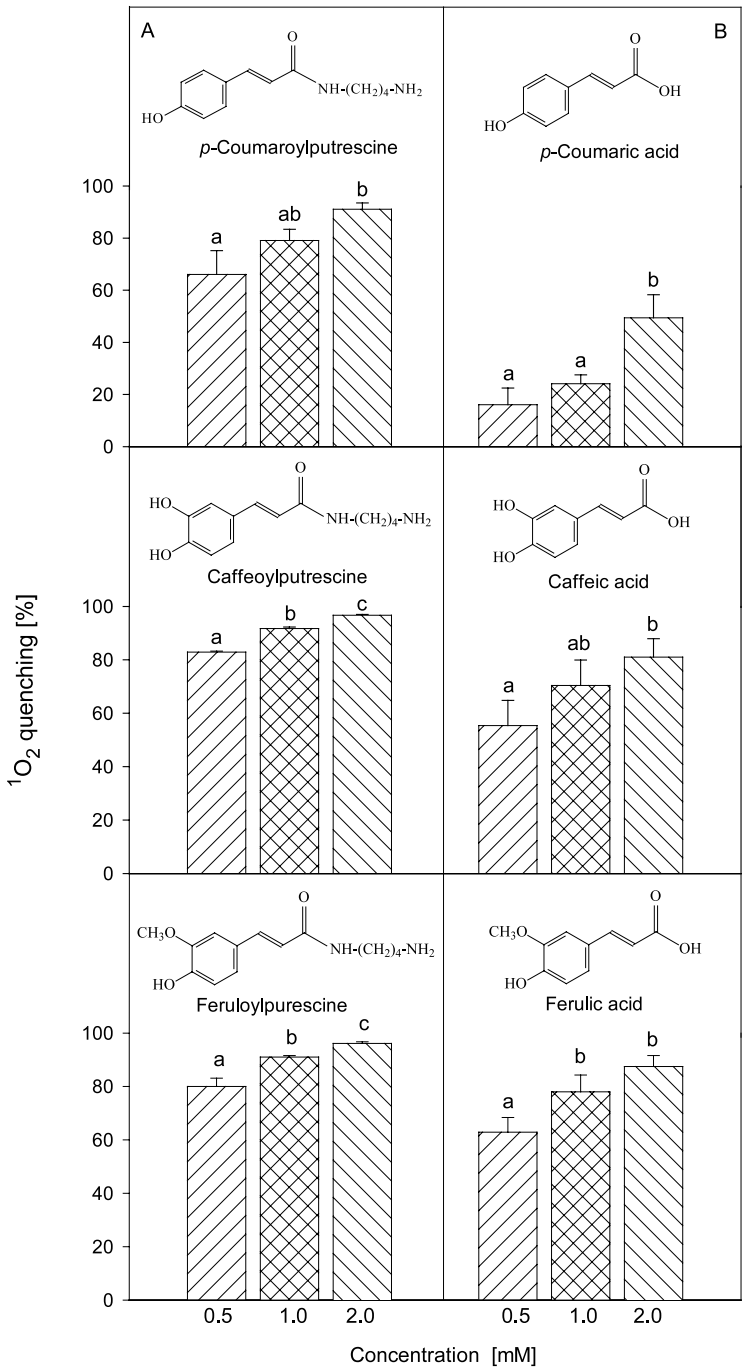

Fig. 2. $^1O_2$ quenching (%) by different concentrations of (A) *p*-coumaroylputrescine, caffeoylputrescine and feruloylputrescine conjugates and (B) their acidic parent compounds, *p*-coumaric acid, caffeic acid and ferulic acid. The values with the same lower case letters in the group of every compound are not significantly different with $P < 0.05$.

tained by other methods, indicating a lower $^1O_2$ quenching ability of the diamines Put and Cad as compared to the triamine Spd and the tetraamine Spm. The rate of superoxide radical scavenging of the above PA was observed to be of the same order (Drolet *et al.*, 1986).

The experimental data proved that the hydroxycinnamic acids (Fig. 2B), parent components of phenylamides, afforded a $^1O_2$ quenching ability as also shown by Foley *et al.* (1999). This ability was expressed at a lower rate in monohydroxy-substituted *p*-coumaric acid and potentiated in the *o*-dihydroxy- and *o*-methoxy-substituted caffeic and ferulic acids (Fig. 2B). Similar dependencies were observed relevant to the free radical scavenging ability of *p*-coumaric and caffeic acids (Grace and Logan, 2000; Aaby *et al.*, 2004). The results are in accordance with structure-activity studies of phenolic compounds, showing enhancement of antioxidant and antiradical activity of benzene derivatives as a function of the number and position of H-donating hydroxy groups. The activity of *o*-dihydroxy-substituted phenols was found to be higher relative to monophenols besides being influenced by other substituents (Son and Lewis, 2002; Rice-Evans *et al.*, 1997; Sroka, 2005). Physical quenching of $^1O_2$ by hydroxycinnamic acids can also be operative, given the availability of several conjugated double bonds in the benzene ring and in the side chain of the molecules (Halliwell and Gutteridge, 1999).

In this study, for the first time experimental evidence was obtained that phenylamides are also endowed with $^1O_2$ quenching properties. Conjugation of hydroxycinnamic acids with Put resulted in the enhancement of the $^1O_2$ quenching ability relative to the acidic parent, particularly in case of *p*-coumaroylputrescine (Figs. 2A, B). Similarly, synthetic amide analogues of caffeic acid were found to be more effective at free radical scavenging than the parent acid (Son and Lewis, 2002). As to the other parent compound, Put, it proved inefficient as $^1O_2$ quencher at near-physiological concentrations. $^1O_2$ quenching at these concentrations was performed only by the Put conjugates with hydroxycinnamic acids (Fig. 2A). Hence, conjugation can be considered as a mechanism, mediating the $^1O_2$ quenching activity of Put. Our results are compatible with those of Bors *et al.* (1989) concerning the low ability of Put in free radical scavenging and its enhancement through conjugation with caffeic and ferulic acids. In phenylamide conjugates, chemical and physical quenching of $^1O_2$ proper to hydroxycinnamic acids is combined with the supply of the H-donating antioxidant amine functionality by PA, this resulting in an improved $^1O_2$ quenching ability of the whole molecule. Moreover, by conjugation with PA, the phytotoxicity of free hydroxycinnamic acids can be overcome (Bouchereau *et al.*, 1999), which allows the performance of their antioxidant and antiradical functions without cellular damage. Further research with conjugates of various PA is necessary to shed light on their metabolic functions and relevance to the photosynthetic performance, stress defense, and other important physiological processes in plants.

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