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Oxygen Free Radical Scavenger Capacity in Aqueous Models of Different Procyanidins from Grape Seeds

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Epicatechin 3-*O*-gallate and various procyanidins obtained from grape seeds were tested for their scavenger capacity for superoxide radical ($O_2^{\bullet-}$) and hydroxyl radical (•OH) in aqueous models. Quantification of $O_2^{\bullet-}$ and •OH scavenger capacities was carried out, respectively, by polarography and by the extent of deoxyribose degradation. All the compounds assayed are potent scavengers of these radicals compared to trolox (for $D_2^{\bullet-}$) and ethanol and mannitol (for •OH). Catechin monomers are also potent scavengers, especially of •OH. Gallic acid esterification increased the $O_2^{\bullet-}$ and •OH scavenger capacity of the dimer procyanidins. However, esterification position was also important. A difference in the $O_2^{\bullet-}$ scavenger capacity was noted between dimers having a C_4–C_6 and C_4–C_8 linkage. Procyanidin B_2 3′-*O*-gallate was found to be the most effective compound in trapping oxygen free radicals.

INTRODUCTION

Oxygen-derived species such as the superoxide radical ($O_2^{\bullet-}$) and hydroxyl radical (•OH) play an important role in tissue damage. They cause oxidative degradation of proteins, unsaturated lipids, carbohydrates, and nucleic acids (Calderon and Roberfroid, 1988). Numerous investigations have been carried out to find antioxidative drugs, which not only prolong the shelf life of food products but also participate as radical scavengers in living organisms. Currently there is an increasing interest in the antioxidant activity of natural compounds. Among these, flavonoids are in general considered to have useful antioxidant properties (Affany et al., 1987; Robak and Gryglewski, 1988; Su et al., 1988; Torel et al., 1986; Zhao et al., 1989). However, few studies with procyanidins on scavenging active oxygen radicals have been done (Ariga et al., 1988; Ariga and Hamano, 1990; Masquelier, 1988; Uchida et al., 1987). This may be explained by difficulties encountered in their isolation. Several improvements in the separation techniques have allowed us to purify a large enough quantity of procyanidins from grape seeds (*Vitis vinifera*) (Ricardo da Silva et al., 1991) to carry out the present study.

The aim was to evaluate the oxygen radical scavenger ability of procyanidins for superoxide and hydroxyl radicals. This scavenger ability is one of the many components of antioxidative action (Pincemail et al., 1985). The procyanidins in the present study were chosen to evaluate the role of the degree of polymerization, gallic acid esterification, and linkage between monomer units in the scavenger capacity of the compounds.

MATERIALS AND METHODS

Materials. Reagents, enzymes, and their sources were as follows: Xanthine (2,6-dihydroxypurine), 2-deoxy-D-ribose, chelating resin (sodium form, dry mesh 50–100), ethylenediaminetetraacetic acid (EDTA), xanthine oxidase (EC 1.2.3.22) from buttermilk, superoxide dismutase (SOD) (EC 1.15.1.1) from bovine erythrocytes, and catalase (EC 1.11.1.6) from bovine liver were all obtained from Sigma Chemical Co. (St. Louis, MO). Tris(hydroxymethyl)aminomethane, L-(+)-ascorbic acid, and 2-thiobarbituric acid were purchased from Merck (Darmstadt, Germany). Ferrous sulfate, D-(−)-mannitol, and ethanol were obtained from Prolabo (France), and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylate) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Standard (+)-catechin was purchased from Sigma and (−)-epicatechin from Sarsyntex (Merignac, France).

(−)-Epicatechin 3-*O*-gallate and procyanidins B_2, B_5, B_2 3-*O*-gallate, B_2 3′-*O*-gallate, C_1 [(−)-epicatechin(4β→8)(−)-epicatechin-(4β→8)(−)-epicatechin], trimer [(−)-epicatechin(4β→8)(−)-epicatechin(4β→8)(+)-catechin], designated trimer 2 in this work, and trimer [(−)-epicatechin(4β→8)(−)-epicatechin(4β→6)(+)-catechin], designated trimer 3, were isolated from grape seed methanol extracts. These extracts were prepared as described by Bourzeix et al. (1986).

Different procyanidin fractions were obtained on a column of Fractogel TSK HW-40 (s) (Merck) with 100% methanol as eluant. Procyanidins and (−)-epicatechin 3-*O*-gallate were isolated with semipreparative reversed-phase HPLC. Their purification level was tested by analytical reverse-phase HPLC and TLC on silica plates using the eluant described by Lea et al. (1979). Enzymatic hydrolysis, complete acid hydrolysis, and partial acid-catalyzed degradation with phenylmethanethiol or phloroglucinol, TLC on silica plates, ^{1}H NMR, and FAB-MS were used as identification techniques. The isolation, purification, and identification techniques applied to these compounds are published in other works (Ricardo da Silva et al., 1991; Rigaud et al., 1991).

Quantification of the Superoxide Radical ($O_2^{\bullet-}$) Scavenger Capacity. The superoxide anion scavenger capacity of various procyanidins was determined according to previous work (Darmon et al., 1990). $O_2^{\bullet-}$ was generated by a xanthine (X)–xanthine oxidase (XO) system:

$$X + H_2O + 2O_2 \xrightarrow{XO} \text{uric acid} + 2O_2^{\bullet-} + 2H^+ \quad (1)$$

O_2 consumption results from the kinetics of the oxidation of X coupled to the spontaneous dismutation of $O_2^{\bullet-}$:

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

These equations show that the oxidation of 1 mol of X leads to a consumption of 1 mol of O_2. The oxygen consumption (called O_2 tot control) was measured at 25 °C by means of a Gilson KIC oxygraph equipped with a Clark electrode and a closed cell of 2-mL volume. Xanthine (0.625×10^{-4} M) in 50 mM Tris hydrochloric acid, 0.2 M NaCl buffer at pH 7.5 or 9.0, was oxidized by addition of 60 or 33 milliunits of XO, respectively. Tris-HCl buffer was chelating resin treated to remove contaminating trace

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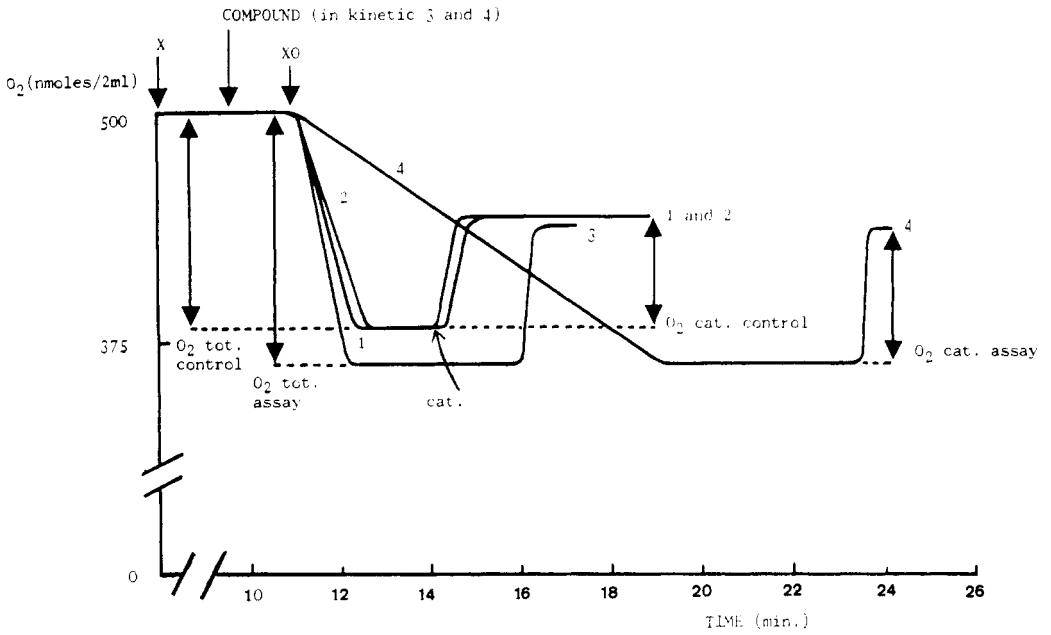


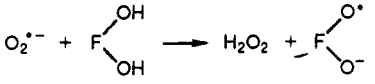
Figure 1. Time course of O_2 concentration, in a xanthine (X)–xanthine oxidase (XO) system, measured with a Clark electrode in various conditions: control at pH 7.5 (1) or pH 9 (2); in the presence of a scavenger (3); in the presence of a scavenger that is also an inhibitor of xanthine oxidase (4).

metals and was air-equilibrated. After total X oxidation, 0.4 unit of catalase (cat) was added to quantify the H_2O_2 yield

$$H_2O_2 \xrightarrow{cat} {}^1/_2O_2 + H_2O \quad (3)$$

by the measurement of O_2 liberated (called O_2 cat control). Kinetics of these total processes at pH 7.5 and 9 are given in Figure 1 (models 1 and 2, respectively) and show that the ratio O_2 tot control/O_2 cat control is close to 2.

Oxygen consumption kinetics were determined in the presence of scavenger added to the reaction mixture before XO. The reaction of scavenging by the flavonoids, $F(OH)_2$, (see eq 4) occurs in competition with the dismutation reaction (eq 2).



(4)

Since spontaneous dismutation is slower at pH 9 than at pH 7.5 (Fridovich, 1970), the ability of a compound to trap $O_2^{\bullet-}$ should be greater at the higher pH. On the other hand, when dismutation is favored by addition of 60 units of SOD, the quantity of $O_2^{\bullet-}$ trapped should be decreased. However, an efficient scavenger could remain active in the presence of SOD.

The trapping effect of $O_2^{\bullet-}$ by the scavenger can be demonstrated by an increase in the initial rate of the kinetics and an increase in total O_2 consumption (O_2 tot assay) (Figure 1, model 3). A decrease of the initial rate (Figure 1, model 4), if insensitive to SOD, could be interpreted as an inhibition of XO activity. By addition of catalase, the O_2 tot assay/O_2 cat assay ratio should stay close to 2.

Finally, this method is especially useful for the direct and easy quantification of $O_2^{\bullet-}$ trapped by a scavenger. According to Darmon et al. (1990), the quantity of $O_2^{\bullet-}$ trapped (x) in the reaction medium may be calculated from O_2 tot control and O_2 tot assay, as follows:

$$O_2^{\bullet-} \text{ produced, by xanthine oxidation} = 2(O_2 \text{ tot control})$$

$$O_2 \text{ tot assay} = x + \left(\frac{O_2^{\bullet-} \text{ produced} - x}{2}\right)$$

$$x = 2(O_2 \text{ tot assay} - O_2 \text{ tot control})$$

Quantification of the Hydroxyl Radical (•OH) Scavenger Capacity. The simple "test tube" assay, described by Halliwell et al. (1987), slightly modified, was used to determine approximate second-order rate constants for reactions of •OH with various compounds to evaluate their scavenger capacity with regard to this radical. 2-Deoxy-D-ribose was degraded on exposure to hydroxyl radicals generated by a Fenton system. The composition of the reaction mixture (in 30 mM phosphate buffer, pH 7.4, in a final volume of 1 mL) was as follows: 2 mM deoxyribose, 100 μM $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$, 104 μM EDTA, and 100 μM ascorbate. An essential reactant for the system, H_2O_2, is a product from Fe^{2+} autoxidation, favored by a neutral pH. The precautions, in the preparation of ferrous and ascorbate solutions described by Halliwell et al. (1987) and Laughton et al. (1989), were observed. Reaction mixtures were incubated at 37 °C for 1 h.

It should be noted that the assay is inapplicable to certain compounds, as the strong metal-chelating agents that can withdraw iron from EDTA, which cause slight anomolous results (Halliwell et al., 1987); this is not the case of procyanidins.

The extent of deoxyribose degradation by •OH was quantified by the thiobarbituric acid method (Halliwell and Gutteridge, 1981). The absorbance (A) at 532 nm was measured. Then the rate constant for the reaction of the compound with •OH can be calculated from the slope of the straight line obtained in a plot of $1/A$ against the scavenger concentration (Halliwell et al., 1987).

RESULTS AND DISCUSSION

Scavenging Capacity of Compounds for Superoxide Radical ($O_2^{\bullet-}$). It was found that for catechins and procyanidins the ratio O_2 tot assay/O_2 cat assay remained close to 2 as in the controls.

At pH 9 and a scavenger concentration of 10^{-4} M (Table I), the initial rates decreased for (−)-epicatechin 3-*O*-gallate, the procyanidins B_5, B_2 3′-*O*-gallate, and C_1, and trimer 2 and increased for the other compounds. Except for (−)-epicatechin and procyanidins B_2 and B_5, at pH 7.5 (Table I) the initial rate decreased in the presence of all the polyphenolic compounds at 10^{-4} M. Since the decrease in the initial rate was SOD-insensitive, the compounds must inhibit XO, especially at pH 7.5. Inhibition of XO by certain flavonoids has already been reported by Iio et al. (1985).

When compared to Trolox, all the compounds tested appear to be potent scavengers of the superoxide radical at pH 7.5 and 9 (Tables II and III). The capacity at the

Table I. Initial Rate of O_2 Consumption (as a Percent of Control)[a]

compd	pH 7.5 10^{-4} M	pH 7.5 10^{-3} M	pH 9
control	100	100	100
Trolox	103	106	113
(+)-catechin	95	58	110
(−)-epicatechin	103	100	141
(−)-epicatechin 3-*O*-gallate	66	20	94
procyanidin B_2	100	97	142
procyanidin B_5	110	106	98
procyanidin B_2 3-*O*-gallate	87	32	110
procyanidin B_2 3′-*O*-gallate	38	15	74
procyanidin C_1	85	37	89
procyanidin trimer 2	83	51	97
procyanidin trimer 3	70	33	106

[a] The values are the means of at least two measurements.

Table II. Values of $O_2^{\cdot-}$ Trapped (nmol/2 mL) at pH 7.5[a]

compd	concn 10^{-4} M	concn 10^{-3} M
Trolox	10.0	25.0
(+)-catechin	1.3	18.8
(−)-epicatechin	3.8	12.5
(−)-epicatechin 3-*O*-gallate	7.5	57.5
procyanidin B_2	11.3	35.0
procyanidin B_5	3.8	10.1
procyanidin B_2 3-*O*-gallate	10.0	65.0
procyanidin B_2 3′-*O*-gallate	20.0	95.0
procyanidin C_1	16.3	72.5
procyanidin trimer 2	5.0	42.5
procyanidin trimer 3	15.0	58.0

[a] The values are the means of at least two measurements.

Table III. Values of $O_2^{\cdot-}$ Trapped (nmol/2 mL) at pH 9.0[a]

compd	concn 0.33×10^{-4} M	concn 0.5×10^{-4} M	concn 10^{-4} M	by monomer equiv
Trolox			37.5	
(+)-catechin			57.5	57.5
(−)-epicatechin			50.0	50.0
(−)-epicatechin 3-*O*-gallate			80.0	
procyanidin B_2		65.0	87.5	65.0
procyanidin B_5		40.0	58.0	40.0
procyanidin B_2 3-*O*-gallate		65.0	75.0	
procyanidin B_2 3′-*O*-gallate		76.0	112.5	
procyanidin C_1	67.5		77.5	67.5
procyanidin trimer 2	58.5		67.5	58.5
procyanidin trimer 3	57.5		73.0	57.5

[a] The values are the means of at least two measurements.

more basic pH was higher and could be related to the lower spontaneous dismutation (Fridovich, 1970). To give a better resolution, the $O_2^{\cdot-}$ trapped at pH 7.5 was also measured at 10^{-3} M. At pH 9, Trolox, (+)-catechin and (−)-epicatechin still show a superoxide radical scavenger ability at 10^{-5} M. (−)-Epicatechin 3-*O*-gallate and procyanidins act as radical scavengers at concentrations to 10^{-6} M (results not shown).

For the monomers, esterification by gallic acid increases the radical scavenger ability of the parent compound. The high scavenger capacity of (−)-epicatechin 3-*O*-gallate has already been demonstrated by Uchida et al. (1987). Of the procyanidins B_2, procyanidin B_2 3′-*O*-gallate has the higher scavenging ability. This suggests that the site of esterification by gallic acid plays an important role.

Our results show that the dimer procyanidin B_2 is more active than the monomers [(+)-catechin and (−)-epicatechin] (Tables II and III); this in agreement with results of Masquelier (1988). The same is observed with procyanidins B_1 and B_3 in the presence of the free radical DPPH (1,1′-diphenyl-2-picrylhydrazyl) (Ariga et al., 1988) or, recently, with peroxy radicals (Ariga and Hamano, 1990).

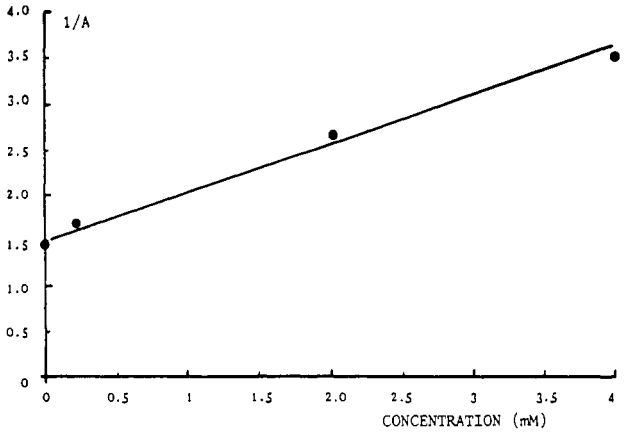


Figure 2. Typical linear plot obtained between $1/A$ (A = absorbance at 532 nm) and the concentration (mM) of procyanidin trimer 2.

Procyanidin B_5, with a C_4–C_6 linkage between monomers, seems to be less active than procyanidin B_2 with a C_4–C_8 linkage. In the results presented by Uchida et al. (1987), the same is observed but with the procyanidins B_2 3,3′-di-*O*-gallate and B_5 3,3′-di-*O*-gallate. For the trimers used in this study, the existence of a C_4–C_8 or C_4–C_6 linkage between the monomer units does not seem to play an important role in the superoxide scavenger capacity of the compound (Tables II and III).

The relative activity of the dimers and trimers can be compared to that of the monomers. In this aim, Table III presents the values obtained at 0.5×10^{-4} M for the dimers and at 0.33×10^{-4} M for the trimers (pH 9). It can be seen that there is only a very slight decrease in scavenger activity on dilution and also that the activity of the oligomers and monomers is almost the same when expressed by monomer equivalents. However, from monomers to trimers our results suggest that there is no proportionality between the $O_2^{\cdot-}$ scavenging ability and the degree of polymerization (Tables II and III). This seems to contradict the results obtained by Uchida et al. (1987), but these authors used procyanidins with both an increase in the degree of polymerization and an increase in the number of gallic acid moieties in the molecule.

In the presence of SOD, at pH 9 and 7.5, O_2 tot and O_2 cat generally return to values very close to those of the control. However, at pH 9, for certain scavengers [(−)-epicatechin 3-*O*-gallate and procyanidins B_2 and B_2 3′-*O*-gallate] SOD does not totally restore the control values. This confirms the high trapping activity of these compounds for superoxide radical. Such a process is similar to the competitive role of SOD on a substance's reduction by $O_2^{\cdot-}$ (Auclair and Voisin, 1987).

Scavenging Capacity of Compounds for Hydroxyl Radical ($^{\cdot}$OH). A typical linear plot of a procyanidin is presented in Figure 2. The different rate constants obtained are presented in Table IV. High rate constants indicate a good scavenger capacity of the compounds. The values obtained with ethanol and mannitol are similar to those obtained by others (Halliwell et al., 1987). Catechins, epicatechin 3-*O*-gallate, and procyanidins are potent scavengers or $^{\cdot}$OH compared to ethanol and mannitol.

C_4–C_8 or C_4–C_6 linkage between monomer units seems, for $^{\cdot}$OH, not to interfere with the scavenger capacity of

Table IV. Second-Order Rate Constants for Reactions of Scavengers with Hydroxyl Radical, by the Deoxyribose Assay[a]

compd	rate constant, M^{-1} s^{-1}
ethanol	1.20×10^9
mannitol	1.15×10^9
(+)-catechin	2.88×10^9
(−)-epicatechin	3.17×10^9
(−)-epicatechin 3-*O*-gallate	1.56×10^9
procyanidin B_2	1.41×10^9
procyanidin B_5	1.44×10^9
procyanidin B_2 3-*O*-gallate	2.40×10^9
procyanidin B_2 3′-*O*-gallate	3.59×10^9
procyanidin C_1	2.78×10^9
procyanidin trimer 2	2.26×10^9
procyanidin trimer 3	2.18×10^9

[a] The values are the means of at least two measurements.

the compound. From monomers to trimers, our results also suggest that there is no proportionality between the •OH scavenging ability and the degree of polymerization.

For dimers, the presence of a molecule of gallic acid esterifying the procyanidin increases the radical scavenger capacity as seen, with $O_2^{\bullet -}$. The esterification in the 3′-position gives a higher radical scavenging capacity, and procyanidin B_2 3′-*O*-gallate was found to be the most effective compound.

These results show that procyanidins and especially procyanidin B_2 3′-*O*-gallate are effective as oxygen radical scavengers. Their potentiality as natural antioxidants and as radical scavengers in food products and in living organisms needs to be carefully investigated.

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Registry No. Superoxide, 11062-77-4; hydroxyl, 3352-57-6; catechin, 154-23-4; epicatechin, 490-46-0; epicatechin 3-*O*-gallate, 1257-08-5; procyanidin B_2, 29106-49-8; procyanidin B_5, 12798-57-1; procyanidin B_2 3-*O*-gallate, 109280-47-9; procyanidin B_2 3′-*O*-gallate, 73086-04-1; (−)-epicatechin-(4β→8)-(−)-epicatechin-(4β→8)-(−)-epicatechin, 65085-09-8; (−)-epicatechin-(4β→8)-(−)-epicatechin-(4β→8)-(+)-catechin, 79813-67-5; (−)-epicatechin-(4β→8)-(−)-epicatechin(4β→6)-(+)-catechin, 79763-28-3.