Cyanidin 3-*O*-β-D-Glucopyranoside is a Potent Antioxidant Against Cytochrome *c*-enhanced 5-(2-Aminoethyl)benzene-1,2,4-triol Oxidation

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A naturally occurring flavonoid, cyanidin 3-*O*-β-D-glucopyranoside, is shown to have potent antioxidant activity against the cytochrome *c*-enhanced oxidation of 5-(2-aminoethyl)benzene-1,2,4-triol (6-OH dopamine), an oxidation assay reaction of neurotoxic relevance.

To identify potentially neuroprotective antioxidants, we have employed an assay based on the cytochrome *c*-enhanced oxidation of 5-(2-aminoethyl)benzene-1,2,4-triol (6-OH dopamine; 6-OHDA). There are two potential neurotoxicity-related components to this oxidation assay: (i) the in vivo neuronal oxidation of dopamine (4-(2-aminoethyl)benzene-1,2-diol; DA), and of its metabolite, 6-OHDA, and (ii) the in vivo abnormal release of mitochondrial cytochrome *c* (Cyt*c*).[1] Moreover, a reason for DA and 6-OHDA neurotoxicity may be the interfere of these compounds with normal Cyt*c* redox cycling,[2] e.g., maintaining the cytochrome in a reduced state that promotes generation of highly destructive hydroxy radicals by Fe(II) Cyt*c* and hydrogen peroxide.

The Cyt*c*-enhanced 6-OHDA oxidation reaction was used to test a phytochemical, cyanidin 3-*O*-β-D-glucopyranoside (C3G), and to compare its activity to that of an important physiological antioxidant, ascorbate. C3G is an anthocyanin compound, a glycosylated anthocyanidin flavonoid. Anthocyanins are common naturally occurring plant pigments that readily donate hydrogen and form stable unpaired-electron-structures.[3] As antioxidants, their potential health benefits are currently under study, especially in relation to neuroprotective functions. There is evidence for such neuroprotective functions of anthocyanins,[4,5] but the basis of this protection remains poorly understood.

Assays with 6-OHDA and Cyt*c* were performed using similar procedures and reagent concentrations as those reported for assays with *N*,*N*,*N*′,*N*′-tetramethy-1,4-phenylenediamine as the indicator.[6] The final concentrations of putative antioxidant, 6-OHDA, cytochrome *c*, and hydrogen peroxide are given in the figure legends.

The structure and purity of C3G is shown in Figure 1. In the time course of the reaction (Figure 2a, up to 140 s), 6-OHDA + H_2O_2 did not undergo substantial oxidation compared to 6-OHDA + H_2O_2 + Cyt*c* (upper curve in Figure 2a). Other controls (not shown) including 6-OHDA alone, 6-OHDA + H_2O_2 + anthocyanin, 6-OHDA + Cyt*c*, and 6-OHDA + Cyt*c* + anthocyanin all exhibited lower oxidation than 6-OHDA + H_2O_2 (bottom line in Figure 2a). The inhibitory activity of C3G is shown in both Figures 2a and 2b. While the presence of 25 μM ascorbate did not significantly decrease the Cyt*c*-enhanced oxidation reaction (Figure 2b upper line), lower concentrations of C3G produced a significant inhibition (Figure 2b lower line). These data provide evidence for differences between the anthocyanin and ascorbate in terms of direct antioxidant potency in this assay; the possibility remains, however, that the difference between the two is related to some other property such as protein affinity, e.g., polyphenols have been reported to interact with protein surfaces.[3] The IC_{50} value for C3G was calculated to be 9 μM. In the same assay (data not shown), an anthocyanin-rich crude extract from a *Vaccinium* species yielded a similar IC_{50} value of 6 μM total anthocyanins.

Anthocyanins and other polyphenols can chelate transition metals.[3,7] Chelation of metals such as iron and copper is expected to increase with increasing pH; and, thus, if such chelation is an important contributor to antioxidant activity, the corresponding antioxidant activity would be expected to increase with increasing pH. Our results show the opposite, i.e., increase in antioxidant activity of C3G with decreasing pH (data not shown).

These results suggest hydrogen donation accounts for most of the antioxidant activity of the anthocyanin in the assay. Previous studies of the effects of C3G on oxidation of low-density lipoproteins also indicated that metal chelation makes a very minor contribution to the antioxidant activity.[8] The decreasing antioxidant activity with increasing pH also suggests that anionic quinoidal base structures of C3G are less potent antioxidants

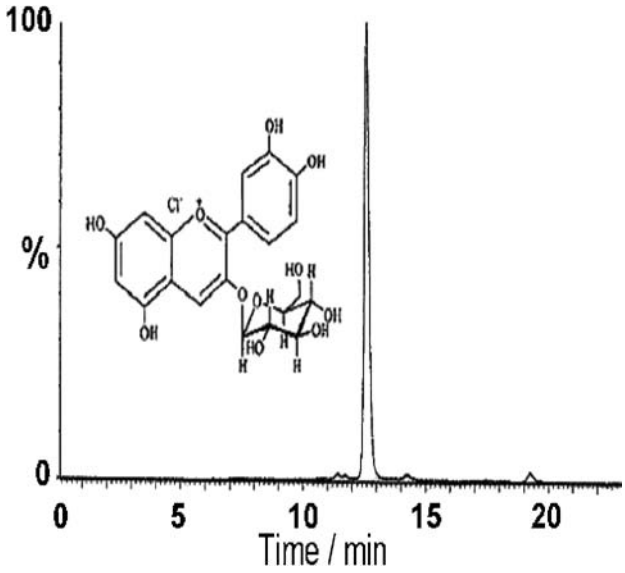

Figure 1. Structure of cyanidin 3-*O*-β-D-glucopyranoside and HPLC analysis of its purity. The flavylium cation structure of monoglycosylated cyanidin is shown. Cyanidin 3-*O*-β-D-glucopyranoside was of HPLC purity grade as analyzed using a 10–100% linear gradient of (v/v) HCO_2H H_2O MeOH (1:9:10) into HCO_2H H_2O (1:10) (Polyphenols SA); the solvent flow rate was 1.2 mL/min with detection at 520 nm.

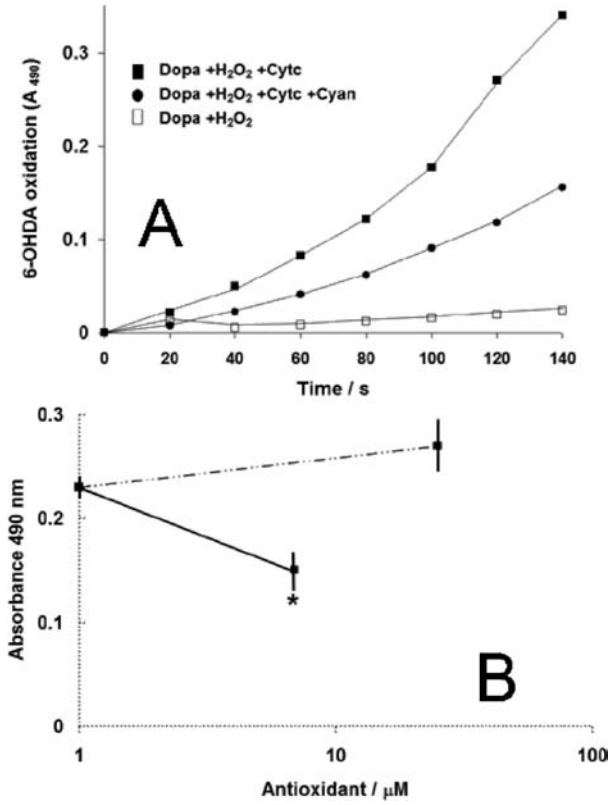

Figure 2. Oxidation kinetics of 5-(2-aminoethyl)benzene-1,2,4-triol (6-OHDA) and the effects of purified cyanidin 3-*O*-β-D-glucopyranoside and ascorbate. (**A**) The oxidation of 6-OHDA in the presence of the reagents indicated in the figure was followed over a time period of 140 s. The final concentrations of 6-OHDA, cytochrome *c*, and hydrogen peroxide, and cyanidin 3-*O*-β-D-glucopyranoside in the assay were 200, 10, 5, and 6.9 μM respectively. (**B**) Absorbance changes for the oxidation reaction with the above concentrations of 6-OHDA, cytochrome *c*, and hydrogen peroxide and the addition of either 25 μM ascorbate (upper, dashed line), or 6.9 μM cyanidin 3-*O*-β-D-glucopyranoside (lower, solid line); the data is related to the control without added antioxidants (0 μM). Antioxidant concentration is shown in log scale. Statistical significance is indicated by $*$ for $p = 0.05$–0.01.

in the assay than the flavylium cation; a similar result has been reported.[9]

Anthocyanins such as C3G are abundant, non-toxic plant metabolites that may have important health benefits related to aging and diseases involving oxidative damage (e.g., Refs. 4 and 10). The results presented herein establish a purified anthocyanin, C3G, as a direct and potent inhibitor of cytochrome *c*-enhanced 6-OHDA oxidation. Inhibition of such oxidation by this flavonoid may be protective against Fe(II) cytochrome *c* reactions with hydrogen peroxide (e.g., peroxide from in vivo catecholamine oxidation) that generate highly destructive hydroxy radicals.

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