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Protection against peroxynitrite by cocoa polyphenol oligomers

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Abstract Flavonoids, natural plant constituents, protect against peroxynitrite and can thereby play a role in defense against this mediator of inflammation. Procyanidin oligomers of different size (monomer through nonamer), isolated from the seeds of *Theobroma cacao*, were examined for their ability to protect against peroxynitrite-dependent oxidation of dihydrorhodamine 123 and nitration of tyrosine. By molarity, oligomers were more effective than the monomeric epicatechin; the tetramer was particularly efficient at protecting against oxidation and nitration reactions. These results suggest that epicatechin oligomers found in cocoa powder and chocolate may be a potent dietary source for defense against peroxynitrite.

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Key words: Flavonoid; Inflammation; Procyanidin; Cocoa; Peroxynitrite; Antioxidant

1. Introduction

Flavonoids occur in different classes, including procyanidins, as natural products in plants, and these polyphenols are ingested with the diet [1,2]. Flavonoids are general free radical scavengers [3] and chelate transition metals [4,5]. Procyanidins (e.g. epicatechin) are exceptionally efficient radical scavengers [6,7]. Possible health benefits of polyphenols include suppression of inflammatory cytokine production [8,9], protection against cardiovascular disease [10,11], and anticarcinogenic effects [12,13].

Peroxynitrite, a potent oxidizing and nitrating species, leads to tissue damage in a number of pathological conditions in humans and in experimental animals [14,15]. The physiological and pharmacological strategies for protection against peroxynitrite are organized into three categories: *prevention*, *interception*, and *repair* (see [16,17]). Flavonoids react with nitric oxide [18], superoxide [19,20] and protect against peroxynitrite-dependent oxidation [21] and nitration [22], and can thereby play a direct role in interception of peroxynitrite and its precursors. Here, the procyanidin, (−)-epicatechin, and the respective procyanidin oligomers ranging up to the nonamer, isolated from *Theobroma cacao*, are examined for their ability to protect against peroxynitrite-dependent oxidation and nitration reactions.

2. Materials and methods

2.1. Reagents

Procyanidin oligomers were purified from Cocoapro cocoa [23,24], kindly supplied by Mars, Inc. (Hackettstown, NJ, USA). The oligomeric composition of the respective procyanidin preparations is given in Table 1. Stock solutions (10 mg/ml) of the tested procyanidin preparations were made in methanol, but were readily soluble in aqueous solutions used in the studies. The absorbance at 280 nm of 25 μg/ml aliquots of these stocks was similar in all preparations (0.460 ± 0.040 absorbance units, mean value ± S.D. ($n = 9$)). Diethylenetriamine pentaacetic acid (DTPA), (−)-epicatechin, and (−)-epigallocatechin gallate were from Sigma (Deisenhofen, Germany). Dihydrorhodamine 123 (DHR-123) was from Molecular Probes (Eugene, OR, USA). MnO_2 was from Fluka (Buchs, Switzerland). 2-Phenyl-1,2-benzoselenazol-3(2H)-one (ebselen) was kindly provided by Rhône-Poulenc-Rorer (Cologne, Germany). Peroxynitrite was synthesized from sodium nitrite and H_2O_2 using a quenched-flow reactor [25], and H_2O_2 was eliminated by passage of the peroxynitrite solution over MnO_2 powder. The final peroxynitrite concentration was determined spectrophotometrically at 302 nm ($\varepsilon = 1700\ M^{-1}\ cm^{-1}$).

2.2. Assay of peroxynitrite-mediated oxidation of dihydrorhodamine 123

The peroxynitrite-mediated oxidation of dihydrorhodamine 123 was performed as described by Kooy et al. [26] using minor modifications [27]. Briefly, peroxynitrite (100 nM) was added to 0.5 μM dihydrorhodamine 123 and different concentrations of procyanidin oligomers in 0.1 M phosphate buffer, 0.1 mM DTPA, pH 7.3, under intense stirring at room temperature, and fluorescence was detected with a fluorescence spectrophotometer LS-5 (Perkin-Elmer, Norwalk, CT, USA) with excitation and emission wavelengths of 500 nm and 536 nm, respectively. There was no significant interference of the test compounds in fluorescence determination of DHR-123. The effect of vehicle (0.3–1.5% methanol) under these conditions was negligible.

2.3. Assay of peroxynitrite-mediated nitration of tyrosine

Protection against peroxynitrite-mediated nitration of tyrosine was performed as described by Pannala et al. [22] with minor modifications. Peroxynitrite (500 μM) was added by bolus addition under constant vortexing to 100 μM tyrosine in 0.1 M phosphate buffer (pH 7.3) containing 0.1 mM DTPA. Under these conditions, effects of the tested compounds (0–20 μM) were determined. Samples (50 μl; containing 100 μM 3-hydroxy-4-nitrobenzoic acid as an internal standard) were injected onto a C-18 reverse-phase column (150 × 4.6 mm; Merck, Darmstadt, Germany) with a Waters 720 WISP autosampler. Separation was performed with a 50 mM potassium phosphate buffer (pH 7.0)/acetonitrile step-gradient on a Merck-Hitachi L-655A 12 HPLC unit coupled with a Merck-Hitachi L-5000 controller unit, at a flow rate of 1.0 ml/min. The initial buffer/acetonitrile ratio was 95/5, followed by a stepwise decrease to 50/50 at 5 min; after 13 min, the ratio was returned to 95/5 and maintained for an additional 13 min. Such a step-gradient was necessary to achieve separation of the compounds of interest and then to elute the flavonoids. The 3-nitrotyrosine formation was monitored with a Merck-Hitachi L-4200 UV/Vis detector equipped with a D-2500 Chromato-Integrator at 430 nm. Calibration curves of the ratio of peak area of 3-nitrotyrosine standard vs. internal standard were used to determine concentrations.

2.4. Estimations of molar concentrations of oligomers

Although the oligomeric preparations contained contributions from other oligomers (see Table 1), the purity of a number of the preparations was sufficient (> 90%) to approximate half-maximal inhibitory concentrations for the inhibition of dihydrorhodamine 123 oxidation

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and tyrosine nitration by peroxynitrite in terms of molarity. For these estimations, the purity of the major oligomer in question was used (e.g. dimer = 99%).

3. Results and discussion

3.1. Protection by procyanidin oligomers against the oxidation of dihydrorhodamine during bolus addition of peroxynitrite

Fig. 1A illustrates the protective effect of some polyphenols, and Table 2 summarizes the protection of all tested compounds against peroxynitrite-mediated oxidation of DHR-123. For comparison, the results obtained with ebselen are also shown (Table 2). The half-maximal inhibitory concentration of ebselen was ~150 nM, similar to our previous findings [27,28]; it should be noted that in a different assay system, another group observed higher values (e.g. 2 μM [21]), underlining the necessity to consider assay conditions when comparisons are performed. When concentrations of the various compounds are compared by weight, epigallocatechin gallate (Table 2) was the most effective of the catechin polyphenols, and epicatechin (Table 2; Fig. 1A, open circles) was more effective than the oligomers (Table 2, Fig. 1A). However, when concentrations were expressed in terms of molarity (Table 2), the procyanidin oligomers were more effective than epicatechin (224 nM), and a number of the oligomers were more effective than ebselen (160 nM) and epigallocatechin gallate (111 nM). The tetrameric compound had a half-maximal inhibitory concentration less than 100 nM (Table 2).

3.2. Protection by procyanidin oligomers against nitration of tyrosine by peroxynitrite

Fig. 1B depicts the protective effect of some polyphenols, and Table 2 summarizes the protection of all tested compounds against nitration of tyrosine (100 μM) by peroxynitrite (500 μM). Nitration of tyrosine in the absence of test compounds, but in the presence of vehicle (0.3% methanol) was ~30 μM; this value was set at 100%. Epicatechin and epigallocatechin gallate were relatively efficient inhibitors of tyrosine nitration by peroxynitrite (12 and 9 μM, respectively; Table 2). The tetramer again was particularly efficient, inhibiting tyrosine nitration more than the trimer or the pentamer (Fig. 2; Table 2).

For the pentamer and smaller oligomers, an association was observed between the ability of the tested compounds to inhibit DHR-123 oxidation and tyrosine nitration by peroxynitrite (Fig. 2); however, while the hexamer, heptamer, octamer, and nonamer were only moderately effective at preventing the oxidation of DHR-123, they were relatively efficient at inhibiting tyrosine nitration (Table 2; Fig. 2). Since the chemical mechanisms of oxidation of DHR-123 and of nitration of tyrosine by peroxynitrite are different [26,29], compounds do

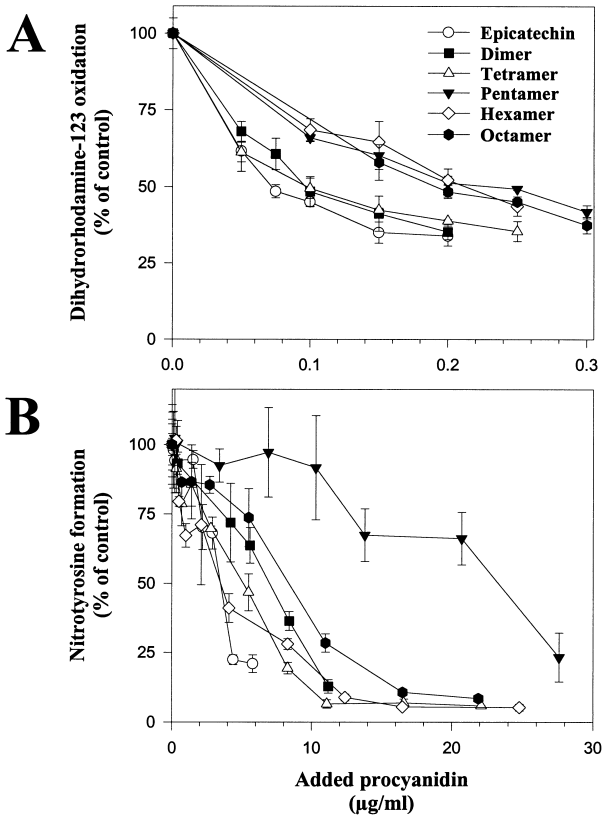

Fig. 1. Protection by procyanidin oligomers against dihydrorhodamine 123 oxidation and tyrosine nitration caused by peroxynitrite. Oxidation of dihydrorhodamine 123 (panel A) was determined fluorometrically as described in Section 2. Peroxynitrite (100 nM) was added to 0.5 μM dihydrorhodamine 123 and different concentrations of procyanidin oligomers in 0.1 M phosphate buffer, 0.1 mM DTPA, pH 7.3, under intense stirring at room temperature. 3-Nitrotyrosine formation (panel B) was determined by HPLC with absorbance detection as described in Section 2. Peroxynitrite (500 μM) was added by bolus addition to 100 μM tyrosine and different concentrations of procyanidin oligomers in 0.1 M phosphate buffer (pH 7.3) containing 0.1 mM DTPA. Key: epicatechin (open circles), dimer (closed squares), tetramer (open triangles), pentamer (closed upside-down triangles), hexamer (open diamonds), octamer (closed hexagons). Results are means ± S.D. (n = 3–6). See Table 2 for summary data.

Table 1
Procyanidin oligomer preparations isolated from cocoa (in percent)

	Oligomeric preparation							
	Dimer	Trimer	Tetramer	Pentamer	Hexamer	Heptamer	Octamer	Nonamer
Monomer	1.0							
Dimer	**99.0**	5.2	0.5	0.3	0.2	0.4	0.3	0.3
Trimer		**94.8**	4.1	0.6	0.4	0.4	0.3	0.3
Tetramer			**95.4**	5.8	2.9	1.5	0.8	0.9
Pentamer				**92.0**	7.2	4.9	1.4	1.4
Hexamer				1.3	**86.2**	25.1	9.1	5.5
Heptamer					1.3	**61.0**	12.9	4.1
Octamer					0.8	2.8	**64.6**	10.8
Nonamer					1.0	3.9	10.6	**76.7**

The bold numbers designate the major oligomer of the fraction. Top row indicates preparation used, oligomer composition is shown on the vertical axis. Data are as provided by supplier; see also [23,24].

Table 2
Half-maximal inhibitory concentrations of epicatechins, procyanidin oligomers, and ebselen in peroxynitrite-mediated oxidation of dihydrorhodamine 123 and nitration of tyrosine

Compound	MW (Da)	DHR-123 oxidation		Tyrosine nitration	
		(half-maximal inhibitory concentration)			
		ng/ml	nM	μg/ml	μM
Epigallocatechin gallate	458	51	111	4.1	9.0
Epicatechin	290	65	224	3.5	12.0
Dimer	578	95	162[a]	7.0	12.0[a]
Trimer	866	125	137[a]	9.0	9.8[a]
Tetramer	1154	100	83[a]	5.2	4.3[a]
Pentamer	1442	235	150[a]	23.3	14.8[a]
Hexamer	1730	210	–	3.5	–
Heptamer	2018	285	–	6.5	–
Octamer	2306	190	–	8.3	–
Nonamer	2594	180	–	5.4	–
Ebselen	274	45	160	n.d.	–

The oxidation of dihydrorhodamine 123 and nitration of tyrosine by peroxynitrite was determined as described in Section 2. See also Figs. 1 and 2.
n.d., not determined.
[a]Calculated using oligomer composition data (see Table 1 and Section 2). Contributions of other procyanidin oligomers to the hexamer, heptamer, octamer and nonamer preparations were >10%, so calculation of molarity was inappropriate for these.

not necessarily have to be equally effective in both assays. Previous studies have shown that polyphenolic compounds may be both oxidized and nitrated by peroxynitrite [30,31]. The observed differences could therefore result from the hexamer and larger oligomers not being as efficient protecting against oxidation by peroxynitrite, but more efficient targets for nitration reactions. Indeed, as described previously, many of the compounds tested here exhibited an increase in absorbance at 430 nm upon reaction with peroxynitrite (data not shown), suggesting nitration of the polyphenols [22].

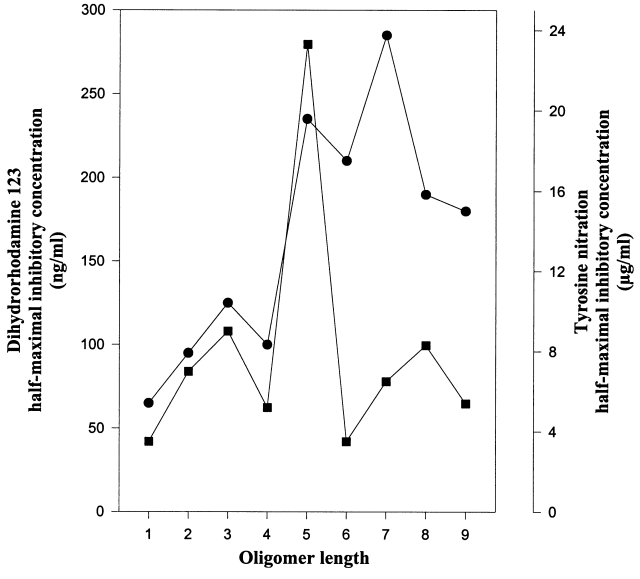

Fig. 2. Comparison of the half-maximal inhibitory concentrations of epicatechin and procyanidin oligomers. Data are as summarized in Table 2 and represented in Fig. 1. The half-maximal inhibitory concentrations for dihydrorhodamine 123 formation (ng/ml; closed circles) and 3-nitrotyrosine formation (μg/ml; closed squares) plotted vs. oligomer length (e.g. '2' = dimer).

3.3. Implications

The role of dietary polyphenols in health and disease has received recent attention (see [32,33]). These compounds have been shown to inhibit oxidation [21] and nitration reactions [22] as well as DNA damage and strand breakage [34,35] caused by peroxynitrite. The presence of procyanidin oligomers in chocolate products has been previously described [23,36]. These compounds were shown here to be highly effective at preventing oxidation and nitration reactions caused by peroxynitrite, some more effective than monomeric (−)-epicatechin on a molar basis. Recent studies have also demonstrated differential effects of procyanidin oligomers compared to the monomeric compounds. For example, procyanidin oligomers (dimer and trimer) isolated from apple juice were found to exhibit higher growth-promoting activity for hair epithelial cells than the monomeric (−)-epicatechin [37]. Results of a Dutch nutritional study suggested that while tea contributed 55% of dietary catechin intake, chocolate contributed 20% [38], indicating that catechins from chocolate may be a significant source of antioxidant defense against peroxynitrite; however, this study did not take into account the more abundant oligomers found in chocolate, present in only minor concentrations in tea. Further, since cocoa generates less hydrogen peroxide than tea [39], cocoa may be a preferable source of antioxidant polyphenols. While epicatechin from chocolate was found to reach a concentration of 0.7 μM in plasma after intake of 80 g black chocolate [40], it is not yet known how well cocoa procyanidin oligomers are absorbed into the bloodstream. Recent work by Spencer et al. [41] using the isolated rat intestine showed that for certain flavonoids, glucuronidation and possibly other metabolism may occur at the level of the intestinal mucosa. The physicochemical basis for the special properties described here for the tetrameric compound will be a challenging topic of research.

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