Radical Scavenging Action and Its Mode in Procyanidins B-1 and B-3 from Azuki Beans to Peroxyl Radicals†

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Procyanidins B-1 and B-3 from azuki beans showed remarkable scavenging activities, especially to hydrophilic radicals. The inhibition rate constants for the scavenging of hydrophilic peroxy radicals generated from 2,2'-azobis(2-amidinopropane)hydrochloride in aqueous media were calculated at 37°C to be $k_{inh} = 6.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $5.9 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $2.7 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $5.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and $1.1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, and the stoichiometric factors as $n = 8.4, 8.0, 3.7, 1.2$ and 1.7 for procyanidin B-1, procyanidin B-3, (+)-catechin, ascorbic acid and α -tocopherol, respectively. Thus, the dimeric procyanidin molecule could trap eight radicals. The production and structures of procyanidin B-3 radicals are discussed on the basis of the ESR spectra observed by autoxidation of procyanidin B-3 in an aqueous alkaline methanol solution.

Recently, radical scavengers have attracted special interest because they can protect the human body from free radicals^{1,2)} which may cause many diseases including cancer and aging.^{3–5)} In a previous paper,⁶⁾ we reported that procyanidins B-1 and B-3 from azuki beans showed remarkable antioxidative activities in aqueous systems, and suggested that they may act as radical trappers. However, their radical scavenging activity and its mode remained unresolved.

In the present study, we quantitatively determined the radical scavenging activities of

procyanidins and elucidated the mode of the radical scavenging action.⁷⁾ Yamamoto *et al.* have reported that the measurement of oxygen uptake is the most useful, versatile and quantitative method for lipid peroxidation compared with others such as the substrate disappearance method, peroxide method, conjugated diene method, and TBA-reactive material method as an indication of the extent of oxidation.⁸⁾ In the oxygen uptake method,^{9,10)} the rate of oxygen uptake would indicate not only the oxidation of methyl linoleate but also the radical scavenging action of the added

Fig. 1. Structures of Dimeric Procyanidins from Azuki Beans Used in This Study.

† Antioxidative Properties of Proanthocyanidins. Part II.

antioxidant. First, we tried to elucidate the radical scavenging action and its mode in dimeric procyanidins B-1 and B-3 to hydrophilic or lipophilic peroxy radicals by the oxygen uptake method used by Niki *et al.*^{9,10} In this method, azo compounds were used as radical initiators in order to obtain constant rates of chain initiation, and methyl linoleate was chosen as a substrate. The procyanidins were evaluated as radical scavengers of the peroxy radicals in the system.

We observed that procyanidins B-1 and B-3 had remarkable radical scavenging activities, especially to hydrophilic peroxy radicals generated from 2,2'-azobis(2-amidinopropane)hydrochloride. Their activities were compared with those of natural antioxidants with respect to the inhibition rate constants and stoichiometric factors (the proportions, in terms of moles, at which antioxidants react with peroxy radicals). We will show that the dimeric procyanidin molecule could trap eight peroxy radicals. In addition, the structures of procyanidin B-3 radicals produced by autoxidation in an aqueous alkaline methanol solution are discussed on the basis of their ESR spectra.

Materials and Methods

Materials. Commercial methyl linoleate (99%, Sigma Chemical Co.), Triton X-100 (Tokyo Kasei Co.), L-ascorbic acid (Wako Chemical Industries), D- α -tocopherol (99%, Serva Chemical Co.) and trolox (97% Aldrich Chemical Company Inc.) were used without further purification. 2,2'-Azobis(2-amidinopropane)hydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylpentanenitrile) (AMVN) were obtained from Wako Chemical Industries. (+)-Catechin (Sigma Chemical Co.) was purified by preparative HPLC (μ -Bondapak C₁₈, 19 $\times$ 150 mm, 7.5% MeOH) before use. Procyanidins B-1 and B-3 were isolated in their pure form from azuki beans in the same way as that described previously.⁶ All other chemicals were purchased from Wako Chemical Industries.

Methods for oxidation testing. AAPH and AMVN were used as hydrophilic and lipophilic radical initiators, respectively. The preparation of methyl linoleate micelles, oxidation testing and measurement of the rate of oxidation were carried out according to the method of Niki *et al.*¹⁰ with a minor modification, the rate of oxidation being

determined by monitoring the oxygen concentration in the head space of the sample bottle and not in solution.

Measurements of K_{inh} and n . The inhibition rate constants (k_{inh}) and the stoichiometric factors (n) for antioxidants were calculated according to the method of Burton *et al.*¹¹

ESR analyses. ESR spectra were recorded on an X-band JEOL FEIX spectrometer at room temperature.

Results and Discussion

Scavenging effect of procyanidin B-3 on the lipophilic peroxy radical in an organic solvent solution

Upon thermal decomposition at 37°C, azo compounds such as AAPH and AMVN give the carbon-centered radical at a constant rate. When a radical scavenger is added to the reaction mixture, the inhibition of oxidation of linoleate is determined by the rate of oxygen uptake. The rate of radical scavenging action was determined quantitatively by following the decrease in the rate of oxygen uptake.

Figure 2 shows the radical scavenging activity of procyanidin B-3 to the lipophilic peroxy radical from methyl linoleate induced by AMVN in *tert*-butanol-methanol (3:1,

Fig. 2. Profile of Oxygen Uptake during the Oxidation of 600 mM Methyl Linoleate in *tert*-Butyl Alcohol-Methanol (3:1, v/v) at 37°C Initiated with 10 mM AMVN in the Presence of Procyanidin B-3 at the Indicated Concentrations.

—, procyanidin B-3; ---, 20 μ M α -tocopherol.

v/v).⁹⁾ In the absence of a radical scavenger, the oxidation initiated by AMVN proceeded without any induction period at a constant rate of oxygen uptake. In the presence of 20 μM α -tocopherol, which is a well-known radical scavenger, the rate of oxygen uptake was suppressed and a distinct induction period was observed. When the induction period was over, the oxidation proceeded at the same rate as that in the absence of a radical scavenger, which suggests that α -tocopherol was exhausted.⁹⁾ On the other hand, in the presence of 20 μM procyanidin B-3, the rate of oxygen uptake was less suppressed, and no induction period was observed. Procyanidin B-3 scavenged the lipophilic peroxy radical generated from methyl linoleate more slowly than α -tocopherol did, but for longer. In the range of concentrations tested, the radical scavenging activity of procyanidin B-3 increased with increasing concentration.

It has been shown that the radical scavengers react mainly with the peroxy radical generated from methyl linoleate under the conditions already described.⁹⁻¹²⁾ The radical scavenging reaction by a phenolic antioxidant under these conditions can be represented by a scheme,⁹⁻¹²⁾ in which $\text{LOO}\cdot$ and IH represent alkylperoxy radicals and the radical scavenger, respectively, and $\text{I}\cdot$ represents the radical generated from the scavenger by donating a hydrogen atom to chain-propagating free radicals. The constant n is the stoichiometric

factor for the radical scavenger.

Chain inhibition

Chain termination

In the presence of an antioxidant which rapidly scavenges free radicals, the chains are mainly terminated by Reactions 1 and 2, instead of by Reaction 3. Since procyanidin B-3 did not rapidly scavenge the lipophilic peroxy radicals, Reactions 2 and 3 would proceed at the same time.

Scavenging activities of procyanidins toward the hydrophilic peroxy radical in an aqueous dispersion

Figure 3 shows that procyanidin B-3 rapidly scavenged free radicals that were induced by AAPH in an aqueous dispersion of methyl linoleate, and gave a distinct induction period. However, when AMVN was used as a radical initiator instead of AAPH in the aqueous dispersion, procyanidin did not act as a radical scavenger (data not presented). AAPH ($\text{A}_1\text{-N}=\text{N-A}_1$) produces hydrophilic radicals such as $\text{A}_1^{\cdot}$ and $\text{A}_1\text{OO}\cdot$ in an aqueous phase,

Fig. 3. Profile of Oxygen Uptake during the Oxidation of 100 mM Methyl Linoleate in a 10 mM Triton X-100 Aqueous Dispersion Initiated with 20 mM AAPH in the Presence of Procyanidin B-3 at the Indicated Concentrations.

whereas AMVN ($A_2-N=N-A_2$) produces lipophilic radicals $A_2\cdot$ and $A_2OO\cdot$, and induces $L\cdot$ and $LOO\cdot$ in the lipid phase.¹⁰⁾ Dimeric procyanidins are easily soluble in water, and slightly soluble in methyl linoleate. Since $A_1\cdot$ reacts extremely rapidly with O_2 to produce $A_1OO\cdot$, the reaction of $A_1\cdot$ with a radical scavenger would be negligible.^{12,13)} On the basis of these properties, it is suggested that procyanidin B-3 scavenges hydrophilic peroxy radical $A_1OO\cdot$ in the aqueous phase.

Fig. 4. Oxygen Uptake during the Oxidation of 100 mM Methyl Linoleate in a 10 mM Triton X-100 Aqueous Dispersion Initiated with 20 mM AAPH in the Presence of Antioxidants.

A, control; B, 20 μ M ascorbic acid; C, 20 μ M α -tocopherol; D, 20 μ M (+)-catechin; E, 20 μ M procyanidin B-3; F, 20 μ M procyanidin B-1.

Table I. INHIBITION RATE CONSTANTS (k_{inh}) AND STOICHIOMETRIC FACTORS (n) FOR ANTIOXIDANTS IN AN AQUEOUS SYSTEM^a

Antioxidant	k_{inh} [$M^{-1}s^{-1}$]	n
Procyanidin B-1	6.0×10^4	8.48
Procyanidin B-3	5.9×10^4	8.03
(+)-Catechin	2.7×10^4	3.65
Trolox ^b	2.9×10^5	2.00
α -Tocopherol	1.1×10^5	1.71
Ascorbic acid	5.0×10^4	1.22

^a The reaction mixture contained 100 mM methyl linoleate in a 10 mM Triton X-100 aqueous dispersion at 37°C and oxidation was initiated by adding 20 mM AAPH.

^b A model compound ($C_{14}H_{18}O_4$) of water-soluble α -tocopherol.

In the range of concentrations tested (5–40 μ M), the radical scavenging activity of procyanidin B-3 increased with increasing concentration. As shown in Fig. 4, procyanidins B-1 and B-3 gave induction periods which were much longer than those of (+)-catechin, ascorbic acid and α -tocopherol.

Table I lists the inhibition rate constants (k_{inh}) and the stoichiometric factors (n) of the dimeric procyanidins (procyanidins B-1 and B-3) and well-known natural antioxidants in an aqueous model system. It is suggested that the dimeric procyanidin molecule can trap eight peroxy radicals, whereas ascorbic acid and α -tocopherol can trap only one and two peroxy radicals, respectively.

The radical scavenging reaction by procyanidin (IH_4) in an aqueous dispersion can be represented by the following scheme:

Chain inhibition

Chain termination

To our knowledge, this is the first report that has determined k_{inh} and n for proanthocyanidins. It is well known that most antioxidants react with one or two peroxy radicals per molecule. The stoichiometric factors (n) for procyanidins were the highest of all for known antioxidants. Furthermore, these results suggest that the higher the degree of procyanidin polymerization, the more procyanidin scavenges the hydrophilic free radical per molecule. Judging from the k_{inh} and n values, it can be said that the radical scavenging activity of procyanidin B-3 to the water-soluble peroxy radical in an aqueous phase was 6.6 times stronger than that of ascorbic acid per molecule.

It was demonstrated that procyanidins are effective as radical scavengers, especially to the hydrophilic peroxy radical in the aqueous phase. This result agrees with the strong antioxidative properties of procyanidins in

aqueous systems that were described in a previous paper.⁶⁾

Detection of procyanidin radicals

Radical scavengers are generally converted into radicals of the respective scavengers during the protection reaction. We attempted to demonstrate the formation of procyanidin radicals in a reaction mixture of 40 μ M procyanidin B-3 and 100 mM methyl linoleate in an aqueous system initiated with 20 mM AAPH, but the life of the radical was too short to detect by ESR. In order to confirm the existence of procyanidin radicals, procyanidin B-3 was exposed to autoxidation in an aqueous alkaline methanol solution, where the procyanidin B-3 radicals produced were stabilized and increased in concentration.

Figure 5(A) shows the ESR signals of

procyanidin B-3 radicals, and has three main lines centered at $g=2.0050$ with fine-coupling constants of $a_{H1}=3.23$ G, $a_{H2}=0.61$ G and $a_{H3}=0.48$ G. (B) shows the signals simulated by computer on the basis of fine-coupling constants of $a_{H1}=3.23$ G, $a_{H2}=0.61$ G and $a_{H3}=0.48$ G.

From the result that the number of main lines was three, it is suggested that the number of hydrogens for the radical-trapping moiety (the bloc linked by the π electron) of procyanidin B-3 radicals molecule is three. Thus, procyanidin B-3 appears to trap free radicals at a position in aromatic ring B.

Figure 6 illustrates the suggested structure of dimeric procyanidin B-3 radicals, which were mainly formed in an alkaline methanol solution. It is suggested that procyanidin B-3 can trap four radicals at its maximum at a

Fig. 5. ESR Spectra of Procyanidin B-3 Radicals Formed in an Alkaline Methanol Solution.

(A) the spectrum obtained by the experiment; (B) the spectrum reconstructed by a computer simulation method. Autoxidation conditions: 0.4% procyanidin B-3 in 1 M NaOH–dimethyl sulfoxide (1 : 9, v/v) at room temperature. Instrument settings: microwave power, 1 mW; modulation amplitude, 0.032G; time constant, 0.1 sec; sweep time, 4 min.

Fig. 6. Suggested Structures of Procyanidin B-3 Radicals Formed in an Alkaline Methanol Solution.

position in aromatic ring B.

Since procyanidin B-3 is one of the (+)-catechin dimers, we tried to confirm the existence of the (+)-catechin radical in the same way as that just described. The ESR spectrum of the (+)-catechin radical obtained had two lines centered at $g=2.0050$ with $a_{H1}=1.85$ G and $a_{H2}=0.20$ G. This result suggests that (+)-catechin trapped the free radical at a position in aromatic ring A. It is speculated that if procyanidin B-3 can trap four radicals at a position in ring A as well as two (+)-catechins do, it can trap eight radicals at its maximum per molecule, which well agrees with stoichiometric factor $n=8$ obtained in this study.

Free radical reactions usually occur in blood,¹⁴⁾ which is a kind of aqueous dispersion, where hydrophilic radicals such as $O_2^{\cdot -}$ and $OH^{\cdot}$ are formed. Barclay has suggested that a hydrophilic chain-breaking antioxidant may have therapeutic effects in the treatment of disorders (e.g., inflammation) that are suspected to involve lipid peroxidation.¹⁵⁾ Of the known proanthocyanidin oligomers, dimeric procyanidin B-types are known to be the most widely distributed in food materials.¹⁶⁻¹⁹⁾ Humans usually take in many of the oligomeric procyanidins B-types through a variety of foods, especially fruits. Our results raise two interesting possibilities: first, procyanidin oligomers may contribute to prevent humans from adult diseases; second, they may have a therapeutic effect in the treatment of disorders suspected to involve lipid peroxidation.

In conclusion, it is now clear that procyanidins B-1 and B-3 act as effective radical scavengers, especially to hydrophilic peroxy radicals generated from AAPH in an aqueous phase, and that they can trap eight peroxy radicals per molecule.

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