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Evaluation of the NO Scavenging Activity of Procyanidin in Grape Seed by Use of the TMA-PTIO/NOC 7 ESR System

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The nitrogen monoxide (NO) scavenging activity of grape seed extract (GSE) was studied in the TMA-PTIO/NOC 7 system. The procyanidin-rich (>95%) GSE showed strong NO scavenging activity in the system. The activity was found to depend on the condensation rate of cyanidin when synthetic oligomers were tested. Investigation of the NO scavenging activities of other polyphenols (catechin, epicatechin, epigallocatechin, and epigallocatechin gallate) in the TMA-PTIO/NOC 7 system revealed that gallocatechin, epigallocatechin, and epigallocatechin gallate exhibited strong activities. From the results, it was suggested that the high condensation rate of and the gallate ester moiety in procyanidin in GSE may play an important role in the NO scavenging activity. The mechanism of the NO scavenging activity of phenolic compounds such as GSE is speculated to be as follows: NO reacts with phenolic compounds directly to generate phenoxy radicals.

KEYWORDS: Nitrogen monoxide; procyanidin; radical scavenger; electron spin resonance

INTRODUCTION

Nitrogen monoxide (NO) is a free radical known to play an important role in human health. It has been implicated in a number of diverse physiological processes, including smooth muscle relaxation, platelet inhibition, neurotransmission, immune regulation, and penile erection (*1*−*9*). The biochemical pathways in these processes share two common features: the enzymatic synthesis of NO from L-arginine and the formation of an iron−nitrosyl complex in a target protein to evoke the functional response. Excess NO is a key mediator of toxicity in various physiological processes such as inflammation and endothelial damage. Therefore, NO scavengers play an important protective role in biological systems. In the absence of adequate NO scavenging activity, the excess NO can form peroxynitrite, a highly reactive compound capable of damaging biological molecules. One physiologically important class of NO scavengers is flavonoids, as described by Saskia et al. (*10*). The NO scavenging activities of other polyphenols have also been reported. The reactive oxygen species (ROS) scavenging activity of grape seed extract (GSE) procyanidin has been revealed (*11*), whereas the NO scavenging activity remains to be clarified.

Recently, an evaluation system for the NO scavenging activities of polyphenols was developed by Yoshimura et al. (*12*). In the system, NO generated from NOC 7 reduces TMA-PTIO to TMA-PTI under neutral conditions. The conversion rate of TMA-PTIO to TMA-PTI can be measured by electron spin resonance spectrometry (ESR). We show here the NO scavenging activity of procyanidin in GSE as an application of the evaluation system.

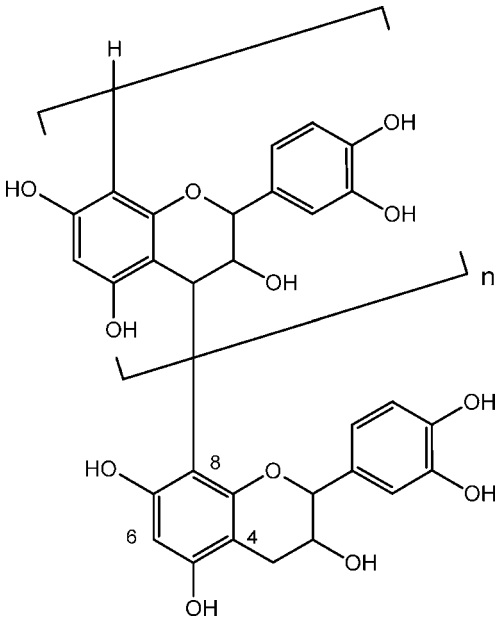

Figure 1. Structure of procyanidin oligomer.

MATERIALS AND METHODS

Chemicals. TMA-PTIO spin-trapping agent was purchased from Dojindo Co. (Kumamoto, Japan). This reagent was dissolved in 40 mM HEPES buffer (pH 7.4, 80 mM), immediately prior to use. 1-Hydroxy-2-oxo-3-(*N*-methyl-3-aminoethyl)-3-methyl-1-triazene (NOC 7) was obtained from Dojindo Co. and dissolved in 0.1 M sodium hydroxide, leading to the formation of NO. Bovine erythrocyte superoxide dismutase was purchased from Sigma (St. Louis, MO). (+)-Catechin, (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epigallocatechin

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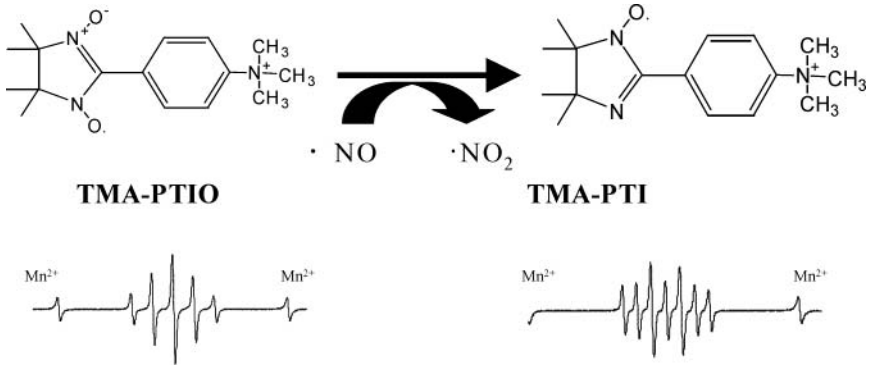

Figure 2. Reaction between TMA-PTIO and NO and their ESR spectra.

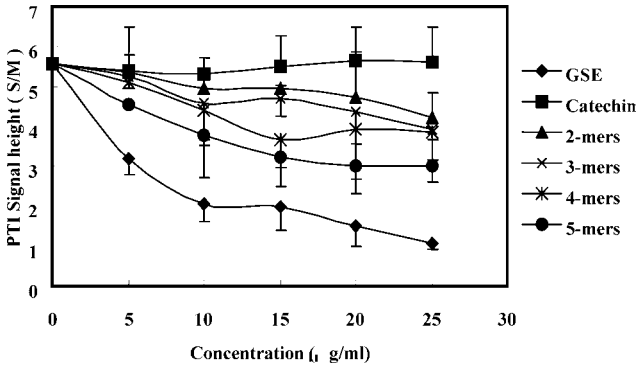

Figure 3. NO scavenging activity of GSE and synthetic oligomers by use of TMA-PTIO. The ordinate indicates the signal height of PTI (g = 2.0098) relative to the MnO_2 internal marker signal. The test sample was dissolved in DMSO at the final concentration indicated in the abscissa. Each point is the mean of three independent measurements, and error bars show the standard deviation of those measurements.

gallate (EGCG), (+)-taxifolin, and naringenin were purchased from Funakoshi (Tokyo, Japan). Chlorogenic acid, gallic acid, and ellagic acid were purchased from Tokyo Kasei Kogyo (Tokyo, Japan).

Preparation of GSE. Procyanidin-rich GSE (Gravinol super) was obtained from Kikkoman Co. (Chiba, Japan). The GSE contained 89.3% procyanidins, and the degree of polymerization ranged from 2 to 15 as revealed by MALDI-TOFMS detection (*13*). The speculated typical molecular structure of procyanidin in GSE is shown in **Figure 1**.

Preparation of Synthetic Procyanidin Oligomers. Synthetic procyanidin oligomers (dimers, trimers, tetramers, and pentamers) were prepared according to our previous paper (*17*). In brief, (+)-catechin and (+)-taxifolin were condensed in the presence of $NaBH_4$ in ethanol. The reactant was separated by Sephadex LH-20 column chromatography.

ESR Settings. The ESR spectrometer used was a JEOL JES-FR30 (JEOL, Tokyo, Japan). Conditions for ESR spectrometry were as follows: magnetic field, 334.6 ± 5 mT; power, 4.0 mW; frequency, 9.425 GHz; modulation width, 0.079 mT; gain, 50; time scan, 1 min.; time constant, 0.03 s.

Procedure for NOC 7/TMA-PTIO Reaction. NO scavengers of various final concentrations (0−100 μg/mL in DMSO) were added to 20 μL of 200 μM TMA-PTIO (final concentration = 20 μM) and 10 μM phosphate buffer solution (pH 7.4). Subsequently, 1 μL of 10 mM NOC 7 was added to this solution. This mixture was transferred to a quartz flat cell of 200 μL for ESR measurement. NOC 7, being unstable, decomposes under neutral or acidic conditions to form NO. TMA-PTI is generated by the reaction between NO and TMA-PTIO. Therefore, NO scavenging activity can be measured from the decrease of the control peak. The effect of the scavenger was determined from the extent of reduction of the TMA-PTI-derived signal (g = 2.0098) compared to that of the control signal. Fifty percent inhibitory concentration (IC_{50}) was obtained from the graph of sample concentration versus percentage inhibition. The reaction scheme and typical ESR signals are shown in **Figure 2**.

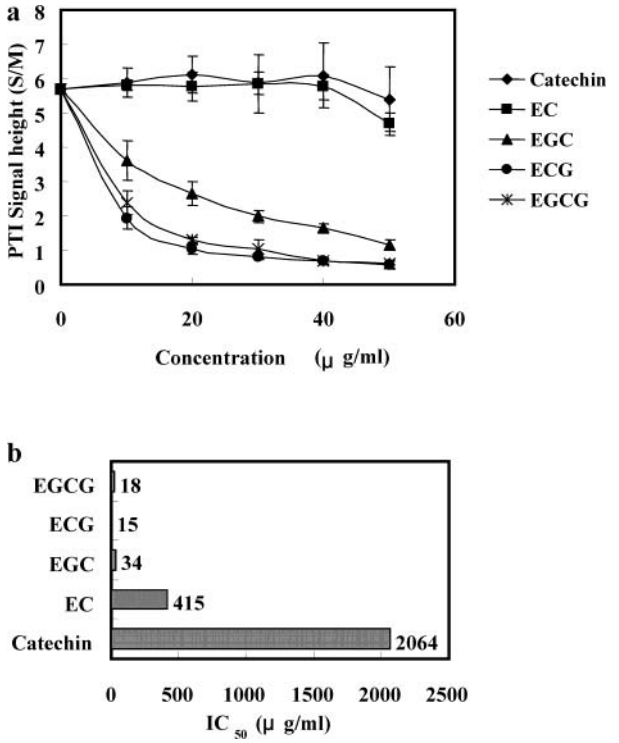

Figure 4. NO scavenging activity of catechins by use of TMA-PTIO. The ordinate indicates the signal height of PTI (g = 2.0098) relative to the MnO_2 internal marker signal. The test sample was dissolved in DMSO at the final concentration indicated in the abscissa. Each point is the mean of three independent measurements, and error bars show the standard deviation of those measurements. EC, epicatechin; EGC, epigallocatechin; ECG, epicatechin gallate; EGCG, epigallocatechin gallate.

RESULTS AND DISCUSSION

Synthetic procyanidin oligomers and procyanidin-rich GSE were tested in the NO scavenging system, and their dose-dependent activities was observed. The NO scavenging activity of each oligomer was dependent on its degree of polymerization. GSE, the average degree of polymerization of which ranges from 6 to 7, showed the strongest activity among the tested compounds (**Figure 3**). The condensation of the phenolic compound may contribute to the radical scavenging activity because the electron density is relatively high and it may stabilize the unpaired electron from the free radical.

For comparison, the NO scavenging activities of tea catechins were tested in the same manner. EGCG, EGC, and ECG showed strong activities, whereas catechin and epicatechin (EC) showed weak activities (**Figure 4a**). The IC_{50} values of the five catechins are shown in **Figure 4b**. The NO scavenging activities of other

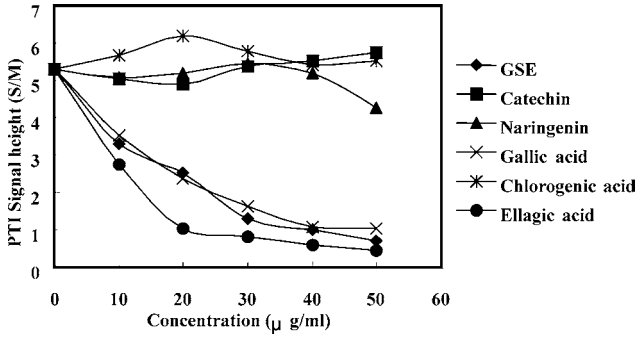

Figure 5. NO scavenging activity of GSE and polyphenol compounds. The ordinate indicates the signal height of PTI ($g = 2.0098$) relative to the MnO_2 internal marker signal. The test sample was dissolved in DMSO at the final concentration indicated in the abscissa. Each point is the mean of three independent measurements.

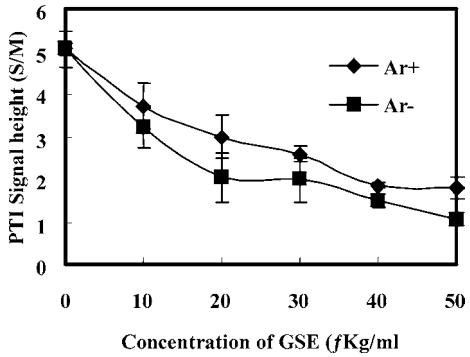

Figure 6. Effect of oxygen on NO scavenging activity of GSE. The ordinate indicates the signal height of PTI ($g = 2.0098$) relative to the MnO_2 internal marker signal. The test sample was dissolved in DMSO at the final concentration indicated in the abscissa. Each point is the mean of three independent measurements. +Ar, argon gas bubbled; –Ar, argon gas not bubbled.

related phenolic compounds were also tested, and gallic acid and ellagic acid showed strong activities (**Figure 5**). It was suggested that the adjacent hydroxyl groups play an important role in the NO scavenging activity. The average degree of polymerization of procyanidin in GSE was estimated to be from 6 to 7 from mass spectrometry and nuclear magnetic resonance spectroscopy as mentioned above, and the existence of a gallate ester moiety was also suggested. These characteristics in the molecular structure of procyanidin in GSE may easily explain its strong NO scavenging activity, as shown in **Figure 3**.

The NO scavenging activities of GSE and other polyphenolic compounds have been revealed in the above experiments, and the mechanism of those activities is predicted to be as follows. One is the direct reaction of phenolic compound and nitric oxide, and the other is an indirect reaction, namely, the reaction of nitric oxide and superoxide anion derived from dissolved oxygen by the reducing activity of the phenolic compound (*14*).

To examine the effect of oxygen, a degassing process was performed. Argon gas was bubbled into the reaction mixture at a flow rate of 1 mL/min for 5 s prior to the addition of NOC 7 (the source of NO) to replace the dissolved oxygen. After that, the NO scavenging activity of GSE was measured in the same manner as that described under Materials and Methods (**Figure 6**). The result revealed that the effect of argon bubbling was very slight and not significant. In the presence of 80 units of superoxide dismutase in the reaction mixture, no significant change was observed, similar to the case of argon bubbling (**Figure 7**). Therefore, it can be concluded that the superoxide

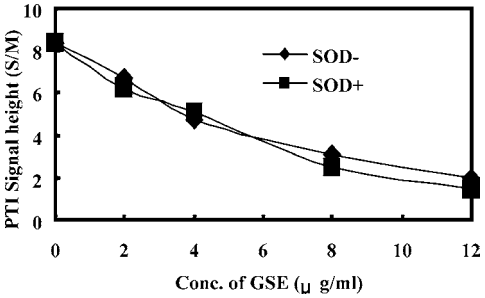

Figure 7. Effect of SOD on NO scavenging activity of GSE. SOD was dissolved in 40 mM HEPES buffer (pH 7.4) at the concentration of 400 units. The solution was added to the reaction mixture prior to the addition of NOC 7 solution. +SOD, bovine SOD added; –SOD, only buffer solution added.

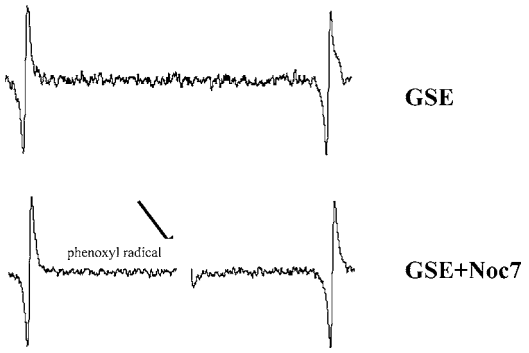

Figure 8. Generation of phenoxyl radical by reaction of NO and GSE. All ESR settings were the same as those of conventional measurements described under Materials and Methods except that the concentration of NOC 7 was 10 times higher and GSE was added at 250 μg/mL.

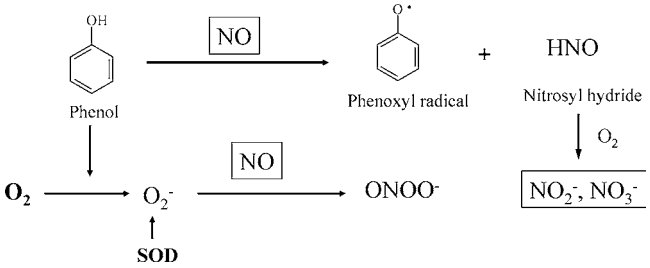

Figure 9. Mechanism of NO scavenging reaction with polyphenol compounds.

anion derived from dissolved oxygen may not play an important role in the NO scavenging activity of GSE.

By contrast, phenoxyl radical generation was observed in the ESR measurement when relatively high concentrations (10 times the usual concentration) of GSE and NOC 7 were used in the reaction system (**Figure 8**).

From those results, the mechanism of the NO scavenging activity of GSE can be speculated, as shown in **Figure 9**: NO can react with a phenolic compound directly to generate phenoxyl radical and nitrosyl hydride. Nitrosyl hydride, being relatively unstable, is easily oxidized by dissolved oxygen to generate nitrate and nitrite ions (*15*).

As shown in the results, procyanidin in GSE is a potential agent that can act as an NO scavenger. In vivo, it may suppress many adverse reactions caused by excess NO in extraordinary biological reactions. It has already been reported that procyanidins in GSE scavenge superoxide anion and hydroxyl radical (*16*), and the preventive effect of GSE on free-radical-related disease models has been shown by Saito et al. (*17*) and Yamakoshi et al. (*18*). The intake of procyanidin in GSE from

the diet may control the production of almost all deleterious radical species and reduce the risk of diseases caused by those radicals.

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