Antiulcer Activity of Grape Seed Extract and Procyanidins

Makoto Saito,* Hiroshi Hosoyama, Toshiaki Ariga, Shigehiro Kataoka, and Nobuyuki Yamaji

Research and Development Division, Kikkoman Corporation, 399 Noda, Noda City, Chiba Prefecture 278, Japan

It is known that procyanidins, which are contained in grape seeds, are antioxidative and have certain biological effects. Antiulcer activities of grape seed extracts (GSE-I and GSE-II) and procyanidins were investigated using rats. GSE-I (with low flavanol content), GSE-II (with high flavanol content), and procyanidins at a dose of 200 mg/kg strongly inhibited the stomach mucosal injury induced by 60% ethanol containing 150 mM hydrochloride. This suppressive effect seems dependent on the content of procyanidin oligomers. Procyanidin oligomers (dimers to hexamers) were prepared and studied for their antiulcer activities at a dose of 200 mg/kg. The gastric protective activity of a series of procyanidins increased with the increasing polymerization of catechin units. Oligomers longer than tetramers showed a strong protective effect against gastric mucosal damage. The binding ability of procyanidin oligomers to bovine serum albumin in the acidified solution was strengthened with the increase of molecules. The mechanism of antiulcer activity may be the protection by radical scavenging activity on the stomach surface against radical injury induced by HCl/EtOH solution and the defense action of procyanidins covering the stomach surface by their strong ability to bind protein.

Keywords: *Grape seed extract; antiulcer activity; procyanidin; oligomer; radical scavenging activity; HCl/EtOH-induced ulcer*

INTRODUCTION

Grape seeds are a rich source of catechins and procyanidins, and they are included in red wine and grape juice. There are a number of reports of these compounds showing a potent antioxidant activity (Ariga et al., 1988; Vinson et al., 1995; Teissedre et al., 1996) and free radical scavenging activity (Uchida et al., 1987; Ariga and Hamano, 1990; Ricardo da Silva et al., 1991; Frankel et al., 1993; Maffei Facino et al., 1994). It was also reported that procyanidins acted to inhibit platelet aggregation (Zafirov et al., 1990) and the growth of cariogenic *Streptococcus mutans* (Toukairin et al., 1991) and also showed activity as antioxidants of human low-density lipoprotein (Meyer et al., 1997), as antimutagenic (Liviero et al., 1994) and antiviral (Takechi et al., 1985) agents, and as inhibitors of some enzymes (Toukairin et al., 1991; Maffei Facino et al., 1994). Recognition of such health benefits of catechins and procyanidins has facilitated the use of grape seed extract as a dietary supplement.

Recognizing this, it is interesting to study other pharmacological applications of grape seed extract in vivo. Vennat et al. (1989) reported the antiulcer activity of the procyanidins prepared by fermentation of an extract of rhizomes of *Fragaria vesca* on a reserpine-induced ulcer model. However, those procyanidins consist mainly of dimers, and their composition is different from that of grape seed extracts, which are composed primarily of longer oligomers. This paper reports the effect of grape seed extracts on gastric mucosal lesion and the antiulcer activity of such extracts (*Vitis vinifera* L.) against stomach injury. It was found that this activity was due to the longer oligomers of procyanidin contained in these extracts.

MATERIALS AND METHODS

Materials. Ethanol (EtOH), hydrochloric acid, and $NaBH_4$ were purchased from Wako Pure Chemical Co. (Tokyo, Japan). (+)-Catechin, (±)-taxifolin,, and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). Pine bark extract (PBE) was obtained from Horphang Research (Geneva, Switzerland), and Sephadex LH-20 was from Pharmacia Biotech Co. Japan (Tokyo).

Grape Seed Extracts. Crude extracts (GSE-I: KPA) from grape seeds (*V. vinifera* L.) were obtained from Kikkoman Co. (Tokyo). GSE-I was extracted by 20% ethanol solution from the seeds. The preparation method for GSE-II (containing a high amount of procyanidins) was as follows. After whole grape seeds were washed with water at 60 °C for 2 h, the extraction was made with water at 90 °C for 2 h. The extract was evaporated to give GSE-II (Ariga et al., 1991).

The compositions of GSE-I, -II and PBE are shown in Table 1. The contents of total flavanols of GSE-I and GSE-II were about 40 and 81%, respectively. The content of procyanidins and monomeric flavanols was tested as follows. The amount of total flavanols was measured according to the vanillin method using (+)-catechin as a reference (Broadhurst and Jaues, 1978). The content of monomeric flavanols was obtained as the sum of each monomeric flavanol's amount such as (+)-catechin and epicatechin by an HPLC method using a Capcellpak C_{18} column (Shiseido Co., Japan) according to a method of Kitao et al. (1993). The content of procyanidins was calculated as the difference between total flavanols and monomeric flavanols.

Animals. Male Wistar/Crj rats (Charles River Japan, Atsugi) weighing 170−190 g were used in in vivo studies. The animals were fasted but were allowed free access to water for 24 h before the experiments.

Antiulcer Test. A gastric injury model based upon a modification of the method described by Mizui and Doteuchi (1983) was induced by acidified ethanol solution (150 mM HCl/absolute ethanol = 40:60 v/v, HCl/EtOH solution) in five rats per group. Test compounds basically dissolved in water were

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Table 1. Composition of GSE-I, GSE-II, and PBE

component	GSE-I (%)	GSE-II (%)	PBE (%)
total flavanols	40.9	81.3	53.9
procyanidins	38.5	73.4	50.7
monomeric flavanols	2.4	7.9	3.2
fat	0.0	0.0	–[a]
fiber	0.0	0.0	–
total sugars	19.3	1.7	–
glucose	7.8	0.2	–
sucrose	trace	1.1	–
fructose	8.9	0.2	–
total organic acids	16.7	6.4	–
citric acid	11.6	5.0	–
malic acid	trace	0.6	–
ash	5.0	3.4	–
moisture	2.5	3.5	–
protein	3.7	3.7	–

[a] Not measured.

administered orally at the dose of 200 mg/kg. Doses of glucose, fructose, and citric acid, which were main the components of GSE-I, were 15.6, 17.8, and 23.2 mg/kg, respectively, in proportion to 200 mg/kg of GSE-I. Thirty minutes later, 1.5 mL of HCl/EtOH solution was orally administered to the rat. After 1 h, the animals were killed by Nembutal anesthesia according to the schedule after the operation. The stomachs were removed and fixed by inflation with 8 mL of 10% formalin solution in phosphate-buffered saline. They were then incised along the greater curvature, and the length (millimeters) of each lesion formed on the glandular portion was measured under a dissecting microscope. The sum of the lengths of lesions in each animal was calculated.

Preparation of Procyanidin Oligomers. Procyanidin oligomers were prepared according to a slightly modified method of Eastmond or Ariga (Eastmond, 1974; Ariga, 1990). A mixture of (+)-catechin (5 g) and (±)-taxifolin (5 g) in the presence of $NaBH_4$ in ethanol was stirred at 40 °C. After 4.5 h, the reaction mixture was added to distilled water (800 mL) and adjusted to pH 5.1 with 5% acetic acid solution. The solution was extracted with ethyl acetate (700 mL), and the organic phase was collected, dried over Na_2SO_4, and evaporated in vacuo. The residue was dissolved in a small amount of ethanol. This solution was applied on a Sephadex LH-20 column chromatograph (200 mL), and procyanidin oligomers were eluted in the order of dimers, trimers, tetramers, pentamers, and hexamers with ethanol followed by methanol. The fraction containing each oligomer was collected and evaporated in vacuo. The final yields of procyanidin oligomers were as follows: dimers (mixed isomers of dimeric procyanidins: catechin−catechin), 1.1 g; trimers (catechin−catechin−catechin), 1.0 g; tetramers (catechin−catechin−catechin−catechin), 0.79 g; pentamers (catechin−catechin−catechin−catechin−catechin), 0.48 g; hexamers (catechin−catechin−catechin−catechin−catechin−catechin), 0.24 g (Table 2). The fraction of dimers was further purified by preparative HPLC on a μ-Bondapak C_{18} column (Waters Co., Japan) with aqueous methanol (5% methanol over 120 min) to obtain a dimer—procyanidin B-3. Their structures (Figure 1) were characterized by R_f values of TLC, mass spectra, nuclear magnetic resonance spectra, and ultraviolet spectra (Lea, 1978; Ariga et al., 1981, 1988; Ariga, 1981, 1990). The characterization of procyanidins was also done using acid hydrolysis according to the method of Thompson et al. (1972). The formation of (+)-catechin, cyanidin, and procyanidins was confirmed by hydrolysis using 5 N HCl solution. Each oligomer obtained showed one spot on TLC.

Measurement of Interaction of Procyanidins and BSA. Procyanidin (1 mg/mL) and BSA (10 mg/mL) were dissolved in acidified water (pH 2.0). Five microliters of procyanidin solution was added to 0.1 mL of BSA solution. After mixing for 30 min at 37 °C, the reaction was stopped by 0.3 mL of absolute ethanol. The mixture was then centrifuged for 15 min at 10000*g*, and procyanidin content in the supernatant was measured spectrophotometrically at a wavelength

Table 2. Characterization of Synthetic Procyanidin Oligomers

	Sephadex LH-20 chromatogr[a]	TLC R_f[b]	*m/z* ($[M + H]^+$) by SIMS[c]	products on acid hydrolysis[d]
(+)-catechin	1.00	0.75	291	(+)-catechin
dimers	1.63	0.60	579	(+)-catechin, cyanidin
trimers	2.63, 3.43	0.46	867	(+)-catechin, cyanidin, UPCs[e]
tetramers	4.66	0.36	1155	(+)-catechin, cyanidin, UPCs[e]
pentamers	5.25	0.29	1443	(+)-catechin, cyanidin, UPCs[e]
hexamers	6.60	0.24	1731	(+)-catechin, cyanidin, UPCs[e]

[a] Ratio of elution volume to that of (+)-catechin. [b] TLC plate: Merck 1.05715. Solvent: toluene/acetone/formic acid (3:6:1 v/v/v). [c] Mass spectra were measured on a Hitachi RMU-7M spectrometer. [d] Hydrolysis by 5 N HCl in ethanol. [e] UPC, unidentified procyanidin.

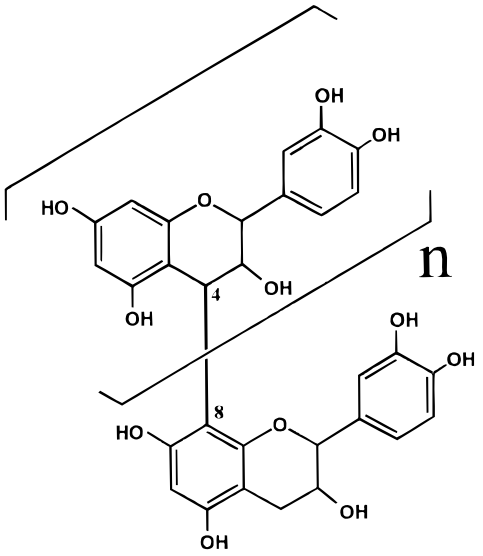

Figure 1. Representative structure of procyanidin oligomers.

of 280 nm. The interaction index was calculated using the expression

$$\text{interaction index} = [\text{UV-I}(\text{UV-II} \times \text{UV-III})]/\text{UV-I}$$

where UV-I is the UV absorption of samples with procyanidins alone, UV-II is the UV absorption of samples with procyanidins containing BSA, and UV-III is the UV absorption of samples with BSA alone.

Statistics. Results were expressed as the means ± SD. Statistical significance was determined by Student's *t* test.

RESULTS

Antiulcer Activity of GSEs and Catechin. The antiulcer activities of crude GSEs were tested. As shown in Figure 2, GSE-I showed potent gastric mucosal protective activity against stomach injury induced by HCl/EtOH solution. It reduced the lesion index by 82%. GSE-II, which contained more procyanidins than GSE-I, showed even more effective protection against stomach damage than did GSE-I. PBE, which was extracted from pine bark, showed relatively weaker activity than

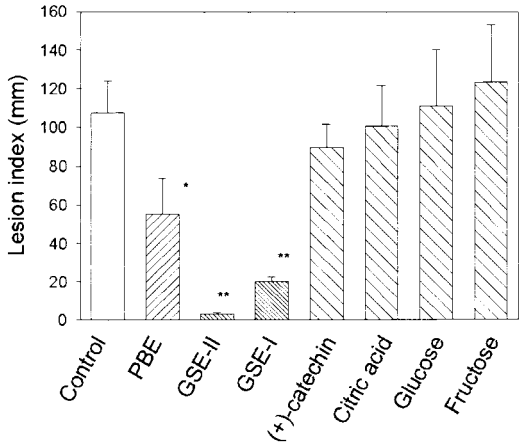

Figure 2. Protective effect of (+)-catechin, GSEs, PBE, and the component of GSE-I (glucose, fructose, and citric acid) against damage to the gastric mucosa by HCl/EtOH solution. The gastric lesion index is shown as the mean value ± SD. Asterisks indicate significant difference from control (*, $p < 0.05$; **, $p < 0.001$).

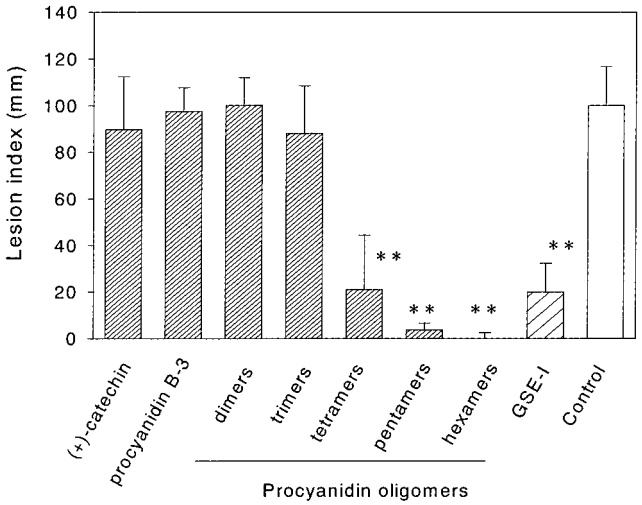

Figure 3. Protective effect of procyanidin oligomers against gastric injury induced by HCl/EtOH solution. The gastric lesion index is shown as the mean value ± SD. Each sample was given orally at a dose of 200 mg/kg. Asterisks indicate significant difference from control (**, $p < 0.001$).

grape seed extracts. Interestingly, (+)-catechin did not show antiulcer activity against HCl/EtOH-induced ulcer. Other main components of GSE-I, such as sugars (sucrose and fructose) and organic acid (citric acid), also did not show antiulcer activity when the same amount included in 200 mg/kg GSE-I was administered (Figure 2). These results suggested that oligomers and polymers of procyanidins might contribute to the mucosal protective activity of grape seed extracts.

Antiulcer Activity of Procyanidin Oligomers. Since the monomer component, (+)-catechin, did not show the antiulcer activity, the active compounds showing mucosal protective activity were thought to be procyanidin oligomers and polymers. Procyanidin oligomers from (+)-catechin and (±)-taxifolin were prepared using the method of Ariga (1990), and the effect on gastric injury induced by HCl/EtOH solution was tested.

As shown in Figure 3, (+)-catechin, procyanidin B-3, and dimeric and trimeric procyanidins showed no protective effect against stomach damage; however, longer oligomers such as tetrameric, pentameric, and hexameric procyanidins did show potent antiulcer activity. That is, this activity of a series of procyanidins increased with the increased degree of polymerization of the catechin unit.

Table 3. Interaction of Procyanidin Oligomers and BSA

oligomer	interaction index[a]	oligomer	interaction index[a]
(+)-catechin	−0.14 ± 0.18	tetramers	0.38 ± 0.11
procyanidin B-3	0.01 ± 0.06	pentamers	1.02 ± 0.03
dimers	0.06 ± 0.02	hexamers	1.05 ± 0.03
trimers	0.24 ± 0.03		

[a]When the interaction index is near zero, procyanidins did not bind to BSA in this experiment. Note: Values are mean ± SD from two independent experiments with duplicate assays.

Binding to BSA. Procyanidins are generally known to bind and precipitate several proteins such as those contained in brewed beer and hemoglobin (Bate-Smith, 1973; Okuda et al., 1985). To clarify the mechanism of antiulcer activity of procyanidin oligomers, the ability of procyanidins to bind BSA was tested.

As shown in Table 3, (+)-catechin and dimeric procyanidins bound only slightly to BSA. However, procyanidin oligomers showed increased interaction index as their molecular weight increased. Procyanidin pentamers and hexamers strongly bound to BSA.

DISCUSSION

Catechin and procyanidin dimers and trimers extracted from grape seeds have recently been shown to possess significant antioxidant activity toward low-density lipoprotein oxidation in vitro (Teissedre et al., 1996). The antioxidant activity of wine is thought to be a major factor in preventing coronary heart disease (Frankel et al., 1993). GSEs containing a large quantity of procyanidins have been focused on as dietary supplements for their health benefits.

In the present study, GSEs of *V. vinifera* at a dose of 200 mg/kg prevented the acute macroscopic lesions in rat gastric mucous induced by oral administration of 60% ethanol solution containing 150 mM HCl. Other main components of GSE-I, such as sugars and organic acid, did not show antiulcer activity when the same amount included in 200 mg/kg GSE-I was administered. (+)-Catechin, a representative monomeric flavanol, did not inhibit ulcer formation by HCl/EtOH solution. The antiulcer activity of GSEs increased with increasing content of procyanidins. Interestingly, despite a high content of procyanidins in PBE, the antiulcer activity was somewhat weaker than that of GSEs at the same dose.

The protective effect of GSEs against gastric mucosal injury was shown to be due to procyanidin oligomers. As shown in Figure 3, the antiulcer activity of a series of procyanidins increased with increasing degree of polymerization of catechin unit. Oligomers longer than three catechin units showed strong protective activity against stomach mucosal injury. The protective effect of GSEs against gastric mucosal injury was, therefore, found to be due to longer molecular procyanidin oligomers contained in GSEs. We found that procyanidins covered the surface of the stomach when the stomach was incised. Therefore, the binding ability of procyanidins to protein was investigated using BSA. Longer procyanidins such as pentamers and hexamers strongly bound to BSA (Table 3). These results support the antiulcer activity of longer oligomers. The lower activity

of PBE may be related to the lower content of longer oligomers compared with that in GSEs.

Though the exact mechanism of pathogenesis by HCl/ethanol is not yet known, recent studies have demonstrated that active oxygen species might be involved in the formation of gastric mucosal damage (Itoh and Guth 1985; Pihan et al., 1987; Smith et al., 1987; Yoshikawa et al., 1990; Matsumoto et al., 1993). Matsumoto et al. reported that superoxide dismutase, catalase, and their combination reduced gastric lesion formation induced by HCl/EtOH solution in mice, while Pihan and his colleagues reported that free radicals were involved in the pathogenesis of acute gastric mucosal injury caused by ethanol. They suggested that oxygen free radicals might contribute to the gastric mucosal lesions formed by HCl/EtOH solution. Ariga and Yuasa (1994) reported that the longer procyanidin oligomers showed potent antioxidant activity in the assay carried out by measuring the β-carotene bleaching rate in the coupled oxidation of linoleic acid and β-carotene in an aqueous solution. Procyanidins, especially longer oligomers, are potent antioxidants (Ariga and Yuasa, 1995). It seems that procyanidins inhibit mucosal injury by trapping and/or quenching of oxygen-derived free radical or radical peroxide via radical scavenging effect of procyanidins on gastric mucous.

In this study, GSEs exhibit a protective effect on acute gastric lesions in rats. This activity may be due to longer procyanidins. Though their antiulcer activity seems to involve their binding ability to protein and their antioxidant activity, the details of the mechanism are not yet well-defined. Another mechanism may be explained in a part by the defense action of procyanidins on the stomach surface due to their strong binding ability to protein. Thus, more research is needed to determine the antiulcer mechanisms of procyanidins against stomach mucosa injury induced by HCl/EtOH solution.

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