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Procyanidin-Rich Extract from Grape Seeds Prevents Cataract Formation in Hereditary Cataractous (ICR/f) Rats

JUN YAMAKOSHI,* MAKOTO SAITO, SHIGEHIRO KATAOKA, AND SHOICHI TOKUTAKE

Research and Development Division, Kikkoman Corporation, 399 Noda, Noda City, Chiba 278-0037, Japan

Antioxidants such as vitamin C, vitamin E, and carotenoids have been reported to prevent the progression of experimentally induced cataracts. However, little is known of the effect of procyanidins, a powerful antioxidant, on cataract formation. This paper investigates the anticataract activity of grape seed extract (GSE, which contains 38.5% procyanidins) in hereditary cataractous rats (ICR/f rats). The ICR/f rats were fed a standard diet containing 0 or 0.213% GSE [0.082% procyanidins in the diet (w/w)] for 27 days. The GSE significantly prevented and postponed development of cataract formation by evaluation of slit lamp observations of the rats' eyes. Lens weight and malondialdehyde concentration in the lens and plasma cholesteryl ester hydroperoxide (ChE-OOH) level induced by $CuSO_4$ were significantly lower in the GSE group compared with the control group. The rats were also fed for 14 days either the diet containing 0.085% procyanidin dimer to tetramer fraction (0.085% as the procyanidins), the diet containing 0.090% procyanidin pentamer to heptamer fraction (0.085% as the procyanidins), or the diet containing 0.093% procyanidin oligomers more than decamer fraction (0.085% as the procyanidins). The ChE-OOH levels in the procyanidin pentamer to heptamer and procyanidin oligomers more than decamer groups were significantly lower than in the procyanidin dimer to tetramer group. These results suggested that procyanidins and their antioxidative metabolites prevented the progression of cataract formation by their antioxidative action. The larger molecular procyanidins in the GSE might contribute this anticataract activity.

KEYWORDS: Procyanidin; grape seed extract; antioxidant; cataract; ICR/f rat

INTRODUCTION

Cataract formation is one of the principal causes of blindness in world populations, and oxidative stress has been suggested to be a common mechanism of cataractogenesis (*1*, *2*). Dietary antioxidants such as vitamin C, vitamin E, and carotenoids may prevent the progression of cataract formation in humans (*3*−*5*). Anticataract activity of these antioxidants has been demonstrated in several studies of experimentally induced cataracts (*6*−*10*). Epidemiological studies in human populations have in general supported the hypothesis that improvement in dietary antioxidant content such as vitamin C, vitamin E, and carotenoids may lower the incidence of cataracts (*11*−*13*).

Proanthocyanidins, which are oligomers and polymers of polyhydroxy flavan-3-ol units, such as (+)-catechin and (−)-epicatechin (*14*), are present in their largest amounts in polyphenols of red wine and grape seeds (*15*, *16*). In grape seeds, only the procyanidin type of proanthocyanidins has been detected (*17*). A few monomeric flavanols have been also detected, but other flavonoid compounds such as anthocyanins and flavonols are not contained in the seeds (*15*). Prieur et al. found that 55% of the procyanidin extracted from grape seeds consisted of more than five monomer units and determined that their mean degree of polymerization ranged from 2.3 to 15.1 (by thiolysis) and from 2.4 to 16.7 (by gel permeation chromatography) (*18*). Procyanidins have been reported that have strong antioxidative and free radical scavenging activities in vitro (*19*−*24*) and have antioxidative activity in vivo (*25*). Therefore, the dietary procyanidins may retard the progression of cataract formation. Procyanidins have been also reported to have a variety of biological activities, including antiatherosclerotic (*26*), anticarcinogenic (*27*, *28*), and antiulcer activities (*29*). Procyanidins have been reported to improve human intestinal microflora (*30*) and have been also reported to have antidiabetic and anticataract activities in diabetic rats (*31*). Nguyen et al. reported that the ingestion of procyanidin-rich grape seed extract [0.5% in diet (w/w)] produced a reduction in blood glucose and glycohemoglobin A_{1c} levels and postponed development of cataract formation (*31*). However, the anticataract activity of procyanidins in hereditary cataract (ICR/f) rats has not been investigated.

On the other hand, the ICR/f rat strain develops hereditary cataracts that appear around 75 days of age (*32*, *33*). Yagi et al. showed that lipid peroxide increased in the serum, and

* Corresponding author (telephone +81-4-7123-5546; fax +81-4-7123-5550; e-mail 7161@mail.kikkoman.co.jp).

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vacuoles appeared in the posterior region of the lens by electron microscopic observation at around day 13 (*34*). They described an increase of lipid peroxide content in the liver, and its circulation into the lens might be responsible for initiating this damage (*34*). Therefore, the increase of lipid peroxides in the liver, serum, and lens may be a direct cause of cataractogenesis in ICR/f rat.

In this study, we investigated the effect of procyanidin-rich extract from grape seeds on cataract formation in the ICR/f rats.

MATERIALS AND METHODS

Grape Seed Extract (GSE). The GSE (Gravinol) was provided by Kikkoman Corp. (Noda City, Chiba, Japan). Briefly, the grape seeds were washed with water and then extracted with water and ethanol under reflux overnight. The extract was condensed to remove solvents, then the concentrate was filtered, and it was spray-dried to obtain a powder of a procyanidin-rich extract. The extract was composed of 38.5% procyanidins, 2.4% monomeric flavanols, 8.9% fructose, 7.8% glucose, 11.6% citric acid, 5% ash, 2.5% moisture, and 3.7% protein (*29*). The rest of the components were organic acids except for citric acid, unidentified sugars, and others. The extract did not contain resveratrol or other phenolic compounds such as anthocyanidins and flavonols.

Isolation of Procyanidin Dimer to Tetramer, Procyanidin Pentamer to Heptamer, and Procyanidin Oligomers More than Decamer. The procyanidin fractions were isolated with the method described elsewhere (*30*, *35*). GSE (31.0 g) containing 89.3% procyanidins was chromatographed on a Sephadex LH-20 column (∅ 4.0 × 55.0 cm; Pharmacia Biotech Co., Uppsala, Sweden) with acetone and ethanol to separate two fractions corresponding to procyanidin dimer to tetramer and procyanidin pentamer to heptamer, and each fraction was freeze-dried; 7.87 g of powder containing procyanidin dimer to tetramer and 4.99 g of powder containing procyanidin pentamer to heptamer were obtained. Each fraction was analyzed by thin-layer chromatography (TLC) (silica gel 60 TLC, 0.25 mm thickness; Merck, Darmstadt, Germany) developed with toluene/acetone/formic acid (3:6:1, v/v/v) and ^{13}C NMR spectroscopy using a Bruker Digital NMR Avance 500 operated at 125 MHz (*36*). We confirmed that one fraction was procyanidin dimer, procyanidin trimer, and procyanidin tetramer, and the other fraction was procyanidin pentamer, procyanidin hexamer, and procyanidin heptamer by TLC and ^{13}C NMR spectroscopy analyses. Although each fraction was a mixture of isomeric flavanols, the fraction of procyanidin dimer to tetramer contained 100% total flavanols, and the fraction of procyanidin pentamer to heptamer contained 94.4% total flavanols by analysis according to the vanillin−HCl method (*37*). Procyanidin oligomers more than decamer were also produced by the GSE containing 89.3% procyanidins followed by separation by regenerated cellulose (Millipore, Bedford, MA). A total of 91.9 g of powder of procyanidin oligomers more than decamer was obtained from 120.0 g of GSE. The fraction was also analyzed by TLC and ^{13}C NMR spectroscopy, and we confirmed that the fraction was procyanidin oligomers more than decamer and contained 92.0% total flavanols by analysis according to the vanillin−HCl method.

Animals and Diets. Thirty-nine male ICR/f rats (Kiwa Experimental Animal Co., Ltd., Wakayama, Japan), weighing 183.1 ± 18.9 (6 weeks old) were used. All rats were kept in an air-conditioned room (23 ± 1 °C, 55 ± 5% humidity) under a 12-h dark/light cycle and were allowed free access to food and water for 1 week to adapt to the new environment. A control group of seven rats was fed a standard diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) for 27 days. The GSE group of six rats was fed the diet containing 0.213% GSE (0.082% as procyanidins) for 27 days. In another study, a control group of five rats was fed the standard diet for 14 days. Seven rats of the procyanidin dimer to tetramer group, procyanidin pentamer to heptamer group, and procyanidin oligomers more than decamer group were fed the diet containing 0.085% procyanidin dimer to tetramer fraction (0.085% as the procyanidins), 0.090% procyanidin pentamer to heptamer fraction (0.085% as the procyanidins), and 0.093% procyanidin oligomers more than decamer fraction (0.085% as the procyanidins) for 14 days, respectively. During the experimental periods, food consumption and body weight were recorded. At the end of experimental periods, blood of all rats was drawn from the abdominal aorta into heparinized tubes under anesthesia of sodium phenobarbital, and the rats were killed with an overdose of sodium phenobarbital. During the experiment, the rats received humane care consistent with institutional guidelines. Plasma was separated by centrifugation at 1000*g* for 10 min at 4 °C and was stored at −80 °C. The lenses and livers of rats of the control and GSE groups were immediately removed and weighed and were stored at −80 °C.

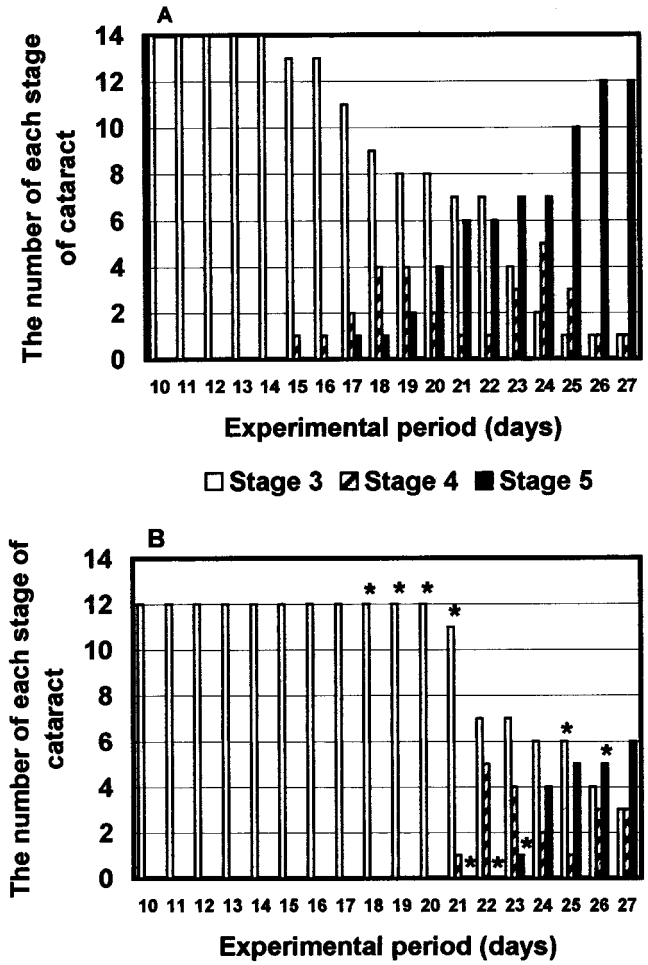

Figure 1. Number of each stage of cataract formation in ICR/f rats fed the standard diet (A, control group) or the diet containing 0.213% GSE (B, GSE group) for 27 days. *, significantly different from the control group, $P < 0.05$.

Cataract Classification. The rats of the control and GSE groups were dilated by topical administration of 0.5% tropicamide, and lens opacity was observed with a slit-lamp microscope FM-3 (Nikon, Tokyo, Japan) and documented by photography with a camera attached to the microscope under anesthesia of sodium phenobarbital. Cataracts were classified as previously described (*33*): stage 3, in addition to a little anterior superficial cortical opacities, a separate opacity was detected in the posterior subcapsular lens cortex (**Figure 2**); stage 4, the opacity in the posterior subcapsular cortex progressed into the deeper posterior lens cortex, and the lens opacity became clearly recognizable with the naked eye, without a slit-lamp microscope (**Figure 2**); stage 5, the opacity extended toward the anterior and posterior lens cortices, lenticular nucleus, and posterior perinuclear cortex, but the anterior deep cortex remained more or less unaffected by the opacity (**Figure 2**). Nishida et al. classified six stages (stages 0−5) according to development of the cataract (*33*). In our experiment, all rats were already stage 3 at the start of experimental period (**Figure 2**).

Oxidation of Rat Plasma and Measurement of Cholesteryl Ester Hydroperoxide (ChE-OOH). The plasma was oxidized by $CuSO_4$,

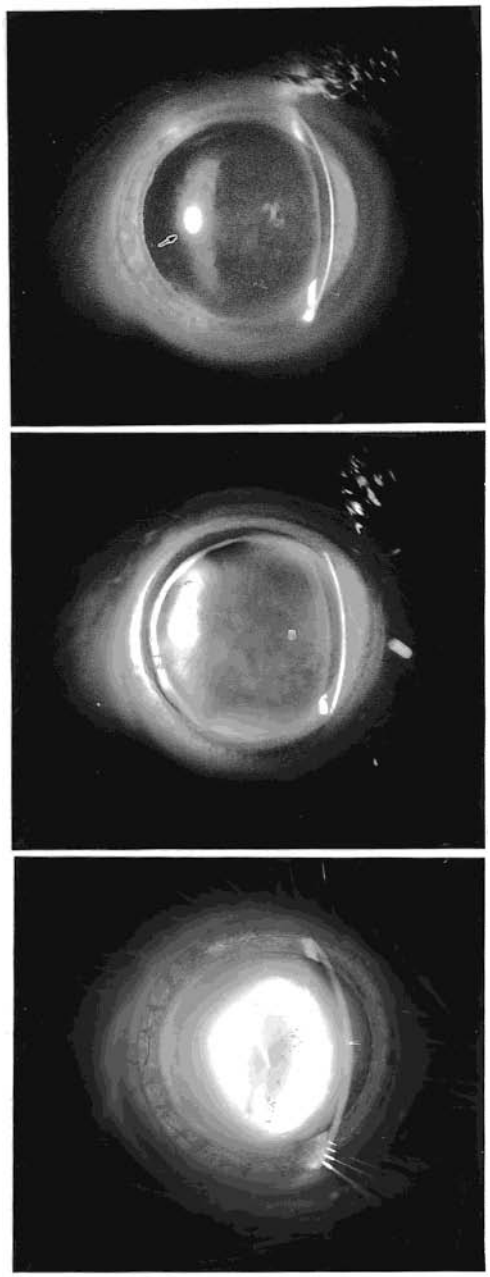

Figure 2. (Top) Stage 3 of cataract formation of an ICR/f rat at the start of the experimental period. Opacity is observed in the posterior subcapsular lens cortex (arrow) by the slit lamp microscope. (Middle) Stage 4 of cataract formation of an ICR/f rat of the GSE group at day 27. Opacity in the posterior subcapsular cortex progresses into the deeper posterior lens cortex, whereas the development of cataract is prevented in the rat compared with an ICR/f rat of the control group at day 27. (Bottom) Stage 5 of cataract formation of an ICR/f rat of the control group at day 27. Opacity extends toward anterior and posterior lens cortices, lenticular nucleus, and posterior perinuclear cortex.

and ChE-OOH in the plasma was measured according to a method described in detail elsewhere (*25*, *26*, *38*). Briefly, $CuSO_4$ was added to plasma in phosphate-buffered saline (PBS; 1:1, v/v). The final concentration of $CuSO_4$ in the reaction mixtures was 500 μmol/L. The reaction mixture was incubated at 37 °C for 4 h, and ChE-OOH was measured by HPLC using a TSK gel Octyl-80 Ts column (∅ 4.6 × 100 mm; Tosoh, Tokyo, Japan). The ChE-OOH concentration was calculated from the standard curve of the hydroperoxy derivative of cholesteryl linoleate (*39*).

Malondialdehyde (MDA) Contents in the Lens and Liver. The lenses and livers of 13 rats of the control and GSE groups were added to 10 volumes (w/v) of cold 0.1 M PBS (pH 7.0) and homogenized. The homogenates were used for the determination of MDA (*40*). MDA was determined by a commercially available colorimetric reagent system (LPO-586 from Bioxytech, OXIS International, Portland, OR).

Table 1. Oxidant and Antioxidant Status in ICR/f Rats Fed the Standard Diet or the Diet Containing Grape Seed Extract (GSE) for 27 Days[a]

	experimental group	
	control	GSE
lens		
MDA, nmol/g of wet tissue	10.5 ± 1.8	7.8 ± 1.4*
GSH, μmol/g of wet tissue	2.0 ± 0.2	2.3 ± 0.4
liver		
MDA, nmol/g of wet tissue	7.1 ± 1.3	6.6 ± 1.2
copper ion-induced plasma oxidation		
ChE-OOH, μmol/L	4.13 ± 0.64	2.99 ± 0.56**

[a] Values are means ± SD, $n = 7$ (control group) or 6. *, significantly different from control group, $P < 0.05$; **, significantly different from control group, $P < 0.01$.

Reduced Glutathione (GSH) Content in the Lens. The lenses of 13 rats of the control and GSE groups were homogenized in 20 volumes of 5% metaphosphoric acid in 0.1 M PBS (pH 7.0). The homogenate was centrifuged at 3000*g* for 10 min at 4 °C, and the supernatant was used for determination of GSH. GSH was determined by a colorimetric reagent system (GSH-400 from Bioxytech, OXIS International).

Statistical Analyses. Student's *t* and Fisher tests were used to evaluate differences between the control and GSE groups. One-way ANOVA followed by Tukey's significant difference test was also used to evaluate differences in the groups: control group, procyanidin dimer to tetramer group, procyanidin pentamer to heptamer group and procyanidin oligomers more than decamer group.

RESULTS

Anticataract Activity of GSE. There were no differences in body weight (bw) among the groups. Mean body weight of the control group was 226.9 ± 10.9 g and that of the GSE group was 232.6 ± 10.3 g. The GSE intake was 165.1 ± 5.4 mg/(kg of bw·day), and the procyanidin intake was 63.6 ± 2.1 mg/(kg of bw·day). The number of each stage of cataract formation in ICR/f rats fed the standard diet (control group) or the diet containing 0.213% GSE (GSE group) for 27 days is presented in **Figure 1**. All rats of both groups were already at stage 3 at the start of the experimental period (**Figures 1** and **2**). Each stage of cataract formation of the GSE group was significantly postponed than that of the control group throughout days 18−26 of the experimental period (**Figure 1**). The mean values of the stages were significantly lower in the GSE group than in the control group throughout days 18−27 ($P < 0.05$). At the end of the experimental period (day 27), the control group showed a mean stage value of 4.79 ± 0.58, whereas a stage value of 4.25 ± 0.87 was seen in the GSE group ($P < 0.05$). The rat's lens of the control and GSE groups at day 27 by slit-lamp observations is shown in **Figure 2**. Mean lens wet weight in the GSE group (38.3 ± 6.3 mg) was significantly lower than in the control group (42.5 ± 1.4 mg) ($P < 0.05$).

Antioxidant Status of the ICR/f Rats Fed GSE. Oxidant and antioxidant statuses of ICR/f rats fed the standard diet or the diet containing 0.213% GSE for 27 days are shown in **Table 1**. MDA concentration in the lenses was significantly lower in the GSE group than in the control group (**Table 1**). The GSH level in the lenses tended to be higher in the GSE group than in the control group, but the difference was not significant ($P = 0.186$) (**Table 1**). MDA concentration in the livers of the GSE group did not differ from that of the control group (**Table**

Table 2. Copper Ion-Induced Plasma Oxidation in ICR/f Rats Fed the Standard Diet, the Diet Containing Procyanidin Dimer to Tetramer Fraction, the Diet Containing Procyanidin Pentamer to Heptamer Fraction, or the Diet Containing Procyanidin Oligomers More than Decamer Fraction for 14 Days[a]

	experimental group			
	control	procyanidin dimer to tetramer	procyanidin pentamer to heptamer	procyanidin oligomers more than decamer
ChE-OOH, μmol/L	3.70 ± 0.77b	2.35 ± 0.90ab	0.70 ± 0.66a	1.23 ± 0.25a

[a] Values are means ± SD, $n = 5$ (control group) or 7. Values without a common letter differ, $P < 0.01$.

1). The plasma ChE-OOH level in the GSE group was significantly lower than that of the control group ($P < 0.01$, **Table 1**).

Plasma ChE-OOH Level of ICR/f Rats Fed Procyanidins. There were no differences in body weight among the groups (date not shown). Although the plasma ChE-OOH levels induced by $CuSO_4$ in the procyanidin groups were significantly lower than in the control group (procyanidin dimer to tetramer group vs control group, $P < 0.05$; procyanidin pentamer to heptamer group vs control group, $P < 0.01$; and procyanidin oligomers more than decamer group vs control group, $P < 0.01$), the levels in the procyanidin pentamer to heptamer group and procyanidin oligomers more than decamer group were significantly lower than in the procyanidin dimer to tetramer group ($P < 0.05$) (**Table 2**).

DISCUSSION

In this study, we showed that a procyanidin-rich extract from grape seeds prevented and postponed development of cataract formation in ICR/f rats. The GSE used our study contained 38.5% procyanidins and 2.4% monomeric flavanols such as (+)-catechin. We also examined the anticataract activity of GSE containing 73.4% procyanidins and (+)-catechin using ICR/f rats. Furthermore, these extracts did not contain other potent antioxidants, resveratrol, and other phenolic compounds such as anthocyanidins and flavonols. The anticataract activity of the GSE [0.0734% proanthocyanidins in the diet (w/w)] was almost the same as that of GSE containing 38.5% proanthocyanidins [0.082% proanthocyanidins in the diet (w/w)], but the activity of (+)-catechin [1 or 0.1% (+)-catechin in the diet (w/w)] did not show for 4 weeks of experimental period (data not shown). These results suggested that the anticataract activity of GSE was due mainly to procyanidins.

Feeding the GSE to ICR/f rats decreased plasma ChE-OOH levels induced by $CuSO_4$. The feeding also inhibited the increase of lens weight, the MDA concentration in the lenses, and the decrease of GSH (a major intracellular antioxidant) level in the lenses. In ICR rats, Yagi et al. reported that liver lipid peroxide levels of the rats impermanently increased on days 9−11 after birth, and serum lipid peroxide levels markedly increased on day 13 and then decreased, reaching the original levels on day 17 (*34*). In accordance with the increase in serum lipid peroxide levels, incipient morphological changes in the lens were observed. Thereafter, lipid peroxide levels in the lens and lens wet weight increased, and the GSH level in the lens decreased and the opacification of the lens occurred (*33*, *41*). Yagi et al. suggested that the increase of lipid peroxides in the liver, serum, and lens might be a direct cause of cataractogenesis in the rat (*34*). Our results suggested that procyanidins and their antioxidative metabolites prevented the progression of cataract by their antioxidative action. Seven-week-old ICR/f rats used in our study were already at stage 3 of cataract formation at the start of the experiment. MDA concentration in the livers did not differ in the GSE and control groups at the end of experimental period. In ICR/f rats, it was reported that the polyol pathway, which has an important role in the pathogenesis of sugar cataract and enhanced the pathway that leads to damage of the lens, had no relationship to cataract formation in the lens (*42*) and blood glucose was at a normal level (*34*). The anticataract activity of procyanidins might be due to their antioxidative action in the lens related with an increase of antioxidative potential of the plasma of the rats.

When procyanidin-rich GSE was administered orally to rats, procyanidins and their metabolites such as (+)-catechin and (−)-epicatechin were detected in the plasma (*25*, *26*). In an in vitro study using Caco-2 cells, (+)-catechin, procyanidin dimer B3, and trimer C3 were absorbed to a similar extent (*43*). Exposure of procyanidin trimer to hexamer to gastric juice has been reported to result in a rapid cleavage/decomposition of the procyanidins to mixtures of lower oligomers and (−)-epicatechin (*44*). High amounts of (−)-epicatechin were detected on the serosal side of the rat small intestine after perfusion with procyanidin dimers B2 and B5, suggesting that (−)-epicatechin released from procyanidins could penetrate through the portal vein and become conjugated with glucuronides and/or sulfates in the liver (*45*). These studies indicated that some procyanidin oligomers and/or catabolites of procyanidins such as procyanidin dimers and trimer might be absorbed as well as catabolites of procyanidins such as (+)-catechin and (−)-epicatechin and that they might play a role in the prevention of the cataract.

Although the plasma ChE-OOH levels induced by $CuSO_4$ in procyanidin groups were lower than in the control group, the levels in the procyanidin pentamer to heptamer and procyanidin oligomers more than decamer groups were significantly lower than in the procyanidin dimer to tetramer group (**Table 2**). These results suggested that the larger molecular procyanidins in the GSE might contribute the anticataract activity. Two mechanisms may be proposed to account for the effect of larger molecular procyanidins. First, the larger molecular procyanidins such as procyanidin pentamer to heptamer might yield to stronger antioxidative catabolites of the procyanidins compared with smaller molecular procyanidins such as procyanidin dimer to tetramer in digestive track, and the antioxidative catabolites might be absorbed and might contribute the increase of resistance of plasma against oxidative stress. Second, some unchanged and/or only decomposed larger molecular procyanidins originated from the larger molecular procyanidins in the digestive track might have stronger hypocholesterolemic activity compared with the smaller molecular procyanidins, and this activity might relate the phenomenon described above. Tebib et al. reported that plasma total cholesterol, LDL cholesterol, VLDL, and MDA concentrations were significantly lower, and fecal cholesterol and bile acid excretions were significantly higher in rats fed procyanidin dimer to tetramer than in rats fed tannin monomers (*46*, *47*). They proposed that the anti-hypercholesterolemic effect by the procyanidins was due to

enhancement of reverse cholesterol transport, reduction of intestinal cholesterol absorption, and increase of bile acid excretion, and especially the level of bile acid excretion was dependent on the degree of polymerization of procyanidins (*46*). Therefore, in our study, larger molecular procyanidins might contribute to a reduction of lipoproteins such as LDL and VLDL containing cholesteryl linoleate and, finally, might reduce the ChE-OOH levels in plasma. Further investigation is necessary for roles of the larger molecular procyanidins in preventing cataracts.

Our results indicate that procyanidin-rich grape seed extract may contribute to the prevention of cataracts associated with oxidative stress.

ABBREVIATIONS USED

ChE-OOH, cholesteryl ester hydroperoxides; GSH, reduced glutathione; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; PBS, phosphate-buffered saline.

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