# Antioxidative Properties of Phenolic Antioxidants Isolated from Corn Steep Liquor

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With the immersion of corn into dilute sulfur oxide during starch-manufacturing processes, corn steep liquor (CSL) remains as leftover material. CSL is often used for fermentation, but its components are not fully understood. To determine the properties of CSL, 12 *p*-coumaric acid-related compounds were isolated from an ethyl acetate extract of CSL with the guidance of antioxidative activity on the rabbit erythrocyte membrane ghost system. The activity of these compounds was compared against oxidative damages, and it was elucidated that the activity of *p*-coumaric acid derivatives was mainly affected by their functional groups at the 3-position and less by the conjugated side chain. Moreover, *p*-coumaric acid derivatives exhibited inhibitory activity stronger than that of tocopherols and ascorbic acid on peroxynitrite-mediated lipoprotein nitration. These findings that *p*-coumaric acid derivatives, which might play a beneficial role against oxidative damage, exist in CSL suggest this byproduct might be a useful resource of phenolic antioxidants.

**Keywords:** *Phenolic antioxidants; corn steep liquor; low-density lipoprotein; peroxynitrite; xanthine oxidase*

## INTRODUCTION

The steeping process is the first step in starch manufacture. It is very effective for softening the corn kernel and facilitating the separation of germ, hull, fiber, and gluten from starch granules. Corn steep liquor (CSL), which is often used as a nitrogen source of fermentation, is produced through this process. However, the chemical identification of components in the exudate has scarcely been mentioned in previous research. In recent studies, much attention has been focused on oxidative stress from the viewpoint of its participation in several diseases such as atherosclerosis (Palinski et al., 1989), cancer (Ames et al., 1993), and aging (Carney et al., 1991). Some edible plants contain certain amounts of antioxidants, and they are expected to prevent these oxidative stresses (Lin et al., 1996; Jang et al., 1997; Ohta et al., 1997). Also, new antioxidants have been isolated from a wide variety of fields (Lo et al., 1994; Murakami et al., 1996; Abe et al., 1998). We searched for antioxidants from CSL with the guidance of the inhibitory activity against the oxidation of rabbit ghost membrane by *tert*-butyl hydroperoxide. Finally, we isolated 12 phenolic compounds, of which 11 antioxidants were *p*-coumaric acid derivatives, and we then evaluated some structure−activity relationships of the functional groups at the 3-position and side chains of *p*-coumaric acid derivatives.

Peroxynitrite ($ONOO^-$), generated from nitric oxide and superoxide anion radical at near-diffusion rate (Huie and Padmaja, 1993; Goldstein and Czapski, 1995), holds interest as a strong plausible oxidant in vivo, and its marker, 3-nitrotyrosine, has been detected in several lesions of some (model) diseases (Beckmann et al., 1994; Kaur and Halliwell, 1994; Matthews and Beal, 1996; Leeuwenburgh et al., 1997). Thus, we have also evaluated the activity of isolated compounds in preventing the protein nitration resulting from this powerful oxidant.

In this paper, the expression of "*trans*" with respect to *p*-coumaric acid derivatives was omitted. We have also noted the hydrogenated compounds such as 3-(4-hydroxyphenyl)propionic acid as *p*-coumaric acid/$H_2$, for the structure−activity relationships.

## MATERIALS AND METHODS

**Chemicals.** *p*-Coumaric acid, caffeic acid, and vanillic acid were purchased from Nacalai Tesque Inc., Ltd. (Kyoto, Japan). L-Ascorbic acid, cinnamic acid, and butylated hydroxyanisole (BHA) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Ferulic acid was obtained from Tokyo Kasei Organic Chemicals Co., Ltd. (Tokyo, Japan). Sinapinic acid and allopurinol were products of Aldrich Chemical Co., Inc. (Milwaukee, WI). *dl*-α-Tocopherol, *d*-γ-tocopherol, *dl*-Trolox, hypoxanthine, and xanthine oxidase (X-4376) were obtained from Sigma Chemical Co. (St. Louis, MO). β-Carotene and *tert*-butyl hydroperoxide were purchased from Merck (Darmstadt, Germany). The highest quality agents available were obtained in all cases.

Peroxynitrite was synthesized (Hughes and Nicklin, 1970) and quantified prior to use as described previously (Hughes and Nicklin, 1968). Briefly, an acidic solution (10 mL, 0.6 M HCl) of $H_2O_2$ (0.7 M) was mixed vigorously with $NaNO_2$ (10 mL, 0.6 M) in an ice-cold bath, and the reaction was immediately quenched with ice-cold NaOH (20 mL, 1.5 M). The solution was then frozen for several hours, and the yellowish top layer was collected.

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**Instruments.** Preparative high-performance liquid chromatography (HPLC) was done with a Wakosil-II 5C18HG column (i.d. 20 × 250 mm; Wako Pure Chemical) with a flow rate of 5.0 mL/min at ambient temperature. $^1$H NMR spectrum was obtained on a JNM GSX-270 (JEOL, Tokyo, Japan). All chemical shifts are reported as $\delta$ values (parts per million) relative to the incomplete signal of the deuterated acetone (2.05 ppm). EI-MS spectrum was measured on a JMS DX-705L (JEOL).

**Antioxidant Purification from CSL.** CSL (500 g), obtained from our plant (Aichi, Japan), was diluted with 500 mL of saturated brine and extracted with 500 mL of ethyl acetate (EtOAc) by centrifugation (3500 rpm, 15 min) in a Teflon vessel, three times. The EtOAc solution was re-extracted with saturated $NaHCO_3$ solution (300 mL × 3), and then the alkaline aqueous phase was acidified by aqueous HCl and extracted with EtOAc (500 mL × 3). The organic phase was washed, dehydrated over anhydrous $Na_2SO_4$, and evaporated in vacuo. This acid fraction (1.9 g) was subjected to a silica gel column chromatography eluted with *n*-hexane/EtOAc (1:2) to get four fractions (A1−A4). Further purification of A2 was accomplished with preparative HPLC equipped with an UV detector at wavelength 254 nm using a solvent mixture of $H_2O$/MeOH/trifluoroacetic acid (TFA) (700:300:1) and gave seven fractions (A21−A27). A23 and A25 were further resolved by preparative HPLC eluted with $H_2O$/acetonitrile/TFA (800:200:1) at a wavelength of 210 nm. Using this method, we obtained caffeic acid (**4**; 4.3 mg) and vanillic acid (**1**; 14.6 mg) from A23 and 3-(4-hydroxyphenyl)propionic acid (*p*-coumaric acid/$H_2$) (**8**; 6.1 mg) and 3-(4-hydroxy-3-methoxyphenyl)-propionic acid (ferulic acid/$H_2$) (**9**; 1.4 mg) from A25 as active compounds. Purification of A27 on preparative HPLC with a solvent mixture of $H_2O$/MeOH/TFA (500:500:1) gave eight fractions (A271−A278). A271, A272, and A273 were subjected to reverse-phase chromatography eluted with $H_2O$/MeOH/TFA (600:400:1) to get *p*-coumaric acid (**5**; 4.1 mg), ferulic acid (**6**; 11.6 mg), *cis*-*p*-coumaric acid (**2**; 0.6 mg), and *cis*-ferulic acid (**3**; 3.3 mg). A3 was purified by preparative HPLC monitoring at UV 210 nm, and we obtained 3-(3,5-dimethoxy-4-hydroxyphenyl)propionic acid (sinapinic acid/$H_2$) (**10**; 2.0 mg), sinapinic acid (**7**; 6.5 mg), 3-(3,5-dimethoxy-4-hydroxyphenyl)-propionic acid methyl ester (sinapinic acid/$H_2$ methyl ester) (**11**; 1.4 mg), and 3-(3,5-dimethoxy-4-hydroxyphenyl)propionic acid ethyl ester (sinapinic acid/$H_2$ ethyl ester) (**12**; 0.9 mg).

The structures of active compounds were deduced from their spectral data as well as from synthesis as described below, and 12 compounds were determined.

**Modification of *p*-Coumaric Acid Derivatives.** *Catalytic Hydrogenation.* Catalytic hydrogenation of *p*-coumaric acid derivatives was proceeded with a catalytic amount of 5% Pd/C (Wako Pure Chemical) under $H_2$ atmosphere for 3 h. When HPLC analysis of these reactants was done using a Wakosil-II 5C18HG column (i.d. 4.6 × 250 mm) on a Shimadzu CLASS-LC10 series HPLC system equipped with a photodiode array detector (SPD-M10Avp, Shimadzu), there was no absorption longer than 300 nm derived from a conjugated double bond. For *p*-coumaric acid, in practice, a reaction mixture that dissolved 100 mg of starting material in MeOH (20 mL) was vigorously mixed with 5% Pd/C (10 mg) in $H_2$ atmosphere. After 3 h, the reaction mixture was filtered and concentrated to dryness under reduced pressure. Then, a purification with preparative HPLC eluted with a solvent mixture of $H_2O$/MeOH/TFA (650:350:1) monitoring at 254 nm gave *p*-coumaric acid/$H_2$.

*p-Coumaric Acid/$H_2$ (**8**):* $^1$H NMR (270 MHz, acetone-$d_6$) $\delta$ 8.12 (1H, br s), 7.08 (2H, d, $J$ = 7.3 Hz), 6.75 (2H, d, $J$ = 7.3 Hz), 2.81 (2H, t, $J$ = 7.6 Hz), 2.55 (2H, t, $J$ = 7.6 Hz).

*Esterification.* Esterification of sinapinic acid/$H_2$ (**10**; 100 mg) was achieved by using a catalytic amount of sulfuric acid (150 $\mu$L) in corresponding alcohol (20 mL) that was refluxed for 4 h. The reaction mixture was cooled, and saturated $NaHCO_3$ was then added for neutralization. Alcohol was evaporated in vacuo, and the remaining residue was partitioned with EtOAc and saturated $NaHCO_3$. The organic layer was washed and dried in a conventional manner. Purification was carried out on Merck precoated glass plates (silica gel $PF_{254}$; 1.0 mm) eluted with a solvent mixture of *n*-hexane/EtOAc (1:1).

*Sinapinic Acid/$H_2$ Methyl Ester (**11**):* $^1$H NMR (270 MHz, acetone-$d_6$) $\delta$ 6.99 (1H, br s), 6.53 (2H, s), 3.80 (6H, s), 3.61 (3H, s), 2.81 (2H, t, $J$ = 7.8 Hz), 2.58 (2H, t, $J$ = 7.8 Hz).

*Isomerization.* Isomerization of *p*-coumaric acid and ferulic acid was done with daylight irradiation. In brief, the sample solution dissolved in MeOH in a round-bottom flask was placed outside to get daylight for 3 h and then evaporated in vacuo. A novel peak emerged after a *trans*-compound on reverse-phase column chromatography and a corresponding fraction were isolated by preparative HPLC eluted with a solvent mixture of $H_2O$/MeOH/TFA (650:350:1).

*cis-p-Coumaric Acid (**6**):* $^1$H NMR (270 MHz, acetone-$d_6$) $\delta$ 8.80 (1H, br s), 7.78 (2H, d, $J$ = 8.6 Hz), 6.86 (1H, d, $J$ = 12.7 Hz), 6.82 (2H, d, $J$ = 8.9 Hz), 5.81 (1H, d, $J$ = 13.0 Hz)

**Antioxidative Activity Measurement.** *Rabbit Erythrocyte Membrane Ghost System.* Antioxidative activity was measured by the thiobarbituric acid reactive substances (TBARS) method on rabbit erythrocyte membrane ghost treated with *tert*-butyl hydroperoxide (Osawa et al., 1987) with slight modification. In brief, rabbit erythrocyte membrane (0.5 mL) prepared from commercially available rabbit blood, by washing with isotonic buffer solution and lysing in 10 mM phosphate buffer (pH 7.4), was treated with 25 mM *tert*-butyl hydroperoxide (50 $\mu$L) with or without samples dissolved in MeOH (2.0 mM, 100 $\mu$L). These reaction mixtures were incubated for 20 min at 37 °C, and TBARS of each sample were determined using UV absorption at 532 nm on a spectrophotometer (U-2000, Hitachi, Tokyo, Japan).

*Detection of 3-Nitrotyrosine with Enzyme-Linked Imunosorbent Assay (ELISA).* Fresh low-density lipoprotein (LDL) was obtained from human volunteers (with informed consent) by density gradient ultracentrifugation (Sparrow et al., 1989). The detection of 3-nitrotyrosine in protein treated with $ONOO^-$ was accomplished as previously described (Kato et al., 1997). In the case of LDL, protein was dissolved in phosphate buffer at 1.0 mg/mL (final amount), and the following procedure was accomplished in the same manner. In brief, 90 $\mu$L of protein solution was placed in a 1.5-mL microtube, and 10 $\mu$L of sample solution (3.0 mM) dissolved in dimethyl sulfoxide (DMSO) was added, and the mixture was treated with $ONOO^-$ (final concentration = 1.0 mM) with vigorous vortex mixing. This modified protein was treated with antiserum against 3-nitrotyrosine and peroxidase-labeled anti-rabbit IgG goat antibody, successively. Then, *o*-phenylenediamine was added as a substrate to peroxidase, and the amount of 3-nitrotyrosine was evaluated by UV absorption at 492 nm using a multiplate reader (Spectra Max 250, Molecular Devices Corp., Sunnyvale, CA).

*Inhibitory Activity against Xanthine Oxidase.* Ferulic acid methyl ester was prepared in a manner similar to that used with sinapinic acid/$H_2$ methyl ester (**11**) and purified with preparative HPLC. Enzymatic reaction was generated using a method of Watanabe et al. (1997) with slight modification. Briefly, 20 $\mu$L of samples dissolved with DMSO, 140 $\mu$L of phosphate-buffered saline (PBS, pH 7.4), and 20 $\mu$L of hypoxanthine (3.0 mM) dissolved in PBS with a minimum amount of 1.5 M NaOH was placed on a 96-hole plate. The reaction was started with the addition of 20 $\mu$L of xanthine oxidase (150 milliunits) and proceeded with gentle shaking at ambient temperature. After 0.5 h, reaction was terminated with the addition of 20 $\mu$L of HCl solution (1.0 M). The reaction mixtures were diluted 5-fold with distilled water, and 10 $\mu$L of each sample was analyzed with HPLC using a Wakosil-II 5C18HG column (i.d. 4.6 × 250 mm). An aqueous 50 mM ammonium dihydrogenphosphate was used as the mobile phase with a flow rate of 1.0 mL/min at 40 °C, and the remaining hypoxanthine was evaluated by the peak area eluted at 6.7 min with 254 nm. Fifteen minutes of washing with 60% MeOH and 20 min of conditioning followed each 10-min analysis.

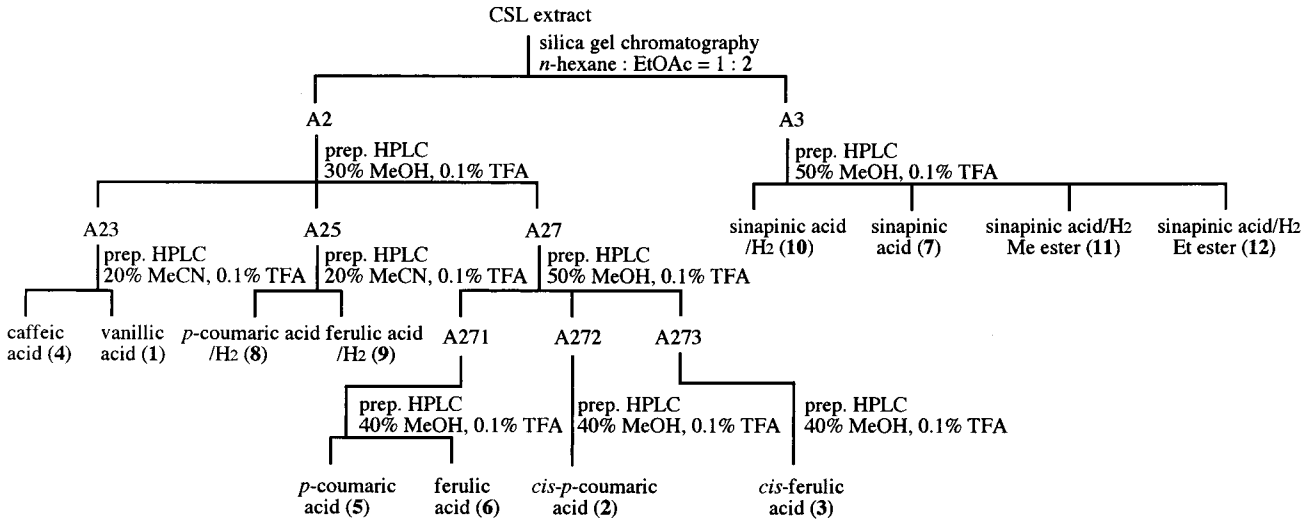


**Figure 1.** Scheme for preparation of antioxidants from CSL.

## RESULTS AND DISCUSSION

To facilitate the fractionation of CSL, we first examined the extractions with some organic solvents (*n*-hexane, EtOAc, or dichloromethane). Among these solvents used, the EtOAc extract showed good results in terms of yield and antioxidative activity (data not shown). CSL components were distributed between EtOAc and saturated brine, and then the organic layer was further separated into acid and neutral fractions. Although both fractions had antioxidative activity, we used the acid fraction for isolation because of its stronger activity as compared to that of the neutral fraction at the same weight concentration. Repeated silica gel and reverse-phase column chromatography of the EtOAc-soluble acid fraction, using an antioxidative activity as a guide, led to the 12 antioxidants (Figure 1). Each isolated compound was analyzed by spectroscopic methods. By comparing the spectrum and the HPLC traces with those of authentic samples, compounds **1** and **4**−**7** were identified as vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and sinapinic acid, respectively. On the other hand, the coupling constant values of olefinic protons of compounds **2** and **3** were smaller than those of the *trans* compounds, although other aromatic signals were similar to those of *p*-coumaric acid and ferulic acid. We concluded that **2** and **3** were *cis* isomers of *p*-coumaric acid and ferulic acid, because each compound had emerged from the corresponding *trans* form by isomerization. Compounds **8**−**10** had no absorption longer than 300 nm in a photodiode array-equipped HPLC analysis, and they had two methylene signals on $^1$H NMR spectra. By catalytic hydrogenation of commercially available chemicals, as described under Materials and Methods, we determined that **8**−**10** were reduced forms of *p*-coumaric acid, ferulic acid, and sinapinic acid, respectively. Compounds **11** and **12** had $^1$H NMR spectra very similar to that of **10** except for the methoxyl or ethoxyl signals, and they were easily prepared from **10** by acid-catalyzed esterification. Finally, 11 *p*-coumaric acid derivatives and vanillic acid were determined (Figure 2).

*p*-Coumaric acid derivatives are components of plant cell wall and contribute to plant growth (Kamisaka et al., 1990). Recent studies have examined these compounds as antioxidants that might be used to prevent oxidative stresses (Laranjinha et al., 1994; Castelluccio et al., 1996). Among CSL-derived antioxidants, sinapinic acid derivatives (**7** and **10**−**12**) and caffeic acid (**4**) strongly inhibited the hydroperoxide-mediated lipid peroxidation, whereas *p*-coumaric acid and ferulic acid derived compounds (**2**, **3**, **5**, **6**, **8**, and **9**) showed <50% inhibition. However, the activities of CSL-derived antioxidants were no more effective than those of α-tocopherol and BHA (Figure 3). This study suggested some structure−activity relationships of *p*-coumaric acid derivatives: (1) The methoxyl group(s) adjacent to the 4-hydroxyl group enhance(s) the inhibitory activity, and (2) the adjacent hydroxyl group (catechol) also enhances the activity more effectively. On the side chain that conjugated to the aromatic ring, (3) the loss of the double bond slightly reduced the activity in *p*-coumaric acid, ferulic acid, and sinapinic acid; however, (4) we could not observe any alteration as a result of the isomeriza-

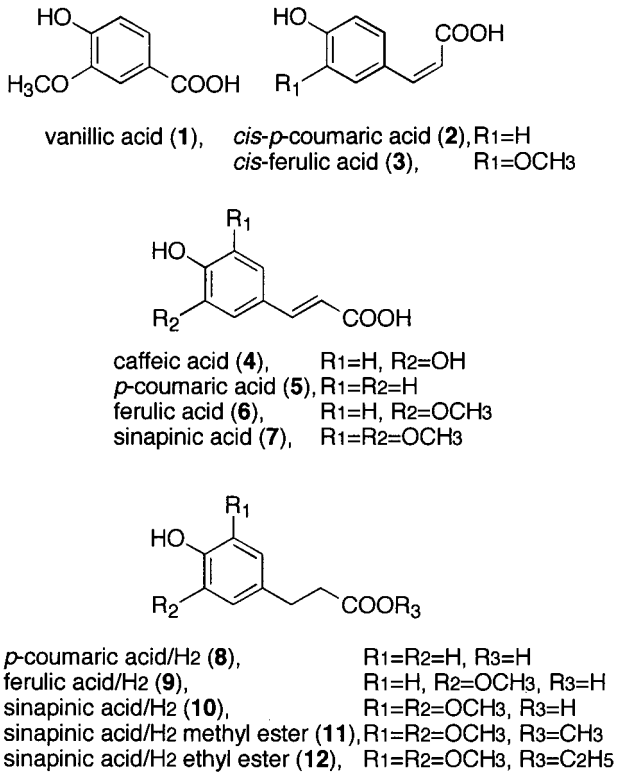


**Figure 2.** Structures of antioxidants isolated from CSL.

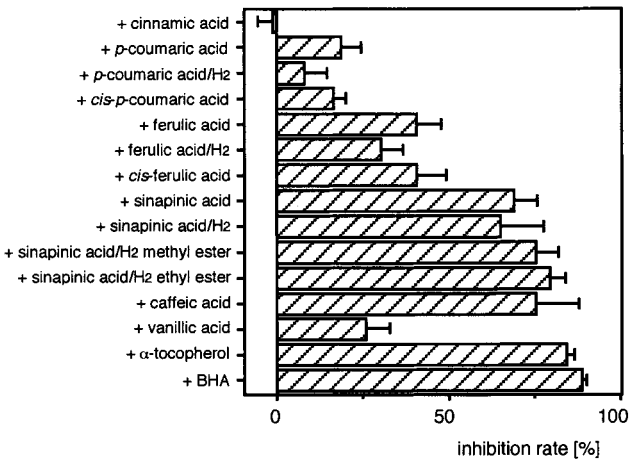


**Figure 3.** Antioxidative activity of the antioxidants isolated from CSL. Inhibitory activity was obtained with a sample concentration at 2.0 mM by comparing the TBARS to results of MeOH alone with or without *tert*-butyl hydroperoxide. Data represent the mean ± SD of three independent measurements.

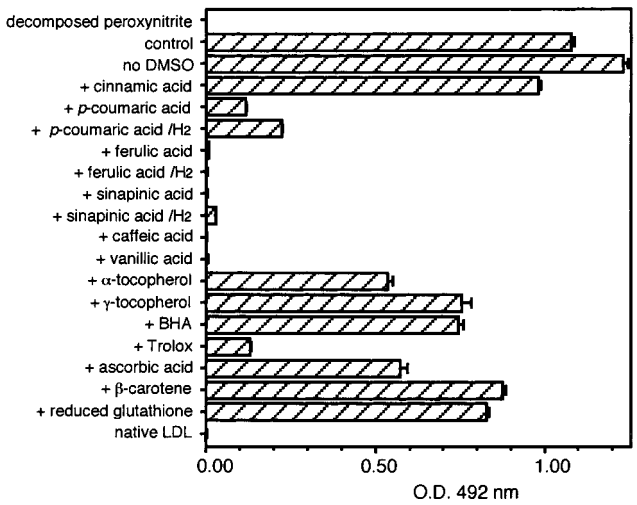


**Figure 4.** Effect of some antioxidants on protein nitration by peroxynitrite. Protein solutions that contained 0.3 mM (final concentration) of each sample were treated with 1.0 mM peroxynitrite, and the nitration was evaluated by ELISA as described under Materials and Methods. Control means the peroxynitrite-exposed LDL in the presence of 10% DMSO without antioxidants. Data represent the mean ± SD of three separate color developments.

tion of the side chain in *p*-coumaric acid or ferulic acid. In this matter, Belguendouz et al. (1997) also observed little change in the antioxidative activity of resveratrol; instead, they described a lowered chelating activity. However, the antioxidative activity of *p*-coumaric acid derivatives against hydroperoxide-induced lipid peroxidation was mainly affected by substituent(s) on the phenolic ring but not the conjugated side chain.

LDL accumulation in macrophage and the subsequent formation of foam cell seem to contribute to atherosclerosis (Palinski et al., 1989). However, native LDL does not make foam cell, and certain oxidative modification seems to be involved, even though the precise mechanism is unknown. $ONOO^-$ is a plausible oxidant in vivo, and this oxidative modification enables LDL to accumulate in macrophage via the scavenger receptor (Graham et al., 1993). Thus, we examined the inhibitory activity of CSL-derived antioxidants against $ONOO^-$-mediated nitrotyrosine formation on LDL evaluated by ELISA, as described under Materials and Methods. The LDL used in our study had a barely detectable amount of nitrotyrosine residue. In our examination in vitro, LDL was nitrated by $ONOO^-$ but not "decomposed $ONOO^-$", indicating that the nitration was not due to the contaminated impurities of $ONOO^-$ synthesis. Phenolic antioxidants obtained from CSL, as shown Figure 2, were potent inhibitors of tyrosine nitration of LDL, whereas typical antioxidants such as BHA, tocopherols, ascorbic acid, and $\beta$-carotene had weak protection against nitrotyrosine formation at this concentration (Figure 4); some previous reports suggested that these endogenous antioxidants play a role in protection from $ONOO^-$-mediated damage (Hogg et al., 1993; Yermilov, V. et al., 1995; Whiteman and Halliwell, 1996). Interestingly, *p*-coumaric acid derivatives produced different types of reaction products when treated with $ONOO^-$ (Kato et al., 1997; Kerry and Rice-Evans, 1998; Pannala et al., 1998, Niwa et al., 1999). Cinnamic acid, which had no phenolic hydroxyl group of *p*-coumaric acid, scarcely exhibited the inhibitory effect, and there was no implication of carboxylic acid-derived effect in our results. Nor did DMSO, used as a dissolving agent, have a remarkable effect, at least under our conditions.

Some *p*-coumaric acid derivatives exert inhibitory activities on enzymatic reactions such as 5-lipoxygenase or arylamine *N*-acetyltransferase (Koshihara et al., 1984; Lo and Chung, 1999). Xanthine oxidase produces superoxide anion radical, a constitute of $ONOO^-$ with nitric oxide, and some studies tied it to vascular dysfunction (Tan et al., 1993; Hamer et al., 1995; White et al., 1996). We aimed to evaluate the isolated compounds of this enzymatic reaction because some *p*-coumaric acid derivatives were described as inhibitors (Chan et al., 1995). According to our evaluation of the inhibitory activity on xanthine oxidase with the remaining hypoxanthine, cinnamic acid and *n*-valeric acid inhibited the enzymatic reaction as well as *p*-coumaric acid and ferulic acid at a range of 1.25−10 mM final concentrations (data not shown). The inhibitory activity of simple organic acids, such as *n*-valeric acid, suggested a carboxylic acid-mediated inhibition of the enzymatic reaction. To avoid an acidic effect derived from free carboxylic acid moiety, we prepared ferulic acid methyl ester and still observed 47.4 ± 6.4% inhibition at 10.0 mM (final concentration), whereas a typical inhibitor, allopurinol, inhibited completely even at 1.25 mM. Chan et al. (1995) previously described the inhibitory activity of cinnamic acid, but the carboxylic acid-derived effect was not mentioned. Recently, Gao et al. (1999) described the superoxide scavenging activity of caffeic acid sugar ester, but they also did not mention any effect on xanthine oxidase. Although further study is required, the inhibitory activity of ferulic acid methyl ester on xanthine oxidase suggested the activity of other *p*-coumaric acid derivatives and that the previously observed "superoxide" scavenging activity of these compounds was derived, at least in part, from the inhibition of the enzymatic reaction itself.

Corn is cultivated almost all over the world and used as a feed ingredient and in the production of food and industrial products (Mehta and Dias, 1999). The isolation of an antifungal benzoxazolinone compound from this common cereal was reported a few decades ago (Beck et al., 1957), and we found the same compound in CSL (data not shown). However, the elucidation of corn-derived chemical components is still in progress (Kuga et al., 1993; Buttery and Ling, 1997). In this study, we revealed 12 phenolic components in CSL that

showed inhibitory activities of oxidative damages in vitro. Our results suggest that an intake of these natural antioxidants might be beneficial to prevent oxidative damages and that CSL might be a useful source of phenolic antioxidants.

## ABBREVIATIONS USED

CSL, corn steep liquor; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; EI-MS, electron impact mass spectrometry; UV, ultraviolet; TFA, trifluoroacetic acid; TBARS, thiobarbituric acid reactive substances; ELISA, enzyme-linked immunosorbent assay; LDL, low-density lipoprotein; $ONOO^-$, peroxynitrite; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline.

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