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Purification and Characterization of Polyphenols from Chestnut Astringent Skin

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ABSTRACT: Polyphenolic compounds from chestnut astringent skin (CAS) were purified by dialysis, using Diaion HP-20 and Sephadex LH-20 columns. During purification, specific α-amylase inhibitory activities were increased about 3.4-fold, and the 50% inhibition value was 5.71 μg/mL in the Sephadex LH-20 fraction (SE-fraction). The SE-fraction contained about 67% of the total polyphenols, 57.3% of the flavanol-type tannins, and 51.3% of the procyanidins. Strong antioxidant activity was observed in the SE-fraction. Oral administration of the SE-fraction in rats fed corn starch significantly suppressed an increase in blood glucose levels. The SE-fraction contained gallic acid and ellagic acid. The MALDI-TOF spectrum showed a peak series exhibiting a mass increment of 288 Da, reflecting the variation in the number of catechin/epicatechin units. Our results suggest CAS contains polyphenols with strong α-amylase inhibitory activity. The data also suggest CAS polyphenols might be oligomeric proanthocyanidins with gallic acid and ellagic acid.

KEYWORDS: chestnut astringent skin, polyphenol, amylase inhibitor, oral carbohydrate tolerance test, antioxidants

INTRODUCTION

The prevalence of diabetes is increasing worldwide, and most diabetic patients suffer from type 2 diabetes.[1,2] Diet is one of the key factors in preventing and treating type 2 diabetes. Maintenance of healthy blood glucose levels is of particular importance and is greatly affected by dietary carbohydrates such as starches. In mammals, dietary carbohydrate digestion is mediated by digestive enzymes such as α-amylase and α-glucosidase. If these enzymes are inhibited, dietary carbohydrate absorption is also inhibited, and the subsequent increase in blood glucose concentration is inhibited. Many inhibitors of carbohydrate digestive enzymes from natural products have been marketed for regulation of blood glucose concentration, for example, wheat protein, kidney bean protein, guava leaf extract, and mulberry leaf extract.[3−5] We previously reported chestnut astringent skin (CAS) extract inhibited human and porcine pancreatic α-amylase and suppressed the increase in blood glucose levels in rats and humans.[6,7]

Chestnut is a deciduous timber tree of the genus *Castanea* in the beech family. Native chestnuts are distributed in the temperate regions of the Northern Hemisphere. Chestnuts are cultivated over large areas in these temperate regions, especially in southern Europe (Spain, France, and Italy) and in East Asia (China, Korea, and Japan). Chestnut part extracts, such as bark, leaf, bur-spine, shell, and astringent skin extracts, inhibit α-amylase activity. In particular, astringent skin extract strongly inhibited α-amylase activity.[7] Chestnut seed consists of the shell, seed skin, endosperm, and so on. The seed skin is called astringent skin because it has a bitter taste and must be removed for eating. Therefore, the astringent skin of chestnut is generated in large quantities as waste product during industrial CAS-free pea processing. CAS could be considered an edible fraction, free from agrochemicals. Therefore, we used CAS as a safe and inexpensive functional food material. CAS was extracted with 75% aqueous ethanol, and the effect of the extract on carbohydrate absorption was investigated.[6,7] The CAS extract strongly retarded absorption of carbohydrate and reduced postprandial hyperglycemia. We presumed an active component of the CAS extract may be a polyphenol, because the CAS ethanol extract contained about 20% polyphenolic material and amylase inhibitory activity increased when the content of polyphenolic material was increased.[6] However, we did not purify and elucidate the chemical component of the CAS. In the present study, we focused on the separation and chemical characterization of polyphenolic compounds isolated from CAS. We also focused on the effects of the purified materials on digestive enzyme activities and nutrition absorption.

MATERIALS AND METHODS

Materials. CAS was obtained from Chuon Co., Ltd. (Matsuyama, Japan). α-Amylases from porcine pancreatic and human pancreas and human saliva, pancreatic lipase (type VI-S) from porcine pancreas, trypsin from porcine pancreas, chymotrypsin from bovine pancreas, pepsin from porcine stomach mucosa, intestinal acetone powder from rat, cellulase from *Aspergillus niger*, tannase from *Aspergillus ficuum*, Folin−Ciocalteu reagent, ellagic acid, (+)-catechin, fluorescein, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich Japan (Tokyo, Japan). α-Amylases from *Bacillus* sp., epicatechin gallate, 1-dodecanethiol, and gallic acid were obtained from Nacalai Co. (Kyoto, Japan). Lipase from *Pseudomonas fluorescence* was obtained from Amano Enzyme Ltd. (Nagoya, Japan). Glucoamylase from *Rhizopus* sp. was obtained from Seikagaku Biobusiness Co. (Tokyo, Japan). Cyanidin

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chloride was obtained from ChromaDex (Irvine, CA). Trolox was obtained from Calbiochem (Darmstadt, Germany). Diaion HP-20 was obtained from Mitsubishi Chemical Co. (Tokyo, Japan). Sephadex LH-20 was obtained from GE Healthcare Japan (Tokyo, Japan). Amicon ultrafiltration membranes were obtained from Nihon Millipore K.K. (Tokyo, Japan).

Preparation and Purification of Polyphenol from CAS Extract. Five hundred grams of CAS was added to 75% aqueous ethanol (1 L), followed by shaking at 37 °C for 12 h. The mixture was filtered, and the filtrate was concentrated and lyophilized to generate CAS ethanol extract. One gram of the CAS ethanol extract was added to 200 mL of water, followed by stirring at 60 °C for 4 h. The mixture was centrifuged at 10000*g* for 20 min, and the supernatant was concentrated to 50 mL by rotary evaporation in vacuo and lyophilized. The lyophilized powder was dissolved in 30 mL of water and dialyzed against 2 L of water using a Spectra/Pro membrane (MWCO = 10000 Da, Spectrum Japan, Otsu, Japan.). The inner and outer dialyzed materials were concentrated and lyophilized. The inner dialyzed material (100 mg) was dissolved with 10 mL of water and applied to a Diaion HP-20 column (300 mm × 20 mm i.d.) equilibrated with water using an Automated Flash Chromatography system (YFLC-AI580, Yamazen Science Inc., Osaka, Japan). The main active compounds were eluted with 60% aqueous ethanol after washing with water. The eluate was concentrated and lyophilized. The eluate (100 mg) was dissolved with 10 mL of water and applied to a Sephadex LH-20 column (300 mm × 20 mm i.d.) equilibrated with water. The active compounds were eluted with 70% aqueous acetone after washing with water. During purification of the CAS extract, amylase inhibition and the amount of polyphenols were monitored.

Assay Methods. α-Amylase activity was determined by measuring the reducing power of released oligosaccharide from soluble starch using the method of Miller[8] with the following minor modifications:[6] The assay system was composed of the following components in a total volume of 1 mL: 100 mM sodium phosphate, pH 6.8, 17 mM NaCl, 5 mg of soluble starch, 100 μL of inhibitor solution, and 10 μL of enzyme solution. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 0.1 mL of 2 N NaOH and 0.1 mL of color reagent (4.4 μmol of 3,5-dinitrosalicylic acid, 106 μmol of potassium sodium (+)-tartrate tetrahydrate, and 40 μmol of NaOH), followed by 3 min of incubation at 100 °C and subsequent absorbance measurement at 540 nm.

Maltase and sucrase activities were determined using maltose and sucrose as substrates, and glucose produced in the reaction was measured with a commercial assay kit (Glucose C II-test, Wako Pure Chemical Industries, Ltd., Osaka, Japan).[9,10] Pancreatic lipase activity was measured using 4-methylumbelliferyl oleate as a substrate.[11] Lipase activity was also determined by measuring the rate of release of oleic acid from trioleoylglycerol.[12] The rate of enzyme inhibition was calculated as a percentage of the control (without inhibitor) using the formula

$$\text{enzymeinhibition}\ (\%) = [(A_0 - A_i)/A_0] \times 100$$

where A_i = activity with inhibitor and A_0 = control activity (activity without inhibitor).

One unit of enzyme inhibition (U) was expressed by the weight of IC_{50} (concentration of inhibitor to inhibit 50% of its activity) value per mL.

Polyphenols (total phenolics) were determined using the method of Folin−Ciocalteu using (+)-catechin as a standard.[13,14] In addition, other colorimetric procedures of the vanillin assay[15] for flavan-3-ol and the butanol−HCl assay[16] for procyanidins were employed for the determination of polyphenolic substances using (+)-catechin and cyanidin chloride as standards, respectively.

Antioxidant activities were determined using the superoxide anion scavenging activity assay, DPPH radical scavenging capacity assay, and oxygen radical absorbance capacity (ORAC) assay. The superoxide anion scavenging activity was determined using the SOD assay kit WST (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), and activities were expressed as 50% inhibition amount of superoxide dismutase-like material.[17] The DPPH radical scavenging capacity of each fraction was determined according to the method of Miliauskas.[18] Radical scavenging activity of the sample or trolox calibration solutions against stable DPPH radicals was determined spectrophotometrically, and the activities were expressed as μmol trolox equivalents (TE) per g of sample. The ORAC assay was applied according to the method of Ou as modified by Davalos.[19,20] The fluorescence was recorded for 90 min using a FlexStation 3 Microplate Reader (Molecular Devices Japan, Ltd., Tokyo Japan) at excitation and emission wavelengths of 485 and 520 nm, respectively. The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve. Samples and trolox calibration solutions were always analyzed in replicates of five. The activities were expressed as μmol TE per g of sample.

Oral Carbohydrate Tolerance Test in Rats. The effect of the SE-fraction on the carbohydrate absorption in rats was investigated. The experimental animal protocol was approved by the Animal Study Committee of Ehime University. Male Wistar King rats, weighing 150−200 g, were starved overnight (15 h) and divided into two groups. The test group received 2 mL of corn starch suspension (2 g/kg body weight) and 1 mL of SE-fraction, whereas the control group received corn starch suspension and 1 mL of water via a stomach tube. After administration, blood samples were collected from the tail vein or artery at regular intervals. Blood glucose was measured using a blood glucose test meter, GLUCOCARD (Arkray Inc. Kyoto, Japan).[6] After a 1 week interval, the test and control groups were switched and the experiment was repeated.

Oral Lipid Tolerance Test in Rats. A suspension of 6 mL of corn oil, 80 mg of cholic acid, and 2 mL of cholesteryl oleate in 6 mL of water was sonicated for 5 min. Male Wistar King rats, weighing 160−210 g, were starved overnight (15 h) and divided into two groups. The test group received the lipid suspension (10.5 mL/kg body weight) and the SE-fraction, whereas the control group received the lipid suspension and water via a stomach tube. After administration, blood samples were collected from the tail vein or artery into heparinized microcapillary tubes, at regular intervals, and centrifuged immediately at 10000 rpm for 5 min.[12,21] After a 1 week interval, the test and control groups were switched, and the experiment was repeated. Plasma triacylglycerol concentrations were determined using the Triglyceride E-test (Wako Pure Chemical Industries).

Analysis of Polyphenol Component. To determine whether the galloyl moiety of the polyphenol was esterified, the sample (10 mg of SE-fraction) was incubated with 0.5 unit of tannase at 25 °C for 30 min.[22] The enzyme reaction was stopped by boiling for 5 min, and gallic acid was detected using analytical reverse-phase HPLC and LC-ESI/MS. (−)-Epicatechin gallate was also incubated with tannase under the same conditions, for use as a positive control.

To analyze free ellagic acid, 250 mg of SE-fraction was dissolved in 2 mL of 2 N HCl and subjected to hydrolysis at 100 °C for 1 h.[23] The mixture was neutralized by the addition of 2 mL of 2 N NaOH, and released ellagic acid was extracted with 6 mL of methanol and detected using analytical reverse-phase HPLC and LC-ESI/MS.

The SE-fraction was degraded by acetic acid in the presence of 1-dodecanethiol. A mixture of sample (1 mg), 5 μL of 1-dodecanethiol, and 12.5 μL of acetic acid in 125 μL of ethanol was heated in a sealed vial at 100 °C for 6 h.[24] The reaction products were analyzed using analytical reverse-phase HPLC and LC-ESI/MS.

HPLC analysis was performed on a UPLC-quadrupole(Q)-TOF-LC/MS system (Waters Corp., Milford, MA). The separation was performed at 40 °C using an Acquity UPLC BEH C18 column (2.1 mm × 150 mm, 1.7 μm), and the solvents were 0.02% aqueous formic acid (solvent A) and 0.02% formic acid in acetonitrile (solvent B).

Table 1. Purification Steps of CAS Polyphenols[a]

purification step	dry wt (mg)	amylase activity			polyphenol[b]		
		IC_{50} (μg/mL)	total activity (U)	specific activity (U/mg)	Folin−Ciocalteu (mg (%)[c])	vanillin assay (mg (%)[c])	butanol−HCl (mg (%)[c])
ethanol extract	1000	19.5	51200	51.2	225 (100)	96.0 (100)	160 (100)
10000*g*							
supernatant	826.8	16.5	50109	60.7	237 (105)	84.3 (88)	140 (88)
precipitate	129.7	182	713	5.49	18.0 (8)	10.5 (11)	19.3 (12)
dialysate, inner	286.2	7.03	40710	142.2	186 (83)	81.5 (85)	141 (88)
outer	658.4	982	698	1.06	63.9 (28)	37.5 (39)	23.7 (15)
Diaion HP-20	171.5	5.92	28970	168.9	119.4 (53)	54.0 (56)	82.3 (51)
Sephadex LH-20	154.4	5.71	26890	174.2	100.7 (45)	47.7 (50)	81.4 (51)

[a] Results are expressed as the mean for three assays. [b] The method of Folin−Ciocalteu and the vanillin assay are expressed as (+)-catechin equivalents, and the butanol−HCl assay is expressed as cyanidin chloride equivalents. [c] (%) was the percent of polyphenol dry weight compared with the ethanol extract.

The gradient program was 0−2 min, 65% B; 2−30 min, 65−100% B. The flow rate was 0.2 mL/min, and the injection volume was 1 μL. Eluted polyphenolic compounds were detected by monitoring both the absorbance at 280 nm and the total ions of the mass spectra as analyzed by ESI/MS (Q-TOF micro AP system, Nihon Waters K.K.).

Analysis of Polyphenol by MALDI TOF/MS. MALDI-TOF mass spectra were collected on a JEOL-MALDI Spiral TOF mass spectrometer (JEOL Ltd., Tokyo, Japan), equipped with a N_2 laser set at 349 nm. An accelerating voltage of 20 kV was used in the detection of positive ions in the mass spectra, which was calibrated with a peptide mixture (bradykinin fragment 1−7 (m/z 754.400), angiotensin II (m/z 1046.542), angiotensin I (m/z 1296.542), $P_{14}R$ (m/z 1533.858), ACTH fragment 1−17 (m/z 2093.087), and ACTH fragment 18−39 (m/z 2465.199)). The sample (1 mg of SE-fraction) was dissolved in 1 mL of a solution consisting of aqueous 0.1% trifluoroacetic acid/acetonitrile (1:2, v/v). The sample solution was mixed with the matrix solution containing 10 mg/mL 2,5-dihydroxybenzoic acid in 80% ethanol, at a ratio of 3:1 (v/v). The resulting 1 μL was placed on a target plate and dried.

Statistical Analysis. Results are expressed as the mean ± SE. The statistical significance of differences with and without (control) the samples was assessed using the paired Student's t test.

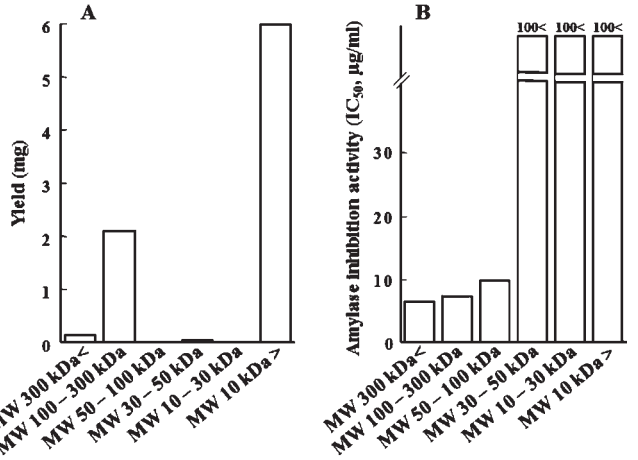

Figure 1. Fractionation of 10000*g* supernatant by Amicon ultrafiltration membranes. One gram of CAS extract was extracted at 60 °C for 4 h and centrifuged at 10000*g* for 20 min. The supernatant was fractionated stepwise using Amicon ultrafiltration membranes (diameter = 63.5 mm; molecular weight cutoffs 10, 30, 50, 100, and 300 kDa). Each fraction was lyophilized, and yield (A) and α-amylase inhibition (B) were determined.

RESULTS AND DISCUSSION

Fractionation of Polyphenolic Compounds from CAS Extract. CAS polyphenols were purified by a dialysis, followed by chromatography on Diaion HP-20 and Sephadex LH-20 columns. CAS extract is a bitter-tasting dark brown powder, which has been found to strongly inhibit porcine pancreatic α-amylase activity.[6,7] The CAS ethanol extract was re-extracted with water at 60 °C and centrifuged at 10000*g*. Most amylase inhibitory activities (about 98%) and dry materials (about 83%) were observed in the supernatant (Table 1). When the supernatant was fractionated using Amicon Ultrafiltration membranes, about 70% of the materials (dry weight) was observed in the fraction under 10 kDa. However, α-amylase inhibitory activity was very weak in this fraction; the IC_{50} value was >100 μg/mL. Most active materials with high α-amylase inhibitory activity were observed in the fraction from 100 to 300 kDa (Figure 1). Although the molecular standards of the ultrafiltration membranes are globular proteins, our results suggest the active materials of the CAS extract are high-polymer substances. Therefore, the supernatant from the CAS water extract was dialyzed using a membrane with a molecular weight cutoff of 10 kDa. The dialysis was an effective separation step (Table 1). Most of the α-amylase inhibitory activity (81%) was recovered in the inner dialysate, and about 70% materials (dry weight) with low amylase inhibitory activity (IC_{50} = 982 μg/mL) was observed in the outer dialysate. The specific α-amylase inhibitory activity of the outer dialysate was about $^1/_{130}$ when compared with the inner dialysate. The specific α-amylase inhibitory activity and specific polyphenol content of the inner dialysate were increased about 2.8-fold compared with the CAS extract.

The inner dialysate was applied to a Diaion HP-20 column, and the adsorbed α-amylase inhibitory activities were eluted with 60% aqueous ethanol (Figure 2A). The eluate was applied to a Sephadex LH-20 column, and most amylase inhibitory activities were eluted with 70% aqueous acetone (Figure 2B). Using 1000 mg of CAS extract, we derived 154.4 mg of SE-fraction using the chromatographic procedures described (Table 1).

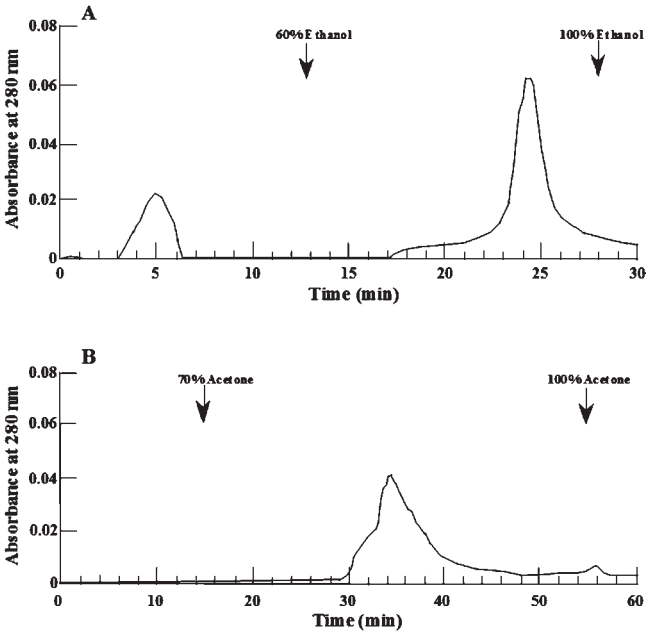

Figure 2. Diaion HP-20 (A) and Sephadex LH-20 (B) column chromatography. Details are given under Materials and Methods.

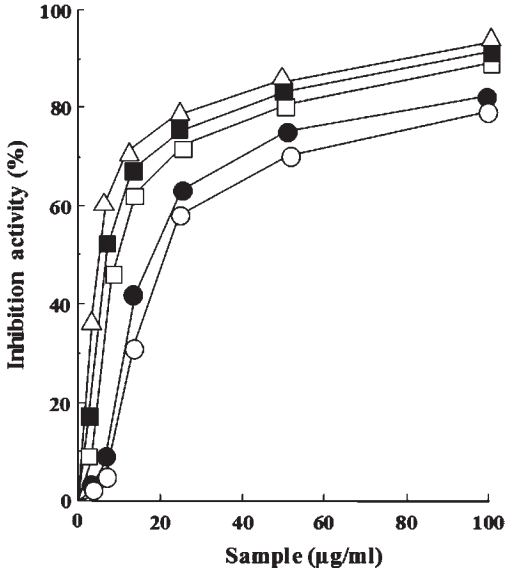

Figure 3. Effect of increased concentration of samples (CAS ethanol extract (○), 10000*g* supernatant fraction (●), inner dialyzate fraction (□), Diaion HP-20 fraction (■), and SE-fraction (△)) on α-amylase inhibition activity. Results are expressed as the mean for four assays.

During purification, each fraction inhibited α-amylase in a dose-dependent manner (Figure 3); the specific α-amylase inhibitory activity was increased about 3.4-fold, and the IC_{50} value was 5.71 μg/mL (Table 1). This IC_{50} value was comparable with that of acarbose, a prescribed α-amylase inhibitor used in the control of type II diabetes, at 7.22 μg/mL. The amount of total polyphenols in the fraction was determined using catechin as a standard according to the Folin−Ciocalteu method. The total polyphenol content of the SE-fraction was about 65% of the dry weight. The contents of flavan-3-ol and procyanidins in each fraction were determined using the vanillin assay and the butanol−HCl assay, respectively. Flavan-3-ol and procyanidins were detected in each fraction; in the SE-fraction, they were about 30.9 and 52.7% of the dry weight, respectively. Similar polyphenol ratios were observed in the dialysate fraction and Diaion HP-20 fraction (Table 1).

We have tried further separation of the SE-fraction. When the SE-fraction was applied to an ODS-S-50W column, most α-amylase inhibitory activities were adsorbed and eluted as a single peak with a gradient of 0−50% methanol (data not shown). The eluted specific α-amylase inhibitory activity and polyphenol contents were similar to those in the SE-fraction. These results suggested the SE-fraction did not separate by the ODS column and the main components of α-amylase inhibitory activity in the CAS extract would be polyphenolic substances consisting of higher polymers. Polyphenolic compounds are widely distributed in plants. They are not essential for the survival of plants but are produced to enhance the plant's ability to fight disease or damage, such as oxidation.[25] Furthermore, in vitro studies have shown many plant polyphenols inhibited carbohydrate-hydrolyzing enzymes. These polyphenols include theaflavin from tea, which inhibited small intestinal sucrase and α-glucosidase activities;[26] gallotannin, which inhibited human salivary α-amylase activity;[27] polyphenols from evergreen mangrove seeds, which inhibited α-glucosidase and amylase activities;[28] polyphenols from Japanese horse chestnut, which inhibited α-glucosidase activities;[22] and polyphenols from berry, which inhibited α-glucosidase and α-amylase activities.[29] Inhibitors of carbohydrate-hydrolyzing enzymes play an important role in controlling postprandial blood glucose levels.

Enzyme Inhibition by the SE-Fraction. CAS polyphenols (the SE-fraction) specifically and strongly inhibit α-amylase. Table 2 shows enzyme inhibition by the SE-fraction. This fraction strongly inhibited human pancreatic and saliva α-amylase. The IC_{50} value for human pancreatic α-amylase was about 3 times lower than that for porcine pancreatic α-amylase. The fraction also inhibited α-amylase from *Bacillus* sp. in a concentration-dependent manner. However, the IC_{50} value was about 4 times higher than that of porcine pancreatic α-amylase. Inhibitions of carbohydrate-hydrolyzing enzymes, other than α-amylase, were weak; the IC_{50} values of the fraction for intestinal α-glucosidase and bacterial glucoamylase activities were about 70−110 times higher than that of porcine pancreatic α-amylase. The SE-fraction did not inhibit bacterial cellulase activity up to 1000 μg/mL.

The SE-fraction also inhibited pancreatic lipase activity. The CAS extract strongly inhibited the hydrolyzing activity of pancreatic lipase toward monomeric substrates such as 4-methylunbelliferyl oleate. During purification, each fraction inhibited the hydrolysis of 4-methylumbelliferyl oleate in a dose-dependent manner (Figure 4), and the specific inhibitory activity was increased about 2.7-fold with an IC_{50} value of 0.797 μg/mL (data not shown). However, inhibition of *P. fluorescence* lipase was weak: the IC_{50} value was >1000 μg/mL. The IC_{50} value of the SE-fraction for the hydrolyzing activity of pancreatic lipase toward emulsified substrate such as trioleoylglycerol phosphatidylcholine emulsion was about 360 times higher than for a monomeric substrate (4-methylunbelliferyl oleate) (Table 2). The CAS polyphenols strongly inhibited the esterase activity of pancreatic lipase, but it did not strongly inhibit the lipase activity of pancreatic lipase. Typical physiological substrates for pancreatic lipase are water-insoluble long-chain triacylglycerols, suggesting emulsified substrates, such as trioleoylglycerol-phosphatidylcholine emulsion, are more physiologically important

Table 2. Enzyme Inhibition by the SE-Fraction[a]

	IC_{50} value (μg/mL)	
α-amylase from porcine pancreas	5.71	
α-amylase from human pancreas	1.89	
α-amylase from human saliva	3.17	
α-amylase from *Bacillus* sp.	23.9	
glucoamylase from *Rizopus* sp.	451	
maltase from rat intestine	634	
sucrase from rat intestine	417	
cellulase from *A. niger*	>1000	
lipase from porcine pancreas	0.797	(4-methylumbelliferyl oleate)
lipase from *P. fluorescence*	>1000	(4-methylumbelliferyl oleate)
lipase from porcine pancreas	286	(trioleoylglycerol)
trypsin from porcine pancreas	>1000	
chymotrypsin from bovine pancreas	>1000	
pepsin from porcine stomach	642	

[a] Results are expressed as the mean for three assays.

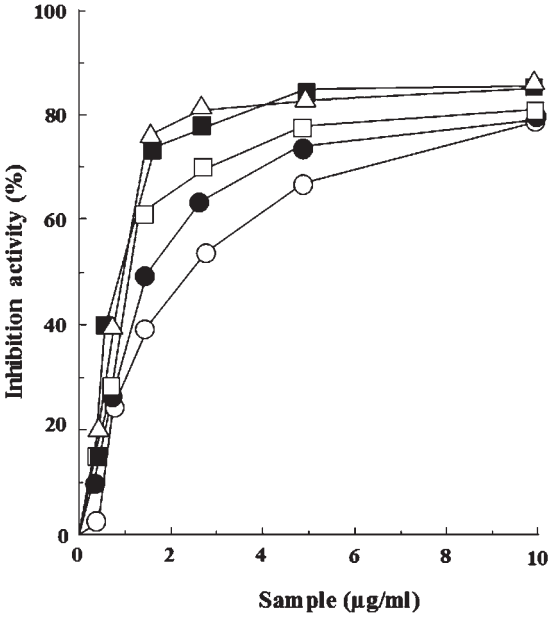

Figure 4. Effect of increased concentration of samples (CAS ethanol extract (○), 10000*g* supernatant fraction (●), inner dialysate fraction (□), Diaion HP-20 fraction (■), and SE-fraction (△)) on pancreatic lipase inhibition activity toward hydrolysis of 4-methylumbelliferyl oleate. Results are expressed as the mean for three assays.

than monomeric substrates, such as 4-methylunbelliferyl oleate. Inhibitory activities of the SE-fraction for the digestive proteases were weak: IC_{50} values of pancreatic trypsin and chymotrypsin were >1000 μg/mL and that for stomach pepsin was 642 μg/mL.

The main components of CAS polyphenols might be (+)-catechin or (−)-epicatechin. We compared the inhibitory property of catechin with that of the SE-fraction: amylase and pancreatic lipase inhibition activities of catechin were very weak; IC_{50} values were >500 μg/mL (data not shown).

Antioxidant Activity of the SE-Fraction. CAS polyphenols (the SE-fraction) contain powerful antioxidants, which may act as superoxide anion scavengers, DPPH radical scavengers, and oxygen radical absorbents. Antioxidant activities were determined using three methods: superoxide anion scavenging activity assay, DPPH radical scavenging capacity assay, and ORAC assay. A strong superoxide anion scavenging activity was observed in the SE-fraction; the IC_{50} value was 7.58 μg/mL. Similar IC_{50} values were observed in the Diaion and the inner dialysate fractions, 7.62 and 8.26 μg/mL, respectively. However, the IC_{50} value of the CAS ethanol extract was about 2 times higher (17.6 μg/mL) than that of the SE-fraction. An effective DPPH radical scavenger was present in the SE-fraction; DPPH radical scavenging capacity was 6612 μmol TE/g sample. Similar values were observed in the Diaion and the inner dialysate fractions, 7205 and 6585 μmol TE/g sample, respectively. The CAS extract was about one-third of the scavenging value: 2038 μmol TE/g sample. We also determined antioxidant activity using the ORAC assay, which measured antioxidant scavenging activity against the peroxyl radical. The antioxidant scavenging activities were increased with the proceeding fractionation: the activities were 1914, 2908, 3656, and 4251 μmol TE/g sample of fractions from the CAS ethanol extract, inner dialysate, Diaion fraction, and SE-fraction, respectively.

We compared the antioxidant activity of catechin with the SE-fraction. Catechin had strong antioxidant activity; superoxide anion scavenging activity, DPPH radical scavenging capacity, and ORAC were 1.5-fold (IC_{50} = 5.0 μg/mL), 1.7-fold (11400 μmol TE/g), and 1.2-fold (5089 μmol TE/g) stronger than the SE-fraction values, respectively.

Polyphenols are the most abundant antioxidants in our diet. They may protect cell constituents against oxidative damage. Previous papers have suggested that the consumption of polyphenol-rich foods may limit the risk of various degenerative diseases associated with oxidative stress such as cardiovascular disease, cancer, diabetes mellitus, and arteriosclerosis.[30−32]

Effects of the SE-Fraction on Increased Blood Glucose Concentrations in Rats. CAS polyphenol(s) (the SE-fraction) suppressed the increase of blood glucose levels after corn starch administration in a dose-dependent manner. We examined whether the amylase inhibition observed in vitro could exert an inhibitory effect on carbohydrate absorption in vivo. In control rats, blood glucose levels increased from a baseline of 48.8 ± 1.30 mg/dL at 0 min to a peak of 113.4 ± 4.04 mg/dL (increased blood glucose value 64.6 ± 4.59 mg/dL) at 30 min after starch administration (2 g/kg body weight). The rise in blood glucose was suppressed in a dose-dependent manner when the SE-fraction (25, 50, 100, 200, and 300 mg/kg body weight) was given with starch (Figure 5A). The AUC for the SE-fraction administration was also found to decrease with increasing concentrations of the SE-fraction, compared to the administration of starch alone (Figure 5B). The IC_{50} value of the AUC was 118 mg/kg body weight. The value was about 3.2-fold lower than the CAS ethanol extract value,[6] and this magnification was comparable to α-amylase inhibition (3.4-fold). We previously reported CAS ethanol extract also suppressed the rise in plasma insulin and the fall in plasma nonesterified fatty acid levels in normal rats.[6] In addition, the CAS ethanol extract also suppressed the rise in plasma glucose levels in diabetic rats.[7] Furthermore, the CAS ethanol extract suppressed any increase in plasma glucose, in a dose-dependent manner, after administration of boiled rice to humans.[6] These data suggest CAS polyphenol(s) might exert an antidiabetic effect by inhibiting α-amylase and suppressing carbohydrate absorption from the intestine, thereby reducing a postprandial increase in blood glucose.

Effects of the SE-Fraction on Increased Plasma Triacylglycerol Concentrations in Rats. CAS polyphenol(s) (the SE-fraction)

suppressed the increase of blood triacylglycerol levels after corn oil administration. Figure 6A shows the time course of the plasma triacylglycerol concentration when corn oil emulsion, with or without the SE-fraction (300 and 1000 mg/kg body weight), was orally administered to rats. In control rats, plasma triacylglycerol levels increased from a baseline of 91.9 ± 2.65 mg/dL at 0 min to a peak of 212.6 ± 18.85 mg/dL (increased plasma triacylglycerol value 120.7 ± 20.17 mg/dL) at 4 h after lipid suspension administration (10.5 mL/kg body weight). After administration of 1000 mg/kg body weight of the SE-fraction, the rise in plasma triacylglycerol concentration was significantly suppressed compared with the control. The AUC for 1000 mg/kg body weight of the SE-fraction administration was also significantly decreased (Figure 6B). However, the rise in plasma triacylglycerol concentration and the AUC was not significantly decreased by administration of 300 mg/kg body weight of the SE-fraction. Compared with the inhibition of carbohydrate absorption, the SE-fraction was a weak inhibitor of lipid absorption: the fraction at 300 mg/kg body weight strongly inhibited carbohydrate absorption, but it did not significantly inhibit lipid absorption. This difference might be caused by enzyme inhibition: porcine pancreatic α-amylase inhibition by the SE-fraction was about 50-fold stronger than porcine pancreatic lipase inhibition (trioleoylglycerol hydrolyzing activity) (Table 2).

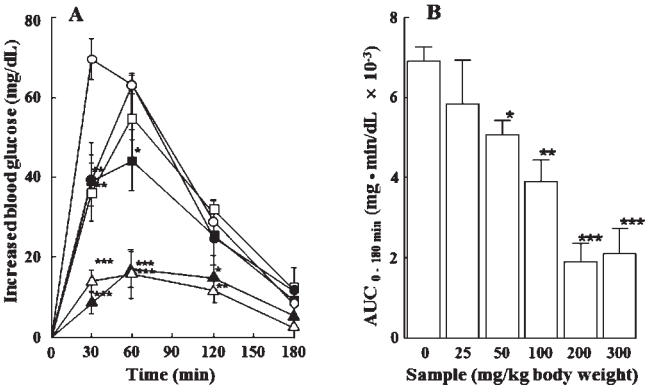

Figure 5. Effects of the SE-fraction on increased blood glucose concentrations (A) and the AUC (B) in rats. (A) Rats were fasted for 15 h before the SE-fraction (25 (●), 50 (□), 100 (■), 200 (△), and 300 (▲) mg/kg body weight) and starch (2 g/kg body weight) were administered. As a control, rats were given only starch (2 g/kg body weight) (○). (B) AUC $_{0-180\ min}$ is the area under the curve of the incremental blood glucose level up to 180 min. Results are expressed as the mean ± SE, $n = 8$. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ versus control.

Structural Elucidation of the SE-Fraction. CAS polyphenols (the SE-fraction) might be oligomeric proanthocyanidins with gallic acid and ellagic acid. We analyzed the polyphenol components of the SE-fraction. When the sample was treated with tannase, released free gallic acid was detected in the reaction mixture. The peak of the reverse-phase HPLC was identical to the peak from the tannase-treated epicatechin gallate, and these peaks were characterized by molecular ions of $[M + H]^-$ at m/z 168.9 (data not shown). When the sample was hydrolyzed by 2 N HCl at 100 °C for 1 h, released free ellagic acid was detected in the reaction mixture. The peak of the reverse-phase HPLC was identical to the peak from the standard ellagic acid peak, and these peaks were characterized by molecular ions of $[M + H]^-$ at m/z 300.9 (data not shown). Figure 7 shows the chromatogram of the SE-fraction degradation products, treated with acetic acid in the presence of 1-dodecanethiol. The three peaks were detectable at 6.98 min (I), 7.40 min (II), and 7.86 min (III). The mass spectra derivatives **I**, **II**, and **III** were characterized by molecular ions of $[M + H]^+$ at m/z 641.8, 777.6, and 490.2, respectively. According to the data, compound **I** was estimated as a dodecyl sulfide derivative of catechin gallate or epicatechin gallate, and compounds **II** and **III** were estimated as dimeric and monomeric dodecyl sulfide derivatives of catechin or epicatechin, respectively. The MALDI-TOF spectrum of the SE-fraction shows a peak series exhibiting a mass increment of 288 Da (Figure 8), corresponding to what is known about the structure of the catechin/epicatechin unit with the repeat unit. The data may indicate a series of polyflavan-3-ol polymers termed the B-type linkage.[33,34] Our results suggest CAS polyphenols might be oligomeric proanthocyanidins with gallic acid and ellagic acid. In other words, they might be highly polymerized mixtures containing hydrolyzable and condensed tannins.

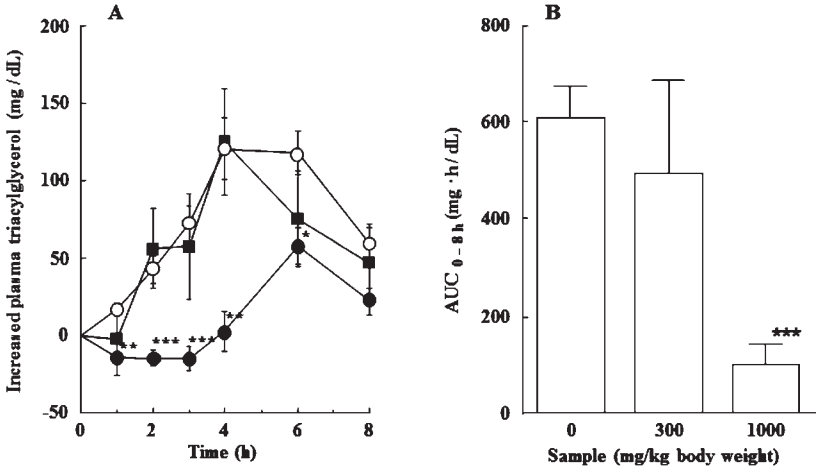

Figure 6. Effects of the SE-fraction on increased plasma triacylglycerol concentrations (A) and the AUC (B) in rats. (A) Rats were fasted for 15 h before the SE-fraction (300 (■) and 1000 (●)) mg/kg body weight) and lipid suspension (10.5 mL/kg body weight) were administered. As a control, rats were given only lipid suspension (○). (B) AUC $_{0-8\ h}$ is the area under the curve of the increased plasma triacylglycerol level up to 8 h. Results are expressed as the mean ± SE, $n = 6$. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$ versus control.

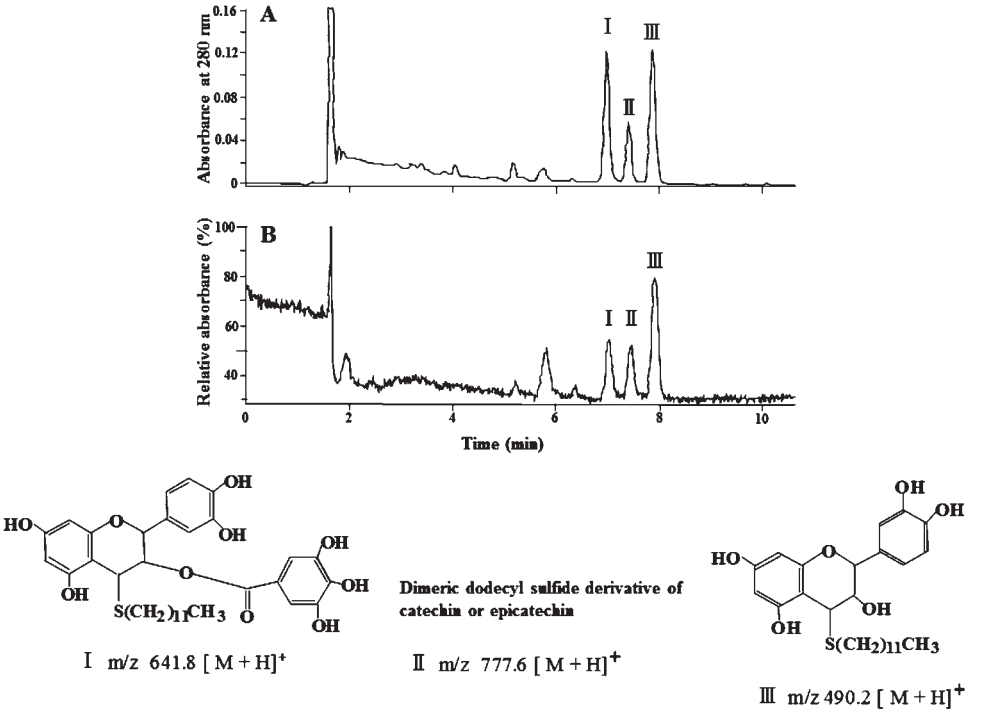

Figure 7. LC-ESI/MS spectra of dodecyl sulfide derivatives following hydrolysis of the SE-fraction. Chromatograms were recorded by monitoring the absorbance at 280 nm (A) and total ions of mass spectra in the positive ion mode (B).

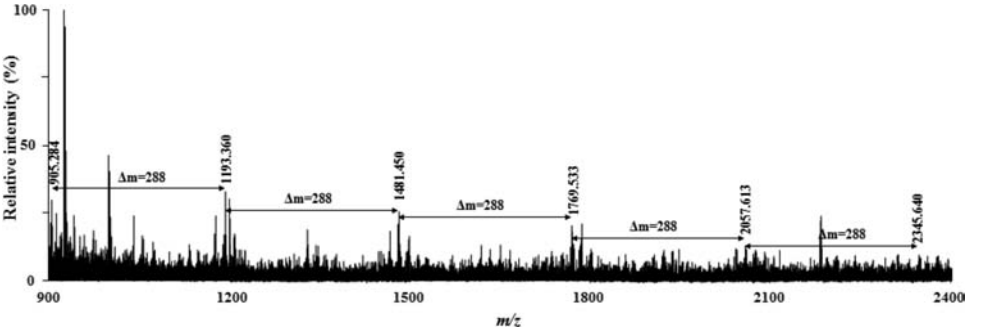

Figure 8. MALDI-TOF positive ion mode spectrum of the SE-fraction.

It is known that not all polyphenols (tannins) are effective α-amylase inhibitors. The α-amylase inhibitions by marketed hydrolyzable tannin (chestnut tannin, polyphenol concentration = 59.4%, obtained from Kawamura Commerce Co., Tokyo, Japan) and marketed condensed tannin (quebracho tannin, polyphenol concentration = 78.4%, obtained from Kawamura Commerce Co.) were very weak: the IC_{50} values of chestnut and quebracho tannins were over 1000 and 250 μg/mL, respectively. The mechanism of enzyme inhibition by tannin is likely due to nonspecific interaction with the enzyme protein. CAS polyphenols can bind to proteins (data not shown). However, enzyme inhibition by polyphenols is not always comparable with measurements from protein interaction. Despite the fact that α-amylase inhibition of the SE-fraction was >200 times higher than that of chestnut tannin, protein precipitation of the SE-fraction was only 2 times higher than that of chestnut tannin using the bovine serum albumin precipitation assay[35] (data not shown). Furthermore, if CAS polyphenols nonspecifically bind to any available proteins within the small intestine, they might nonspecifically inhibit the intestinal digestive enzymes. However, the CAS polyphenols strongly inhibited α-amylase when compared with other intestinal digestive enzymes such as α-glucosidases and proteases (Table 2). Therefore, the mechanism of enzyme inhibition by CAS polyphenols might not be nonspecific binding to the digestive enzymes. Kandra et al. suggested that the inhibition of α-amylase by gallotannin is a mixed noncompetitive type and that only one molecule of tannin binds to the active site or the secondary site of the enzyme.[27] The CAS polyphenols might specifically inhibit α-amylase.

CAS contains high concentrations of polyphenols such as proanthocyanidins or tannins, and the role of these polyphenols might be to defend seeds against a variety of herbivores and pathogenic microbes. It might not only produce an unpalatable bitter taste but also inhibit digestive enzymes to prevent seeds from being eaten.[36] Therefore, CAS may contain polyphenols as an antinutrient such as the α-amylase inhibitor as a defense mechanism. Antinutrients are sometime recognized as toxins. We previously assayed for subchronic toxicity of CAS ethanol

extract using rats and humans.[6] CAS ethanol extracts (1000 or 2000 mg/kg body weight/day) were administered to rats via a stomach tube for 28 days. During the study, the treatment had no effects on clinical signs or survival: food intake, water intake, blood biochemistry, and organ weights exhibited no differences of toxicological significance between control and treated rats. CAS extract (600 or 1200 mg/day) was also administered to humans for 28 days. During the study, the treatment had no effects on clinical signs. There were no significant differences in the clinical data for the urine and blood biochemistry between the study and control group with and without CAS extract administration.

In conclusion, CAS polyphenols consist of oligomeric proanthocyanidins with gallic acid and ellagic acid. They have strong α-amylase inhibition, retard carbohydrate absorption, and reduce postprandial hyperglycemia. Therefore, they are strong candidates as antidiabetic materials.

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ABBREVIATIONS USED

AUC, area under the curve; CAS, chestnut astringent skin; DPPH, 2,2-diphenyl-1-picrylhydrazyl; IC_{50}, concentration of inhibitor needed to inhibit 50% of the activity; ORAC, oxygen radical absorbance capacity; SE-fraction, Sephadex LH-20 fraction; TE, trolox equivalents.

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