Tea constituents that had a preventive effect on d-galactosamine-induced liver injury in rats were partially purified by column chromatography from a n-butanol-soluble fraction of green tea. The fraction containing glycosidic flavonoids was found to suppress the d-galactosamine-induced increase of plasma alanine aminotransferase and aspartate aminotransferase activities. These results indicate that glycosidic flavonoids contribute, at least in part, to the liver injury-preventive effect of green tea.

Key words: green tea; flavonoids; glycosides; liver injury; d-galactosamine

A number of studies have shown that green tea or its constituents have a wide range of biological effects such as antioxidation, anti-mutation, anticarcinogenesis, antibiotic action, anti-hypercholesterolemia, anti-hyperglycemia, anti-hypertension, and anti-inflammation. Furthermore, we recently reported that green tea had a liver injury-preventive effect in rats. When a green tea extract was fractionated into five fractions by successive extraction with organic solvents such as chloroform, ethyl acetate, n-butanol, and 70% ethanol, and these fractions were fed to rats, several fractions were found to have preventive effects on liver injury (unpublished results). These results indicate that different types of tea constituents contribute to the effects of green tea. One of the tea constituents responsible for the prevention of liver injury was soluble dietary fibers, but other constituents of low molecular weights have not yet been identified.

This report describes the partial purification of tea constituents that had a preventive effect on d-galactosamine-induced liver injury in rats from a n-butanol-soluble fraction of green tea.

Green tea (sen-cha) of upper middle grade was extracted by adding 10 volumes (volume/weight) of boiling water to the tea, standing for 30 min at room temperature, and filtering through five sheets of gauze. The extract was lyophilized to give powdery materials by a mixer. The powder of the green tea extract was dissolved in water and extracted successively with equal volumes of chloroform, ethyl acetate, and n-butanol. The yield of the butanol-soluble fraction was 15.7 g per 100 g of green tea extract. The butanol-soluble fraction was fractionated into four fractions (fractions I to IV) by silica gel 60 (Merck, Darmstadt, Germany) column chromatography using chloroform-methanol-water (10:5:1, by volume) as an elution solvent. Since only fraction II had a significant effect on liver injury, as described later, it was further fractionated into three fractions (II-1, II-2, and II-3) by Toyopearl HW-40 (Toso, Tokyo, Japan) column chromatography using methanol-water (1:9, by volume) as an elution solvent. The scheme of fractionation and the yield of each fraction are summarized in Fig. 1. Since only Fraction II-3 had a significant effect on liver injury, this fraction was characterized. Fraction II-3 was very soluble in water and had a light yellow color. This fraction reacted with phenol-H2SO4 to give a brown color, and had a major band at the Rf value of 0.38 when the fraction was analyzed by thin-layer chromatography on silica gel 60F254 (Merck) using butanol-acetate-water (8:2:3, by volume) as developing solvents, made visible with vanillin-H2SO4. Fraction II-3 was further analyzed by HPLC (Model PU-980; Jasco, Tokyo, Japan), equipped with a column (YMC-Pack ODS-AM, 4.6 x 250 mm; YMC, Kyoto, Japan). The mobile phase was aqueous acetic acid (20 ml/l)-acetonitrile (85:15, by volume), the flow rate was 1 ml/min at 40°C, and the elution was monitored at 200, 250 and 350 nm with a multichannel detector (Model MD-910; Jasco). The absorbance spectrum (200 to 500 nm) of each major peak was simultaneously measured. Five major peaks were detected in the HPLC analysis and the absorbance spectra were summarized.
of these and one minor peak were similar (Fig. 2); the values of $\lambda_{max}$ (nm) were 271 and 335 (peak 1; retention time = 12.8 min), 256 and 355 (peak 2; 15.7 min), 259 and 355 (peak 3; 18.2 min), 267 and 347 (peak 4; 23.6 min), 271 and 336 (peak 5; 25.4 min), and 267 and 347 (peak 6; 32.1 min). When quercetin-3-rutinoside (Wako Pure Chemical, Osaka, Japan) was likewise analyzed by HPLC as an authentic glycosidic flavonoid, the values of $\lambda_{max}$ (nm) of the peak (22.9 min) were 256 and 355.

Male rats of the Wistar strain (Japan SLC, Hamamatsu, Japan), 5 weeks old (90-100 g) were used as experimental animals to assess liver injury-preventive effects. The rats were fed on a stock diet (Type MF; Oriental Yeast, Tokyo, Japan) for 3 or 4 d, and then they were given free access to the experimental diets for 14 d in a temperature (23-25°C) and humidity (40-60%)-controlled room with a 12 h cycle of light (06:00-18:00 h) and dark. The composition of the control diet (25% casein diet) was as follows (g/100 g): casein, 25.0; corn starch, 40.1; sucrose, 20.0; corn oil, 5.0; AIN-76 mineral mixture (Oriental Yeast), 3.5; AIN-76 vitamin mixture (Oriental Yeast), 1.0; choline bitartrate, 0.4; and cellulose, 5.0. Supplements were added to the control diet at the expense of starch. Food and water were renewed daily, and the body weight and food intake were also measured daily. Three separate in vivo assays were done in this study. In the first assay, the effects of dietary supplementation with a butanol-soluble fraction (0.47% of diet) on d-galactosamine-induced liver injury was compared with that of a green tea extract (3% of diet); the amount of butanol-soluble fraction introduced into the diet corresponded to that of the 3% green tea extract. In the second and third assays, effects of dietary supplementation with each fraction obtained by column chromatography were assessed; lyophilized powder of each fraction was added to the control diet based on the yield of each fraction. After feeding the experimental diets for 14 d, $\gamma$-galactosamine (Sigma, St. Lois, USA) was injected intraperitoneally at a dose of 350 mg/kg body weight between 14:00 and 14:30 h without starvation before and after injection of the drug. After 22 h, the rats were killed by decapitation between 12:00 and 12:30 to obtain blood and liver. Plasma was separated from heparinized whole blood by centrifugation at 2000 x g for 20 min at 4°C. The activities of plasma alanine aminotransferase (glutamic-pyruvic transaminase, GPT) and aspartate aminotransferase (glutamic-oxaloacetic transaminase, GOT) were measured with a kit (Transaminase C II-Test; Wako Pure Chemicals), the enzyme activity being expressed as I.U. ($\mu$mol/min per l of plasma at 25°C). Data were tested by an analysis of variance, and the difference between
means were tested using Duncan’s multiple range test.  

A p value of 0.05 or less was considered statistically significant.

All of the supplements, except for the green tea extract, did not have any deleterious effects on the body weight gain or food intake; the green tea extract brought about a slight decrease in these variables (data not shown). The effects of green tea extract, butanol-soluble fraction, and each fraction separated by column chromatography on d-galactosamine-induced increase of plasma GPT and GOT activities are depicted in Fig. 3. The d-galactosamine-induced increase of plasma GPT activity was significantly suppressed by the butanol-soluble fraction as well as by the green tea extract (Fig. 3A). Of four fractions obtained by silica gel column chromatography, only fraction II suppressed the increase of plasma GPT activity (Fig. 3B). The effects on plasma GOT activity were essentially similar to those on GPT activity (data not shown). Of three fractions obtained by Toyopearl HW-40 column chromatography, only fraction II-3 suppressed the d-galactosamine-induced increase of plasma GPT and GOT activities (Figs. 3C and 3D).

Thus, this study confirmed that the butanol-soluble fraction of green tea had a preventive effect on d-galactosamine-induced liver injury in rats, and that this effect could be ascribed to constituent(s) included in fraction II-3. The fraction II-3 is assumed to be a mixture of glycosidic flavonoids, since the fraction gave rise to five major peaks of which absorption spectra were all similar to that of authentic flavonol glycoside (quercetin-3-rutinosides) and to the reported data for kaempferol-3-rhamnodiglucosides ($\lambda_{\text{max}}$: 265 and 340 nm) and quercetin-3-rhamnodiglucoside ($\lambda_{\text{max}}$: 255 and 351 nm). Light yellow color, high solubility in water and reactivity with phenol-H$_2$SO$_4$ of fraction II-3 support these assumptions. However, it is currently unclear about the exact structure of each constituent of fraction II-3 and about the structure-activity relationship of green tea flavonoids, because we could not separate fraction II-3 into each constituent on a scale large enough to analyze the structure and to conduct in vivo assays. Further studies are now in progress.

Acknowledgment

This study was supported by the Program for Promotion of Basic Research Activities for Innovative Bioscience (PROBRAIN).

References