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Thermal Effects on the Stability and Antioxidant Activity of an Acid Polysaccharide Conjugate Derived from Green Tea

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A technique of high-performance gel permeation chromatography (HPGPC)−evaporative light-scattering detection and circular dichroism (CD) was developed for the measurement of thermal effects on the homogeneity and conformation of polymeric carbohydrate conjugates and was applied to an acid polysaccharide conjugate (GTa) isolated from the composite enzyme extract of green tea. Incubations in water at 40 and 70 °C for 1.0, 2.5, and 5.0 h have no effects on GTa. In contrast, when incubated in water for 1.0, 2.5, and 5.0 h at 98 °C, a single symmetrical peak corresponding to GTa in HPGPC was split into two adjacent peaks representing two different components formed, and CD spectra revealed an additional positive Cotton effect at 216 nm. To contribute toward our understanding of thermal effects of this polymeric carbohydrate conjugate on antioxidant activity, GTa and related heat-treated samples (GTa-HTI, GTa-HTII, and GTa-HTIII), the latter being obtained from 1.0, 2.5, and 5.0 h incubations at 98 °C, respectively, were subjected to the self-oxidation of 1,2,3-phentriol assay and found to have respective scavenging activities in a concentration-dependent manner. In comparison with GTa, the scavenging potency of heat-treated samples was similar at the dosage range of 50−300 μg/mL but became stronger with continually increasing concentration. Moreover, the present study also provides further insights into the optimal preparation of tea polysaccharide conjugates.

KEYWORDS: Thermal effects; HPGPC-ELSD; CD; tea polysaccharide conjugate; antioxidant activity

INTRODUCTION

From ancient times, tea has extensively been served as a daily beverage and crude medicine in China. A growing body of observations over the years has demonstrated its medical care functions related to antioxidative (*1*), antimutagenic (*2*−*4*), and anticancer effects (*5*). Green tea, a popular type of tea, is solely made utilizing the leaves of *Camellia sinensis*. To the best of our knowledge, catechins and polyphenols present in green tea have been regarded as effective antioxidants and also possess pharmacological activities in association with anticancer, antimutation, and anti-atherosclerosis (*6*−*9*). However, with the exception of these small molecules, abundant attractions have been drawn to tea polysaccharide conjugates (TPCs), which is one of the main functional components in green tea. Investigations on several fronts (*10*−*12*) demonstrate that TPCs contribute to immunological, antiradiation, antiblood coagulation, anticancer, anti-HIV, and hypoglycemic activities. Despite these reports, there is still an important question related to the thermal effects on homogeneous TPCs derived from green tea, to which little attention so far has been given.

The objectives of our work were to investigate the thermal effects of a homogeneous low-grade green tea polysaccharide conjugate (GTa), the latter being released from the composite enzyme extracts, on the homogeneity and conformation utilizing high-performance gel permeation chromatography (HPGPC) and circular dichroism (CD) techniques and on the scavenging powder of the self-oxidation of 1,2,3-phentriol aimed at ascertaining whether a continued heating process was involved in the loss or increase of antioxidant potency.

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MATERIALS AND METHODS

Materials. Low-grade green tea was donated by the Tea Research Institute of China Academy of Agricultural Sciences (Hangzhou, Zhejiang Province). Cellulase, hemicellulase, and pectinase were purchased from Novozymes (China) Biotechnology Co., Ltd. D-Glucose (Glc) and D-galacturonic acid (GalA) were obtained from Sigma Chemical Co. (St. Louis, MO). Diethylaminoethyl (DEAE) cellulose DE-52 was acquired from Whatman (Kent, England). The protein determination kit was purchased from Biyuntian Co., Ltd. (China). All other reagents were of analytical grade unless otherwise specified.

Preparation of TPCs. The powder of low-grade green tea (100 g) was suspended in 2000 mL of distilled water containing 0.05% (w/v) of pectinase, hemicellulase, and cellulase and incubated at 40 °C for up to 4 h under the continuous stirring condition. The extract was filtered, centrifuged (8000 rpm, 6 min), concentrated by a vacuum freeze dryer, and deproteinized with trichloroacetic acid (TCA). After it was centrifuged at 9000 rpm for 5 min, the supernate was adjusted to pH 7.0 by sodium

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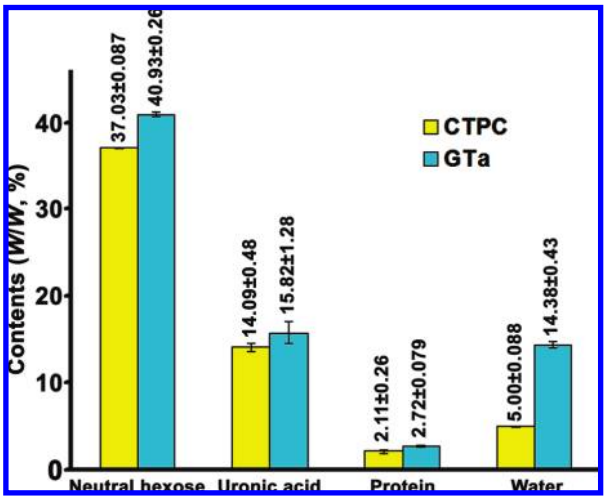

Figure 1. Levels of chemical composition in CTPC and GTa. The data (numerical values, %) were expressed as means ± SDs of three different trials. CTPC, crude tea polysaccharide conjugates; GTa, a homogeneous green TPC obtained by the anion-exchange chromatography of CTPC.

hydroxide (NaOH), bleached with H_2O_2 at 25 °C for 3 h, and precipitated with 4 volumes of absolute ethanol. Subsequent dialysis against distilled water for 24 h gave rise to the retentate, the latter being collected and lyophilized to afford crude tea polysaccharide conjugates (CTPCs; 3.15%, yield). An aliquot (1.6 g) was then loaded for anion-exchange chromatography onto a DEAE-cellulose DE-52 chromatography column (3.6 cm × 40 cm), eluted stepwise with water and 0.25 and 0.40 M NaCl solution to give one main fraction, which was collected, dialyzed, and lyophilized to afford GTa (980 mg) for the subsequent use.

Preliminary Analysis of CTPC and GTa. Neutral hexose contents were measured by the anthrone−sulfuric acid method, using glucose as the standard (*13*). Total uronic acid was estimated by *m*-hydroxydiphenyl method using galacturonic acid as the standard (*14*). The protein contents were determined according to the method of Bradford (*15*) with Coomassie Brilliant Blue and bovine serum albumin (BSA) as the standard. To test the moisture, samples (16 mg) were placed in evaporation dish and heated to constant weight at 115 °C.

Distribution of Homogeneous Polysaccharide Conjugates. Solutions of CTPC and GTa in water (2 mg/mL) were, respectively, applied to an Agilent 1100 high-performance liquid chromatography (HPLC) system (United States) equipped with a gel filtration chromatographic column (7.8−300 mm) of TSKgel G4000PWXL (Tosoh, Tokyo, Japan) under a constant flow (0.6 mL/min) of deionized water at 40 °C. The injection volume was 20 μL, and the eluate was monitored by an evaporative light-scattering detector (ELSD) 2000ES (Alltech, United States) with nitrogen as the nebulizer gas (flow rate, 3.2 L/min). The drift tube temperature was set at 115 °C.

Thermal Assay of GTa. A solution of GTa (50 mg) in 25 mL of deionized water was incubated at 40, 70, and 98 °C, and untreated GTa in water (2 mg/mL) was used as the control. After 1, 2.5, and 5 h of incubations, a portion (10 μL) of the cultures/the control was subjected to HPLC (as described above). To obtain the optimal detection of CD, all of the samples were diluted to 0.5 mg/mL with water.

Spectroscopic Analysis. A portion (120 μL) of solutions (10 mg/mL) of CTPC and GTa in deionized water was diluted to 5 mL with the same solvent, respectively. Following centrifugation, ultraviolet−visible (UV−vis) spectra of their supernates were recorded with a Shimadzu UV-2550 spectrophotometer (Japan) in the range 200−700 nm. CD spectra of samples were recorded with a Jasco J-815 CD spectrophotometer.

Self-Oxidation of 1,2,3-Phentriol Assay. The scavenging potency for the self-oxidation of 1,2,3-phentriol of GTa, as well as its heat-treated samples incubated at 98 °C for 1, 2.5, and 5.0 h (GTa-HTI, GTa-HTII, and GTa-HTIII), was evaluated according to the method of Marklund (*16*) with a minor modification. A total of 2.0 mL of a range of solutions of different concentrations of each sample were mixed with 1.5 mL of 0.05 M Tris−HCl buffer (pH 8.0) containing 1 mM EDTA and 0.5 mL of 6 mM

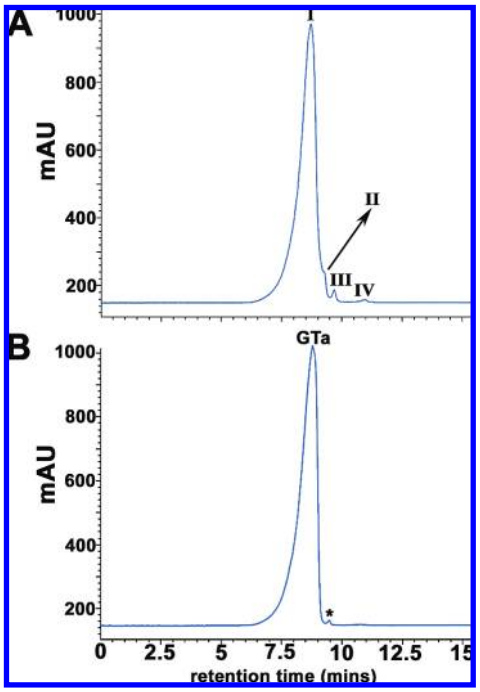

Figure 2. Elution profiles of CTPC (**A**) and GTa (**B**) in HPGPC, using an ELSD 2000ES (Alltech, United States). Four polysaccharide conjugates were observed in CTPC, namely, I (96.37%), II (1.74%), III (1.28%), and IV (0.61%). A negligible trace impurity (*) was present in GTa, probably generated from the matrix of DEAE-cellulose during the process of purification.

1,2,3-phentriol and then shaken rapidly at room temperature. It was accompanied that the final concentrations of tested samples were diluted to 50, 150, 250, 400, and 500 μg/mL. The absorbance of the mixture was determined at 320 nm per 30 s for 4 min against a blank, and a slope was calculated as absorbance/min. The potency to scavenge superoxide radicals was calculated using the following equation: scavenging % = (1 − slope of sample/slope of control) × 100%.

RESULTS AND DISCUSSION

Preliminary Chemical Analysis of CTPC and GTa. A comparison of chemical composition between CTPC and GTa is shown in **Figure 1**. The composite enzyme extracts of green tea underwent the filtration, centrifugation, neutralization, concentration, and precipitation with ethanol to give CTPC, the latter being a white and loose powder. GTa, a bright white and loose powder, was obtained after purifying by DEAE-cellulose DE-52 anion-exchange column chromatography. Interestingly, both of them easily absorbed moisture, in terms of which the moisture content in GTa was almost three times higher than that in CTPC. Following the removal of water, the contents of neutral hexose, uronic acid, and protein were estimated, and in comparison with CTPC, all of the contents in GTa exhibited no significant difference.

Distribution of Homogeneous Components in CTPC and GTa. The profile of CTPC in HPGPC (**Figure 2A**) reflected a total of four homogeneous peaks, that is, I (96.37%), II (1.74%), III (1.28%), and IV (0.61%), in which I as the predominant component was eluted at 8.72 min (retention time, RT). Further separation of CTPC by anion-exchange chromatography gave rise to GTa, the HPGPC of which (**Figure 2B**) exhibited a single symmetrical peak and a RT of 8.79 min. The latter was similar to that of I, demonstrating that GTa and I were the identical carbohydrate conjugates. Here, it was also worthy of mention that at this time the relative molecular weight of GTa could not be

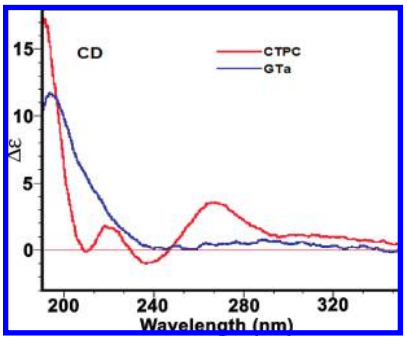

Figure 3. CD spectra of CTPC (red) and GTa (blue). All of the spectra were measured in deionized water.

determined since we failed to prepare the calibration curve by reference to standard dextrans that showed multiple peaks monitored by ELSD.

Spectroscopic Characteristics. The UV−vis scanning spectra (not shown) of CTPC and GTa were carried out in the range of 200−700 nm. A broad absorption peak in CTPC was observed in the 240−300 nm region with the inflection at 257 nm. Conversely, GTa displayed a weak "wavelike" absorption peak in the region of 255−300 nm. To our knowledge, the characteristic absorption peaks of protein and nucleic acid were observed at ∼280 and ∼260 nm, respectively. It was evident that the peaks corresponding to the regions of 240/255−300 nm were associated with protein and nucleic acid. Moreover, in comparison with the $OD_{280\ nm}/OD_{260\ nm}$ ratios of protein and nucleic acid determined to be ∼1.8 and ∼0.5, respectively (*17*), the $OD_{280\ nm}/OD_{260\ nm}$ value of CTPC was calculated as 0.63, while that of GTa was 0.86, supporting the existence of protein and nucleic acid. To provide further insight into the physical and chemical character, CD experiments were performed on CTPC and GTa. As depicted in **Figure 3**, the CD spectrum of CTPC (red) revealed positive Cotton effects at 191, 218, and 266 nm as well as a negative Cotton effect at 237 nm. Conversely, the CD spectrum of GTa only showed a positive Cotton effect at 194 nm.

Thermal Effects on GTa. To investigate the thermal effects on the stability of GTa, solutions of it in water were incubated at 40, 70, and 98 °C for 1.0, 2.5, and 5.0 h. It was observed that HPGPC and CD patterns of both 40 and 70 °C incubation (**Figure 4A**,**E**) were similar across that of untreated GTa, revealing that the components and conformation were unchanged with a positive Cotton effect at 194 nm. Interestingly, when incubated at 98 °C for 1.0 h, changes represent in HPGPC and CD profiles occurred and were even more notable accompanying the longer heating time (2.5 and 5.0 h), as depicted in **Figure 4**. By comparison with a slightly splited peak present in the 1 h (**Figure 4B**) heat-treated product of GTa (GTa-HTI), 2.5 (**Figure 4C**) and 5.0 h (**Figure 4D**) heat-treated products (GTa-HTII and GTa-HTIII) showed clearer doublet peaks. The latter in GTa-HTII were calculated to account for 82.7 and 17.3%, whereas those of GTa-HTIII were 80.9 and 19.1%, respectively, indicative of continuous heating at 98 °C that may contribute to the formation and isolation of heat-generated doublet peaks. In contrast, with the exception of the positive Cotton effect at 194 nm, CD spectra (**Figure 4F−H**) revealed an additional peak reflecting a positive Cotton effect at 216 nm, the intensity of which was increased in a heating time-dependent manner.

Scavenging Activity of Self-Oxidation of 1,2,3-Phentriol. The scavenging effects of GTa and three related heat-incubated GTa-HT(I−III) on self-oxidation of 1,2,3-phentriol were investigated as depicted in **Figure 5**; all of these tested samples were found to possess the dose-dependent inhibitory activities against

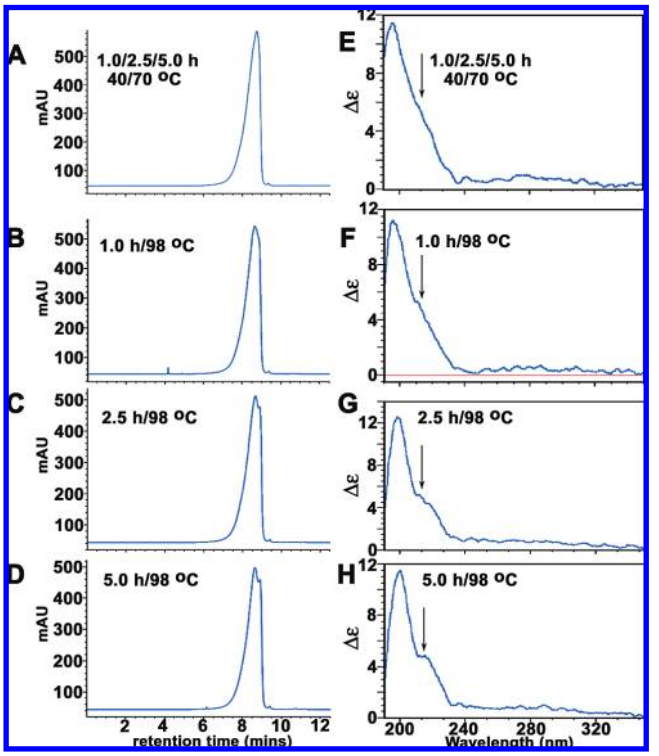

Figure 4. HPGPC (left) and CD (right) analysis of heat-incubated products derived from GTa. Representative HPGPC chromatogram (**A**) and CD spectrum (**E**) of GTa incubated at 40 and 70 °C for 1, 2.5, and 5 h. HPGPC chromatograms (**B**−**D**) and CD spectra (**F**−**H**) of GTa after 1.0, 2.5, and 5.0 h incubations in water at 98 °C. The arrows indicate the site of heat-induced change monitored by CD.

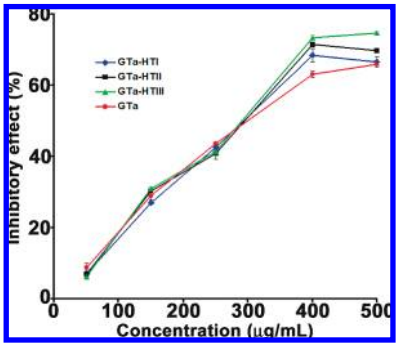

Figure 5. Scavenging activities of self-oxidation of 1,2,3-phentriol of GTa and related heat-treated samples (GTa-HTI, GTa-HTII, and GTa-HTIII) obtained after incubation at 98 °C for 1, 2.5, and 5.0 h, respectively. Each sample was assayed in triplicate for each concentration. Experimental results were means ± SDs of three parallel measurements.

self-oxidation of 1,2,3-phentriol, which grew as their concentrations increased to a certain extent, and to exhibit a similar capacity and rapid increase on the scavenging effects for the concentrations between 50 and 300 μg/mL. However, with continually increasing dosage, it was interesting to note that a little difference could be observed on the scavenging potency of tested samples, which followed the decreased order GTa-HTIII > GTa-HTII > GTa-HTI > GTa.

Prior to drinking, tea is well-known to be soaked in hot water for some time using a covered ceramic pot in China and Southeast Asia. For this traditional procedure, there is no question that it is important to know whether the soaking water temperature and time will be correlated with the quality of tea. Evidence described

in the literature (*18*) has demonstrated that the antioxidant activity of solutions of oolong tea in water increased with the increasing extraction temperature and time of soaking. Therefore, we envisioned that the components present in the water extract of tea vary with incubating temperature and time and thereby resulted in the change of antioxidant activity. In this publication, we introduced thermal effects of an acid polysaccharide conjugate (GTa) isolated from green tea on homogeneity and conformation and inhibitory potency against self-oxidation of 1,2,3-phentriol. On the basis of the above results, with incubating times of 1.0, 2.5, and 5.0 h at 98 °C, GTa was split into two adjacent peaks represented in HPGPC monitored using ELSD as well as new positive Cotton effects at 216 nm observed in CD spectra, and its scavenging activities of self-oxidation of 1,2,3-phentriol were similar at the dosage range of 50−300 μg/mL but increased with continually increasing dosage. It was postulated that thermal effects of GTa on the homogeneity and conformation were, at least in part, responsible for their varying antioxidant activity. A method of HPGPC-ELSD and CD for evaluation of the thermal variation of TPC was described and should be applicable to other homogeneous polysaccharide conjugates. Further research will be directed toward a more detailed characterization of GTa that may contribute to an established CD database for polysaccharide conjugates.

ABBREVIATIONS USED

HPGPC, high-performance gel permeation chromatography; ELSD, evaporative light-scattering detector; CD, circular dichroism; TCA, trichloroacetic acid; NaOH, sodium hydroxide; UV−vis, ultraviolet−visible; RT, retention time; CTPC, crude tea polysaccharide conjugates; DEAE, diethylaminoethyl.

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