The Effects of 1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose on Rat Liver Mitochondrial Respiration

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The inhibitory effects of pure galloylglucose (1,2,3,4,6-penta-*O*-galloyl-β-D-glucose) on the respiratory chain of rat liver mitochondria were investigated. The respiratory control ratio (RCR) decreased by 50% on addition of 20 μM pentagalloylglucose to highly coupled mitochondria, but the adenosine-5′-diphosphate/oxygen (ADP/O) ratio decreased only slightly. The RCR disappeared and the ADP/O ratio could not be measured at concentrations of pentagalloylglucose above 30 μM. On the other hand, the uncoupler-induced oxygen consumption was also inhibited. These findings suggest that pentagalloylglucose at low concentrations inhibits the electron transport system to decrease the RCR, but scarcely impairs the membrane, practically retaining the coupled reaction, while at high concentrations it impairs the structural integrity of the mitochondrial membrane.

Pentagalloylglucose competitively inhibited succinate dehydrogenase activity, and noncompetitively inhibited reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase and ubiquinol-1 oxidase activities of submitochondrial particles (SMP). However, it did not show significant inhibition of the cytochrome c oxidase activity of SMP. It is thus concluded that pentagalloylglucose, which is the lowest-molecular-weight component of tannic acid, exerts its effect on mitochondrial respiration and oxidative phosphorylation through action on the membrane and on succinate dehydrogenase, NADH dehydrogenase and cytochrome bc_1 complex of mitochondria.

Keywords pentagalloylglucose; mitochondria; dehydrogenase; ubiquinol-1 oxidase; submitochondrial particle

Introduction

Tannin, a general term for water-soluble polyphenols contained in plants, is a major component in various oriental medicinal plants. Tannic acid, which is a kind of tannin (also called Chinese gallotannin), is readily available as a commercial reagent prepared from Chinese nutgall. As regards biological activities of tannins, inhibition of lipid peroxidation,[1] decrease of blood urea-nitrogen content[2] and inhibition of plasmin activity[3] have been reported.

We have recently observed that tannic acid exerted its antibacterial effect on *Photobacterium phosphoreum* through inhibition of the respiratory chain,[4,5] and that it also inhibited the oxidative phosphorylation and the respiratory chain of rat liver mitochondria.[6] However, it was necessary to use a pure compound in order to elucidate in detail the inhibitory mechanism of tannic acid, because tannic acid contains many structural analogues composed of glucose and gallic acid.[7] In this work, we therefore studied the inhibitory effect on the respiratory chain of rat liver mitochondria using the lowest-molecular-weight compound, pentagalloylglucose (1,2,3,4,6-penta-*O*-galloyl-β-D-glucose, PGG), purified from the tannic acid.

Materials and Methods

Preparation of Mitochondria Rat liver mitochondria were prepared according to the method of Hogeboom[8] with some modification.[6] Wistar rats (male, 250—300 g weight), purchased from Sankyo Labo. Co., were used in this study.

Preparation of Submitochondrial Particles (SMP) The rat liver SMP were prepared according to the method of Gregg[9] with some modification.[6] The isolated mitochondria were sonicated with a Tomy Seiko UR-200p ultrasonic disrupter.

Assay of Electron Transport Activity of Mitochondria The succinate-dependent respiration rate of mitochondria was determined polarographically in an oxygen monitor equipped with a Clark-type oxygen electrode as described by Estabrook.[10] Freshly prepared mitochondria were incubated in the reaction chamber containing 1 ml of the assay mixture (125 mM sucrose, 20 μM cytochrome c, 50 mM KCl, 6 mM $MgCl_2$, 20 μM rotenone, and 15 mM phosphate buffer, pH 7.0) at 25 °C for 3 min before addition of 10 mM succinate. The substrate-induced oxygen consumption was plotted on a strip chart recorder, and then 200 μM adenosine 5′-diphosphate (ADP) was added to the assay system to induce state 4 respiration. The respiratory control ratio (RCR) was expressed as the ratio of the respiration rate of state 3 to that of state 4.

Ubiquinol-1 Oxidase Activity of SMP Ubiquinol-1 oxidase of SMP was assayed spectrophotometorically. Ubiquinol-1 was added to the assay mixture (0.2 mg of SMP, pentagalloylglucose and 30 mM potassium phosphate buffer, pH 6.8) at 25 °C, and the activity was measured by recording the increase of absorbance of ubiquinone at 278 nm. When the pH of the assay medium of ubiquinol-1 oxidase was higher than 7.0, the absorbance at 278 nm spontaneously increased in the absence of SMP. We adjusted the pH of the assay medium to pH 6.8, at which the spontaneous increase of the absorbance disappeared and the activity of ubiquinol-1 oxidase was retained (91% of the activity at pH 7.4).

Succinate Dehydrogenase and Reduced Nicotinamide Adenine Dinucleotide (NADH) Dehydrogenase Activities of SMP The two dehydrogenase activities were determined spectrophotometrically by measuring the absorbance change of 2,6-dichloroindophenol (DCIP) at 600 nm as described previously.[6]

Cytochrome c Oxidase Activity of SMP The oxidase activity was determined polarographically as described previously.[6]

Determination of Protein Protein concentration was determined by the method of Lowry *et al.*[11] with bovine serum albumin as a standard.

Pentagalloylglucose PGG isolated from Chinese gallotannin was a generous gift from Drs. I. Nishioka and G. Nonaka. This pentagalloylglucose showed a single peak in reverse-phase high performance liquid chromatography (HPLC).

Chemicals Ubiquinol-1 was a generous gift from Eisai Co., Ltd. Other chemical reagents used were of the highest grade commercially available.

Results

The Effects on the Respiratory Control and Oxidative Phosphorylation We have shown previously that tannic acid inhibits the oxidative phosphorylation and the respiratory chain of rat liver mitochondria.[6] The effect of pentagalloylglucose, which is one of the major components of tannic acid, was therefore studied. The respiration of the intact mitochondria is closely controlled by the availability of ADP in the assay medium. The addition of a suitable amount of ADP induced an approximately four-fold increase in the rate of succinate-dependent respiration. However, in the presence of pentagalloylglucose, which consists of five galloyl groups and a D-glucose residue (Fig. 1), the

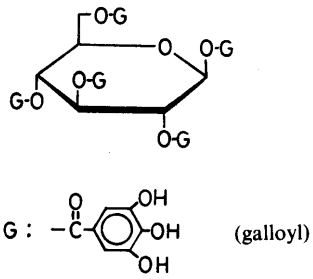

Fig. 1. The Structure of PGG

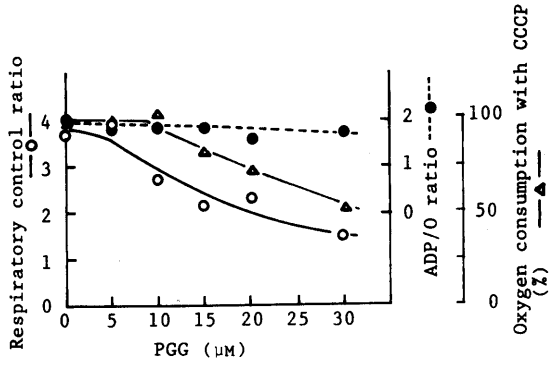

Fig. 2. Effects of Pentagalloylglucose on the Respiratory Control of Rat Liver Mitochondria

(○), RCR; (△), succinate-dependent respiration in the presence of 0.1 μM CCCP; (●), ADP/O ratio. The control uncoupling activity of 0.124 μmol O_2/min/mg of mitochondrial protein was taken as 100%. Mitochondria (0.8 mg of protein/ml) were preincubated in the assay mixture (125 mM sucrose, 20 μM cytochrome c, 50 mM KCl, 6 mM $MgCl_2$, 20 μM rotenone, and 15 mM phosphate buffer, pH 7.0) for 3 min at 25 °C in the presence or absence of pentagalloylglucose before addition of 10 mM succinate. The concentrations of ADP and CCCP added to the assay mixture were 200 and 0.1 μM, respectively.

RCR decreased (Fig. 2). This effect was found to be dose-dependent. On addition of more than 30 μM pentagalloylglucose, respiratory control disappeared. On the other hand, the ratio between the amount of ADP added and the oxygen consumed in state 3 (ADP/oxygen (O) ratio) was slightly decreased from 2.0 to 1.7 by the addition of the inhibitor. The oxygen consumption induced by an uncoupler, carbonylcyanide *m*-chlorophenylhydrazone (CCCP), was also inhibited by pentagalloylglucose (Fig. 2). While these inhibitory patterns of pentagalloylglucose are similar to that of tannic acid as described previously,[6] the inhibitory effect of pentagalloylglucose (50% inhibitory concentration (IC_{50}) = 18 μg/ml) was approximately three times stronger than that of tannic acid (IC_{50} = 50 μg/ml). We also found that the substrate-induced respiration (state 4) was slightly increased by low concentrations of pentagalloylglucose as well as tannic acid (data not shown).[6]

The Effects of Pentagalloylglucose on the Electron Transport Activities of the Respiratory Chain of SMP From the fact that pentagalloylglucose inhibits the oxygen consumption in the presence of CCCP, we presumed that pentagalloylglucose directly inhibited the respiratory chain of mitochondria. In order to determine the inhibitory sites of pentagalloylglucose in the respiratory chain, we studied the effects of pentagalloylglucose on SMP. At first, we investigated the activities of NADH dehydrogenase and succinate dehydrogenase, which catalyze the

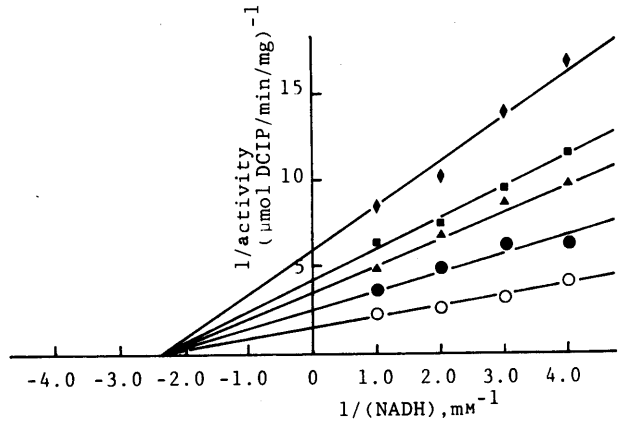

Fig. 3. Double-Reciprocal Plot of the Effect of Pentagalloylglucose on NADH Dehydrogenase Activity of the SMP

The concentrations of PGG were 0 (○), 1 (●), 3 (▲), 5 (■) and 10 μM (♦).

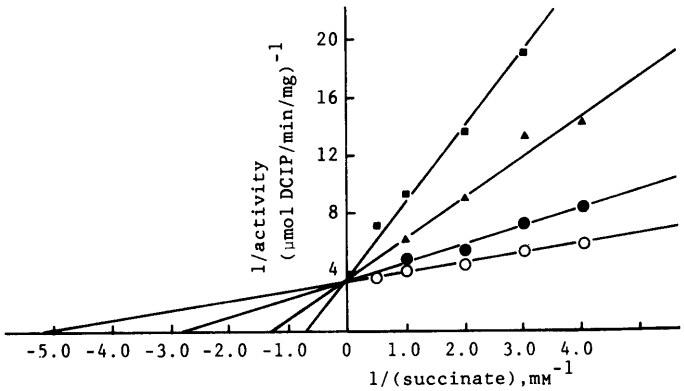

Fig. 4. Double-Reciprocal Plot of the Effect of Pentagalloylglucose on Succinate Dehydrogenase Activity of the SMP

The concentrations of PGG were 0 (○), 5 (●), 10 (▲) and 15 μM (■).

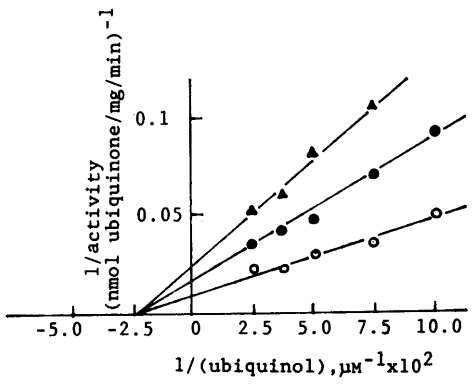

Fig. 5. Double-Reciprocal Plot of the Effect of Pentagalloylglucose on Ubiquinol-1 Oxidase Activity of the SMP

The concentrations of PGG were 0 (○), 10 (●) and 15 μM (▲).

first steps of the electron transfer reaction in the respiratory chain. As shown in Fig. 3, pentagalloylglucose inhibited NADH dehydrogenase activity, and its inhibitory pattern was noncompetitive. Succinate dehydrogenase was also inhibited by pentagalloylglucose, but its inhibitory pattern was competitive (Fig. 4). Furthermore, pentagalloylglucose inhibited ubiquinol-1 oxidase activity, and its in-

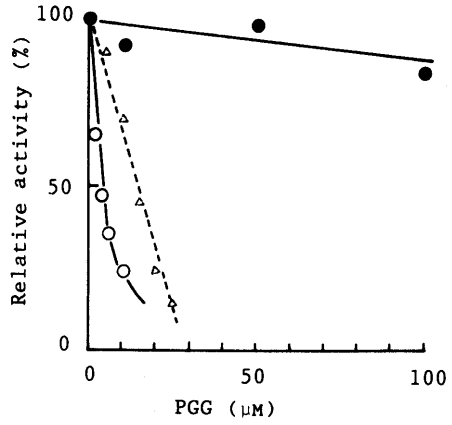

Fig. 6. Effects of Pentagalloylglucose on Cytochrome Oxidase Activity of the Rat Liver SMP

Cytochrome c oxidase activity (●) was compared with NADH dehydrogenase activity (○) and succinate dehydrogenase activity (△). The control activities were 0.02 μmol O_2/min/mg of protein, 0.421 and 0.24 μmol DCIP/min/mg of protein, respectively.

hibitory pattern was noncompetitive (Fig. 5). Therefore, it is indicated that pentagalloylglucose inhibits not only the above dehydrogenases but also the electron transport posterior to cytochrome b. Pentagalloylglucose inhibited the activities of the above enzymes, but did not significantly inhibit the activity of cytochrome c oxidase, the terminal oxidase of the respiratory chain (Fig. 6).

Discussion

We have shown in the previous report that tannic acid inhibits mitochondrial respiration.[6] In this study, it was indicated that pentagalloylglucose, which is the lowest-molecular-weight tannin contained in Chinese gallotannin,[7] also has an inhibitory effect on the mitochondrial respiration. When succinate was used as a substrate, the RCR and the ADP/O ratio of prepared mitochondria were about 3.7 and 2.0, respectively. These data indicated that the mitochondrial preparation was highly coupled and active in oxidative phosphorylation. When pentagalloylglucose was added to the assay mixture, the RCR was decreased dose-dependently, and the ADP/O ratio was slightly decreased. The above tendency was similar to that found with the tannic acid in the previous report.[6] The addition of the uncoupler CCCP did not fully release the depression of the mitochondrial respiration in the presence of pentagalloylglucose. It was suggested that pentagalloylglucose inhibits the electron transport system and this mainly contributed to the decrease of the RCR. At higher concentrations of pentagalloylglucose (above 30 μM), respiratory control was not observed and the ADP/O ratio could not be measured. These results indicate that pentagalloylglucose impairs the mitochondrial membrane, and then directly inhibits the exposed electron transport system. From the result of inhibitory experiments with SMP, the inhibitory sites of pentagalloylglucose on the electron transport system are considered to be succinate dehydrogenase, NADH dehydrogenase and cytochrome bc_1 complex. The inhibitory pattern of pentagalloylglucose on succinate dehydrogenase was competitive, but in other cases it was noncompetitive. The previous report indicated that tannic acid inhibited NADH dehydrogenase and succinate dehydrogenase, in a competitive manner.[6] Since tannic acid contains many structural analogues of gallic acid and glucose, pure pentagalloylglucose isolated from Chinese gallotannin might show a different inhibitory pattern from that of tannic acid. On the other hand, pentagalloylglucose was a competitive inhibitor of succinate dehydrogenase. It is not yet clear how pentagalloylglucose reacts with succinate dehydrogenase. One possible explanation for the competitive inhibition is that the structure of succinate is similar to that of a part of the galloyl group, from the *meta*-hydroxy group to the carboxyl group.

TABLE I. Inhibitory Characteristics of Pentagalloylglucose on the Enzymes of SMP

Enzyme	Inhibitor constant K_i (μM)	Type of inhibition
Succinate dehydrogenase	1.5	Competitive
NADH dehydrogenase	3.7	Noncompetitive
Ubiquinol-1 oxidase	7.5	Noncompetitive

From the above results, we may draw the following conclusions. At lower concentrations, pentagalloylglucose inhibits the electron transport of mitochondria to decrease the RCR, but hardly inhibits the coupled reaction. At higher concentrations, pentagalloylglucose may impair the membrane barrier of mitochondria to dissipate the coupled reaction. Pentagalloylglucose competitively inhibits succinate dehydrogenase and noncompetitively inhibits NADH dehydrogenase and ubiquinol-1 oxidase (probably cytochrome bc_1 complex).

Many inhibitors of the respiratory chain, such as rotenone, antimycin A, and cyanide, have single inhibitory sites.[12-14] On ther other hand, pentagalloylglucose has at least three inhibitory sites on the respiratory chain of mitochondria, and the values of its inhibitor constant (K_i) indicate that it is a strong inhibitor of these enzymes (Table I). Therefore, pentagalloylglucose is a unique inhibitor acting at multiple sites. A study on the effects of pentagalloylglucose on the purified mitochondrial enzymes is in progress in our laboratory.

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