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Inhibitory Effects of Pentagalloylglucose on the Respiratory Chain of *Photobacterium phosphoreum*

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The activity of glucose-dependent oxygen consumption of whole cells of *Photobacterium phosphoreum* was inhibited by purified pentagalloylglucose (1,2,3,4,6-penta-*O*-galloyl-β-D-glucose). Sonicated membrane vesicles were used to study the effect of the inhibitor on the electron transport activity. The activity of reduced nicotinamide adenine dinucleotide (NADH) oxidase in sonicated membrane vesicles of *P. phosphoreum* decreased when pentagalloylglucose was added to the assay system. The results suggested that the targets of inhibitory action were NADH dehydrogenase and terminal oxidase.

The effect of the inhibitor on the terminal oxidase was compared in the case of purified terminal oxidase (cytochrome bd complex) and sonicated membrane vesicles. The oxidase activities toward ubiquinol-1 and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) in the presence of ascorbate were both inhibited by pentagalloylglucose in sonicated membrane vesicles and purified cytochrome bd complex. The inhibition of ubiquinol-1 oxidase activity in sonicated membrane vesicles and purified enzyme was of noncompetitive type. On the other hand, the inhibitions of TMPD plus ascorbate oxidase in both membrane vesicles and purified enzyme were uncompetitive. Thus, the mechanisms of inhibition of the two kinds of oxidase activities by the inhibitor were different.

The inhibitory effect of tetragalloylglucose (1,2,3,6-tetra-*O*-galloyl-β-D-glucose) on the respiratory chain was similar to that of pentagalloylglucose in the case of membrane vesicles and purified enzyme.

Keywords——pentagalloylglucose; cytochrome bd complex; *Photobacterium phosphoreum*; terminal oxidase; respiratory chain

Introduction

Tannins are contained in a number of oriental medicinal plants and drugs, and their effects have been investigated. As regards biological activities of tannins, inhibition of lipid peroxidation,[1] decrease of blood urea-nitrogen content,[2] inhibition of plasmin activity,[3] and several other effects have been reported. However, there are very few reports on the antibacterial activities.

Tannic acid contained in Chinese galls is a mixture of gallotannins (galloylglucoses).[4] Recently we have shown that tannic acid inhibited the growth of *Photobacterium phosphoreum* and that the target sites of tannic acid in the respiratory chain were in reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase and the terminal oxidase complex.[5]

We have previously purified and characterized the terminal oxidase complex of *P. phosphoreum* grown under aerobic conditions.[6] This enzyme, a cytochrome bd complex, is composed of two polypeptides with molecular weights of 41000 and 54000, and contains protoheme (heme b) and heme d as prosthetic groups. The purified oxidase complex showed

N,N,N',N'-tetramethyl-*p*-phenylenediamine dihydrochrolide (TMPD)-dependent oxygen consumption in the presence of ascorbate as a substrate, and also showed ubiquinol-1 oxidase activity.[6]

Commercial tannic acid is a very complex and non-uniform mixture.[4] In this work, we therefore studied the inhibitory effect using one of the major components, pentagalloylglucose (1,2,3,4,6-penta-*O*-galloyl-*β*-D-glucose), purified from tannic acid.

Materials and Methods

Organism——*P. phosphoreum* strain IAM12085 was a generous gift from the Institute of Applied Microbiology, the University of Tokyo, and was grown in the medium described by Watanabe *et al.*[7] Inocula of 10 ml of seed culture were incubated in 2 l of medium in 5-l glass containers at 25 °C for 20 h with vigorous aeration by shaking, and cells were harvested in the exponential phase of growth. The cells were stored at −20 °C before use.

Preparation of Membrane Vesicles——The frozen cell pellet was thawed and suspended in buffer A (10 mM Tris–HCl (pH 7.0) containing 10 mM $MgCl_2$). The suspension was sonicated with a Tomy Seiko UR-200p ultrasonic disruptor with cooling in an ice bath. The sonicated lysate was centrifuged at $20000 \times g$ for 30 min, and the supernatant obtained was centrifuged at $100000 \times g$ for 1 h. The precipitate was washed with buffer A by centrifugation, and suspended in buffer A.

Preparation of Cytochrome bd Complex——Cytochrome bd complex was solubilized and purified from *P. phosphoreum* as described previously.[6]

Assay of Oxidase Activity——Ubiquinol-1 oxidase activity of the purified enzyme was assayed spectrophotometrically as described previously.[8] A mixture (20 μl) of cytochrome bd complex (1 μg), phospholipids (acetone-washed soybean phospholipids, asolectin 2 mM), and 50 mM Tris–HCl (pH 6.8) was incubated at 4 °C for 5 min, and the activity was measured at 25 °C by recording the increase of absorbance of ubiquinol-1 at 278 nm. The activities for oxidation of NADH, and oxidation of TMPD in the presence of ascorbate were measured according to the methods of Kasahara and Anraku,[9] and Kita *et al.*,[10] respectively, using a Clark type oxygen electrode (Rank Brothers, Rank oxygen electrode).

Assay of NADH Dehydrogenase Activity——Assay of NADH-menadione dehydrogenase activity was carried out by the method of Thomson and Shapiro.[11]

Because the activities of ubiquinol-1 oxidase, NADH oxidase, and NADH dehydrogenase in sonicated membrane, and that of ubiquinol-1 oxidase of the purified enzyme, increase by about 20% in the presence of 0.025% Triton X-100, Triton was added to the assay mixture in those assays.

Other Method——Protein was determined by the method of Lowry *et al.* with bovine serum albumin as a standard.[12]

Pure Tetragalloyl- and Pentagalloylglucose——Tetragalloylglucose (1,2,3,6-tetra-*O*-*β*-galloyl-D-glucose) and pentagalloylglucose were generous gifts isolated from Chinese gallotannin[4] and Turkish gallotannin,[13] respectively, by Drs. I. Nishioka and G. Nonaka.

Chemicals——Ubiquinol-1 was a generous gift from Eisai. Other chemical reagents used were of the highest purity commercially available.

Results

Effect on the Electron Transfer Activities of the Respiratory Chain

We have shown previously that tannic acid inhibits the growth of *P. phosphoreum*, and one of the inhibitory sites is probably the respiratory chain.[5] The effect of pentagalloylglucose, which is one of the major components of tannic acid and the one having simplest structure,[4] was studied. In the case of whole cells, glucose dependent oxidase activity was not inhibited in the presence of pentagalloylglucose, if preincubation of cells with the inhibitor was omitted before the assay of activity (not shown). This finding indicates that inhibitor hardly passes through the outer membrane, peptideglycan, or inner membrane of whole cells. However, after incubation for 30 min, the oxidase activity of cells was inhibited by the inhibitor. Therefore, the pentagalloylglucose is probably taken up slowly by simple diffusion into cells.

In order to avoid this type of complication, we used sonicated membrane vesicles for the examination of the effect of the inhibitor on the respiratory chain of *P. phosphoreum*. Figure 1

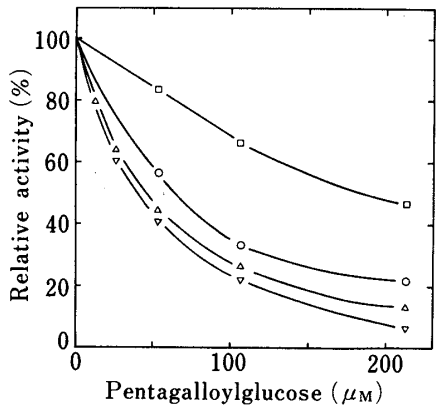

Fig. 1. Inhibition of the Electron Transfer Activities of *P. phosphoreum* by Pentagalloylglucose

Sonicated membrane vesicles were used for the assay of activity. (○), NADH oxidase; (▽), NADH dehydrogenase; (△), ubiquinol-1 oxidase; (□), TMPD+ascorbate oxidase. NADH dehydrogenase activity was determined by NADH-menadione assay. NADH oxidase activity and TMPD+ascorbate oxidase activity were assayed with a Clark-type electrode. Ubiquinol-1 oxidase activity was measured as described in Materials and Methods. The concentrations of ubiquinol-1 and TMPD used in these experiments were 200 μM and 2 mM, respectively. In each assay, the membrane preparation was incubated with the indicated concentration of pentagalloylglucose at 25 °C for 5 min.

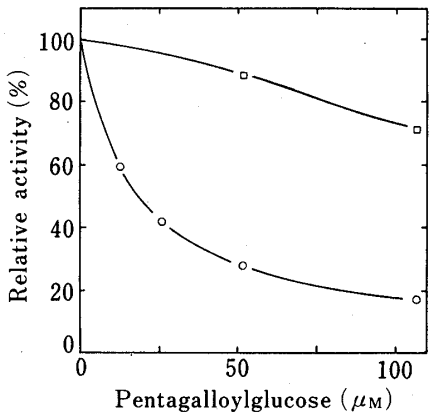

Fig. 2. Inhibition of the Electron Transfer Activity of Purified Cytochrome bd Complex of *P. phosphoreum* by Pentagalloylglucose

The assay procedures were the same as described in the legend to Fig. 1. The ubiquinol-1 oxidase (○) and TMPD+ascorbate oxidase activities (□) were measured.

shows that pentagalloylglucose quite effectively inhibited NADH oxidase, NADH dehydrogenase, and ubiquinol-1 oxidase, and less effectively inhibited TMPD oxidase activity (in the presence of ascorbate) of the respiratory chain of the sonicated membrane vesicles. At the concentration of 100 μM, pentagalloylglucose inhibited about 75% of ubiquinol-1 oxidase and NADH dehydrogenase activities, and about 65% of NADH oxidase activity. However, about 30% of TMPD oxidase activity was inhibited at the same concentration of pentagalloylglucose. Since NADH oxidase includes the NADH dehydrogenase and terminal oxidase reactions as partial reactions, the effect of the inhibitor is comprehensive. These results show that the sites of inhibition are located in NADH dehydrogenase and terminal oxidase of this bacterial respiratory chain. The NADH dehydrogenase of *P. phosphoreum* has not been characterized, and we examined only the terminal oxidase in this work.

Effect of Pentagalloylglucose on the Electron Transfer Activities of the Purified Terminal Oxidase

The terminal oxidase of *P. phosphoreum* was purified and characterized as previously described.[6] This purified terminal oxidase, cytochrome bd complex, was used in the studies of the inhibition of pentagalloylglucose. Phospholipids were essential for full activation of the purified enzyme, and the ubiquinol-1 oxidase and TMPD oxidase activities were measured in the presence of 3 mM asolectin (soybean phospholipid). The ubiquinol-1 oxidase activity was more sensitive to the inhibitor than the activity of TMPD oxidase (Fig. 2). This result was similar to that obtained with the sonicated membrane vesicles. The concentrations giving 50% inhibition of the activity (IC_{50}), estimated from the dose-response curves (Fig. 2), were 20 μM (ubiquinol oxidase) and 167 μM (TMPD oxidase, not shown).

Enzyme Kinetics

We studied the kinetics of inhibition by pentagalloylglucose at various concentrations of

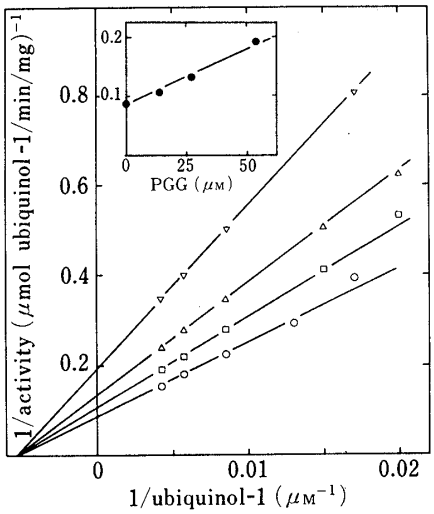

Fig. 3. Double-Reciprocal Plot of the Effect of Pentagalloylglucose on Ubiquinol-1 Oxidase Activity in Sonicated Membrane Vesicles of *P. phosphoreum*

The assay mixture, in a total volume of 1 ml, containing 0.1 mg of membrane protein, 100 mM Tris–HCl (pH 6.8), 0.025% Triton X-100, various concentrations of ubiquinol-1, and/or pentagalloylglucose. (○), no addition; (□), in the presence of 13.3 μM; (△), 26.6 μM; (▽), 53.2 μM inhibitor. The plot is typical of noncompetitive inhibition. The K_m and V_{max} values of the control activity (in the absence of inhibitor) were calculated to be 185 μM and 11.5 μmol ubiquinol-1/min/mg of protein, respectively, and K_m was not altered by addition of pentagalloylglucose. The K_i was estimated to be 45 μM. A linear relationship exists between the intercept values and concentrations of the inhibitor (inset). PGG, pentagalloylglucose.

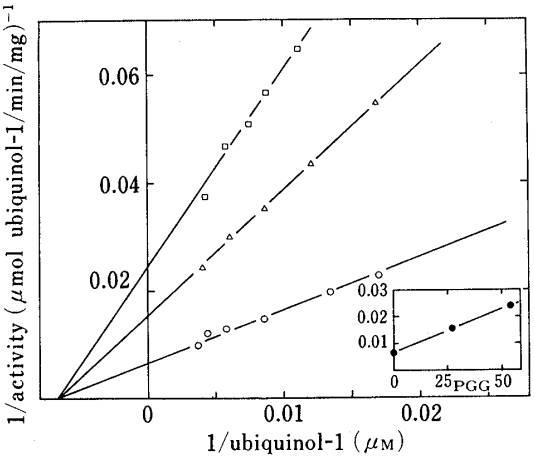

Fig. 4. Double-Reciprocal Plot of the Effect on Ubiquinol-1 Oxidase in Purified Cytochrome bd Complex

The conditions of assay were the same as in the legend to Fig. 3. Purified cytochrome bd complex (0.1 μg) was contained in the assay mixture. (○), no addition; (△), in the presence of 26.6 μM; (□), 53.2 μM pentagalloylglucose. The K_i value was calculated to be 21 μM and K_m for ubiquinol-1 was 147 μM, which was not altered by the addition of the inhibitor. The V_{max} value in the control experiment was estimated to be 149 μmol of ubiquinol-1/min/mg of protein.

ubiquinol-1 by the use of sonicated membrane vesicles. The results are illustrated in a double-reciprocal plot in Fig. 3, which clearly indicates that pentagalloylglucose is a noncompetitive inhibitor of ubiquinol-1 oxidase activity. From this experiment, the inhibitor constant (K_i) was estimated as 45 μM. We then performed similar experiments on the ubiquinol-1 oxidase activity of purified cytochrome bd complex (Fig. 4). The inhibitor constant was 21 μM, which was close to the IC_{50} value estimated from the dose-response curve (Fig. 1). The K_m values (185 μM for membrane and 147 μM for purified enzyme) did not change in the presence of the inhibitor.

The effect of pentagalloylglucose on TMPD oxidase activity was also examined (Fig. 5). The kinetics of inhibition by pentagalloylglucose of TMPD oxidase activity in the sonicated membrane vesicles was uncompetitive, as shown in Fig. 5, and this was different from the case of ubiquinol-1 oxidase. Similar results were obtained with purified cytochrome bd complex (Fig. 6).

Effects of Tetragalloylglucose

Tetragalloylglucose is not contained in Chinese gallotannin but is contained in Turkish gallotannin. It is the lowest-molecular-weight galloylglucose in Turkish gallotannin except for trigalloylglucose (content, only 1%[13]). A study similar to that on the pentagalloylglucose was carried out (data not shown). The activities of NADH dehydrogenase, NADH oxidase, ubiquinol-1 oxidase, and TMPD oxidase in sonicated membrane vesicles were inhibited in a dose-dependent manner by tetragalloylglucose, and the IC_{50} values were 18, 70, 24, and 91 μM,

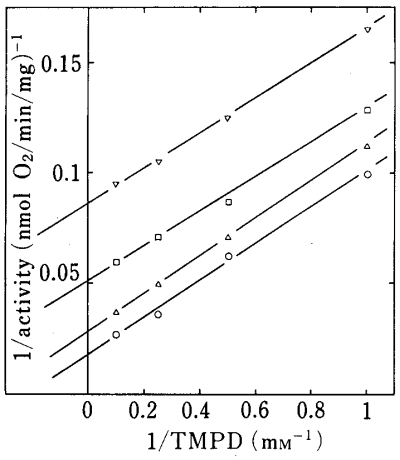

Fig. 5. Double-Reciprocal Plot of the Effect of Pentagalloylglucose on TMPD Oxidase Activity in Membrane Vesicles of *P. phosphoreum* in the Presence of Ascorbate

The assay was carried out with an oxygen electrode. The assay mixture, in a total volume of 1 ml, contained 1.0 mg of membrane protein, 100 mM Tris-HCl (pH 7.4), 5 mM ascorbate, various concentrations of TMPD, and/or inhibitor. (○), no addition, (△), in the presence of 53.2 μM; (□), 106 μM; (▽), 213 μM; pentagalloylglucose. The K_m and V_{max} values of control activity were calculated to be 4.76 mM and 70.0 nmol of O_2/min/mg of protein.

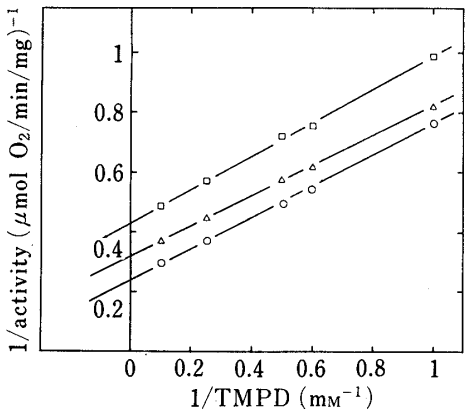

Fig. 6. Double-Reciprocal Plot of the Effect of Pentagalloylglucose on TMPD Oxidase Activity of Purified Cytochrome bd Complex of *P. phosphoreum* in the Presence of Ascorbate

The conditions of assay were the same as in the legend to Fig. 5. Purified cytochrome bd complex (1.0 μg) was contained in the assay mixture. (○), no addition; (△), in the presence of 53.2 μM; (□), 106 μM inhibitor. The K_m and V_{max} of the control activity were estimated to be 2.25 mM and 4.01 μmol of O_2/min/mg of protein.

TABLE I. Kinetic Parameters of Effects of Inhibitors for Oxidase Activity of Purified Cytochrome bd Complex

Substrate	Pentagalloylglucose	Tetragalloylglucose	Type of inhibition
	K_i (μM)		
Ubiquinol-1	21	10	Noncompetitive
Ascorbate + TMPD	133	62	Uncompetitive

respectively. Furthermore, the TMPD and ubiquinol-1 oxidase activities of purified cytochrome bd complex were also decreased by tetragalloylglucose, and the IC_{50} values were estimated to be 121 and 13 μM, respectively. A kinetic study on the oxidase activity was carried out, and we found that the inhibitory effect of tetragalloylglucose on the ubiquinol-1 oxidase activity of sonicated membrane vesicles was typically non-competitive in the same manner as in the case of pentagalloylglucose, and K_i calculated from the double-reciprocal plot was 25 μM. From the result of the experiment using the purified terminal oxidase, tetragalloylglucose was concluded to be a noncompetitive inhibitor for the ubiquinol-1 oxidase activity of cytochrome bd complex, and K_i was 10 μM (Table I).

On the other hand, in the case of TMPD oxidase, the effects of the inhibitor on sonicated membrane vesicles and on the purified cytochrome bd complex were of uncompetitive type. The K_m and V_{max} values were 4.76 mM and 70 nmol O_2/min/mg of protein in control sonicated membrane vesicles (K_m = 2.25 mM, V_{max} = 4.10 μmol O_2/min/mg in the purified enzyme), and were 1.53 mM and 17 nmol O_2/min/mg (1.35 mM, and 2.22 μmol O_2/min/mg in the purified enzyme), respectively, in the presence of 50 μM tetragalloylglucose.

Discussion

In the previous report, we have shown that tannic acid inhibits the growth of *P. phosphoreum* and that one of the inhibitory sites is probably in the respiratory chain. Moreover, the results of experiments on sonicated membrane vesicles and purified terminal oxidase, cytochrome bd complex,[5] indicated that NADH dehydrogenase and the terminal oxidase were sites of inhibition by tannic acid.

In this study, it was indicated that pentagalloylglucose, which is the lowest-molecular-weight compound contained in Chinese gallotannin,[4] also has an inhibitory effect on the terminal oxidase and NADH dehydrogenase of *P. phosphoreum*. The inhibitory pattern of the ubiquinol-1 oxidase of the sonicated membrane vesicles is noncompetitive. The same inhibitory pattern was obtained by the use of the purified cytochrome bd complex. It is interesting that the TMPD oxidase activities of sonicated membrane vesicles and purified terminal oxidase were also decreased by pentagalloylglucose, but the inhibitory patterns were uncompetitive. In the case of *E. coli* terminal oxidase (cytochrome bd complex), Kranz and Gennis reported that the sites for ubiquinol-1 and TMPD oxidation are located on subunits I (large subunit) and II (small subunit), respectively.[14] These conclusions were based on studies using antibodies against this enzyme. Although this type of experiment has not yet been done in the case of *Photobacterium phosphoreum*, results obtained in this work (difference of inhibitory pattern for the reactions with the two substrates) are consistent with those from studies in *E. coli* (difference of sites for the two substrates).

The bacterial oxidases can be grouped into four classes as follows.[15] Class I, cytochrome c oxidases containing heme a and Cu^{2+} (aa_3, caa_3, and a_1-types); Class II, cytochrome c oxidases containing heme b (o- and co-types); Class III, quinol oxidases containing heme b and heme d (chlorine) (bd-type); Class IV, quinol oxidases containing heme b (o-type). In the case of enzymes of Class III, only two oxidases of *E. coli*[16] and *P. phosphoreum*[6]) have been purified and examined in detail. From these results, the inhibitors of the cytochrome bd terminal oxidases include KCN, NaN_3, H_2O_2, HQNO, piericidin A, and NH_2OH. We have shown in this paper that materials isolated from plants have inhibitory activity against Class III oxidase, and are more effective than KCN, NaN_3, and H_2O_2. It was indicated that another inhibitory site is NADH dehydrogenase. Purification of the NADH dehydrogenase involved in the aerobic respiratory chain of *P. phosphoreum* is in progress.

Tetragalloylglucose, which is not contained in Chinese gallotannin but is contained in Turkish gallotannin, was also a potent inhibitor of the oxidase of *P. phosphoreum*. This material is a noncompetitive inhibitor of ubiquinol-1 oxidase and uncompetitive inhibitor of TMPD oxidase in both purified cytochrome bd and sonicated membrane vesicles.

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