Inhibitory Effects of Tannic Acid on the Respiratory Chain of
Photobacterium phosphoreum

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Tannic acid inhibited the growth of Photobacterium phosphoreum in liquid culture and also
decreased the viability, expressed in terms of the colony forming activity.

The activity of glucose dependent oxygen consumption of whole cells was inhibited by tannic
acid. It was found that the activity of reduced nicotinamide adenine dinucleotide (NADH) oxidase
of the sonicated membrane of Photobacterium phosphoreum decreased when tannic acid was added
to the assay system. The results suggested that the targets of tannic acid action were NADH
dehydrogenase and the terminal oxidase.

The inhibitory effect of tannic acid on the terminal oxidase was compared in the cases of
purified terminal oxidase (cytochrome b560-d complex) and sonicated membrane vesicles. The
oxidase activities toward ubiquinol-1 and N,N,N',N'-tetramethyl-p-phenylenediamine dihydro-
chloride (TMPD) in the presence of ascorbate were both inhibited by tannic acid in both sonicated
membrane and purified cytochrome b560-d complex. The inhibition of ubiquinol-1 oxidase activity
in sonicated membrane was biphasic and noncompetitive in both phases. The inhibition of the
ubiquinol-1 oxidase activity of purified terminal oxidase was monophasic and noncompetitive. On
the other hand, the inhibition of TMPD oxidase activity in the presence of ascorbate in both
membrane vesicles and purified enzyme was uncompetitive. Thus, the mechanisms of inhibition of
the two kinds of oxidase activity by tannic acid were different.

Keywords—tannic acid; cytochrome b560-d; Photobacterium phosphoreum; terminal oxidase;
respiratory chain

We have previously purified to near homogeneity and characterized the terminal oxidase
complex of Photobacterium phosphoreum.1) This enzyme contains two polypeptides (54000
and 41000), with protoheme and heme d as prosthetic groups. Moreover, the purified oxidase
showed N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD)-dependent
oxygen consumption in the presence of ascorbate or ubiquinol-1 as a substrate, and the
enzyme is involved in the pathway of oxidative phosphorylation.

Tannic acid contained in Chinese nutgall is a polyphenol and is composed of glucose and
gallic acid.2–5) The known pharmaceutical effects of this compound are astringency and
antimicrobial action.3) However, the antimicrobial effect has not yet been studied from a
biochemical viewpoint.

In this work, we found that the growth of Photobacterium phosphoreum was inhibited by
tannic acid and that the targets of tannic acid in the respiratory chain were reduced
nicotinamide adenine dinucleotide (NADH) dehydrogenase and the terminal oxidase (cyto-
chrome b560-d complex). In order to understand the mechanism of action of this acid,
we carried out some kinetic studies of the enzymes that were affected by tannic acid.
Materials and Methods

Organism—Strain IAM12085 of *Photobacterium phosphoreum* was obtained from the Institute of Applied Microbiology, The University of Tokyo, and was grown in the medium described previously. Inocula of 10 ml of seed culture were incubated in 2-l volumes of medium in 5-l glass containers at 25 °C for 20 h with vigorous aeration by shaking, and cells were harvested in the middle of the exponential phase of growth. The yield was 5 g wet weight of cells/l. The cells were stored at −20 °C before use.

Preparation of Cytochrome b560-d Complex—The procedures for solubilization and purification of the cytochrome b560-d complex were as described previously.

Preparation of Sonicated Membrane—The frozen cell pellet was thawed and suspended with buffer A (10 mm Tris–HCl (pH 7.0), 10 mm MgCl2). 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 × g for 1 h, and the supernatant obtained was centrifuged at 100000 × g for 1 h. The precipitate was washed twice with buffer A by centrifugation. The membrane vesicle preparation obtained was used for the experiments.

Assay of Oxidase Activity—Ubiquinol-1 oxidase activity was assayed as described previously. A mixture (20 µl) of cytochrome b560-d complex (1 µg), phospholipids (acetone washed soybean phospholipids, asolectin 2 mm), and 50 mm Tris–HCl (pH 7.5) 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, and Kita et al., respectively, using a Clark type oxygen electrode (Rank Brothers, Rank oxygen electrode). The NADH oxidase activity of whole cells was assayed after preincubation of the cells in the assay chamber for 30 min.

Assay of NADH Dehydrogenase Activity—Assay of NADH–menadione dehydrogenase activity was carried out by the method described previously. The concentrations of NADH and menadione were 150 and 200 µM, respectively.

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

Chemicals—Ubiquinol-1 was a generous gift from Eisai Co., Ltd. Five lots of tannic acid purchased from 4 companies were used in our experiment (No. 1, Wako Pure Chemical lot No. KWP7592; No. 2, Wako Pure Chemical lot No. EPR7215; No. 3, Nakarai Chemicals lot No. M4R1112; No. 4, Kanto Chemical lot No. 001H5101; No. 5, Sigma-lot No. 64F-0049).

Only the data obtained with tannic acid No. 1 are presented in this paper, but the results with other tannic acids were almost the same (data not shown).

Results

Effect on the Growth of *Photobacterium phosphoreum*

First of all, we examined the effect of tannic acid on the growth of *P. phosphoreum* in liquid culture (Fig. 1). The doubling time of cell growth increased about 1.5-fold when 100 µg/ml of tannic acid was added to the culture medium. The final growth yield of cells decreased drastically above 200 µg/ml of tannic acid. There was no effect on the growth in the presence of tannic acid at concentrations below 10 µg/ml. The cells could not grow at all at about 250 µg/ml of inhibitor. Similar results were obtained in plate cultivation (Fig. 2). Cells were not viable in the presence of more than 500 µg/ml of the inhibitor. No effect was observed in the presence of tannic acid at below 10 µg/ml. It is clear that tannic acid is a potent inhibitor of the growth of *Photobacterium phosphoreum*.

To determine whether the inhibitory effect of tannic acid is bacteriocidal, bacteriostatic, or bacteriolytic, we examined the growth curve of cells after addition of the inhibitor. When 200 µg/ml of tannic acid was added to the culture medium, the increase of turbidity of the culture (monitored by measuring the absorbance at 650 nm) was interrupted and the turbidity remained unchanged for at least 5 h. The viable cell count (based on the ability to form colonies) showed a similar pattern (data not shown). These results indicated that the type of inhibitory effect was bacteriostatic.

The effect of tannic acid on bacterial growth was essentially the same among 5 lots from 4 companies (see Materials and Methods).
Effect on the Electron Transfer Activities of the Respiratory Chain

As shown in Fig. 1 and Fig. 2, tannic acid was an effective inhibitor of the growth of *P. phosphoreum*. The respiratory chain is one of the primary systems which regulate growth, so...
we studied various activities of the respiratory chain of *P. phosphoreum* in an *in vitro* assay system. In the case of whole cells, glucose-dependent oxidase activity was not inhibited in the presence of 200 μg/ml of tannic acid, if preincubation of whole cells with the inhibitor was omitted before the assay (data not shown). This result indicates that it is difficult for tannic acid to pass through the outer membrane, peptide glycan, or inner membrane of whole cells. The inhibitor was probably taken up slowly by simple diffusion into cells. Indeed, after incubation for 30 min, the oxidase activity was inhibited by tannic acid (Fig. 3).

In order to avoid this complication, we used sonicated membrane for this experiment. Figure 3 shows that tannic acid quite effectively inhibited NADH dehydrogenase and NADH oxidase, and less effectively inhibited TMPD oxidase activity (in the presence of ascorbate) and ubiquinol-1 oxidase activity of the respiratory chain. At the concentration of 50 μg/ml, tannic acid inhibited about 90% of NADH oxidase activity, about 70% of NADH dehydrogenase activity, and about 30% of TMPD oxidase activity. Since the NADH oxidase activity includes the activities of NADH dehydrogenase and ubiquinol oxidase, the site of inhibition should be either NADH dehydrogenase or cytochrome b560-d complex. NADH dehydrogenase of *P. phosphoreum* has not been characterized well. NADH—menadione (Fig. 3) and NADH—ferricyanide (data not shown) were detected in sonicated membrane vesicles in this experiment. The NADH dehydrogenase activity was activated by addition of KCN in the absence of tannic acid, but was decreased by addition of KCN in the presence of the inhibitor. We do not know the reason for this at present. In the following study, we examined only the

Fig. 5. Double-Reciprocal Plot of the Effect of Tannic Acid on Ubiquinol-1 Oxidase Activity in Membrane Vesicles of *P. phosphoreum*

The assay mixture, in a total volume of 1 ml, contained 0.1 mg of membrane, 50 mM Tris–HCl (pH 7.5), various concentrations of ubiquinol-1, and/or tannic acid. (○), no addition; (△), in the presence of 20 μg/ml tannic acid; (●), 120 μg/ml tannic acid. The plot is typical of noncompetitive inhibition. The *K*<sub>i</sub> values were calculated to be 58.2 μg/ml in the presence of low concentrations of tannic acid and 40.0 μg/ml in the presence of higher concentrations of tannic acid. The *K*<sub>i</sub> for ubiquinol-1 was 76.9 μM, and this was not altered by addition of tannic acid. One activity unit (U) was equal to 0.001 absorbance unit at 645 nm, and 1 mg of membrane vesicles contained 2.46 U of cytochrome d.

Fig. 6. Double-Reciprocal Plot of the Effect on Ubiquinol-1 Oxidase Activity of Purified Cytochrome b560-d Complex

The conditions of assay were the same as in the legend to Fig. 5. (○), no addition; (●), in the presence of 10 μg/ml tannic acid; (△), 25 μg/ml tannic acid. The plot is typical of noncompetitive inhibition, like Fig. 5. The *K*<sub>i</sub> value was calculated to be 18.8 μg/ml and *K*<sub>m</sub> for ubiquinol-1 was 69.0 μM, which was not altered by the addition of tannic acid. One milligram of purified enzyme contained 28.8 U of cytochrome d.
terminal oxidase.

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

The terminal oxidase of *P. phosphoreum* was purified and characterized as previously described.\(^1\) This purified terminal oxidase, a cytochrome b560-d complex, was used in the studies of the inhibitory effect of tannic acid. Phospholipids were essential for full activation of the purified enzyme, and the ubiquinol oxidase or TMPD + ascorbate oxidase activity was measured in the presence of 3 mM asolectin, soybean phospholipids. The ubiquinol oxidase activity was more sensitive to tannic acid than the activity of TMPD + ascorbate oxidase (Fig. 4). This result was similar to that obtained with the sonicated membrane at relatively high concentrations of tannic acid (Fig. 3). The inhibition pattern of ubiquinol-1 oxidase activity by the inhibitor was monophasic, different from that in sonicated membrane. The concentrations required for 50% inhibition of the activity (ID\(_{50}\)) estimated from the dose response curve (Fig. 4) were 105 \(\mu\)g/ml (TMPD + ascorbate) and 20 \(\mu\)g/ml (ubiquinol oxidase), respectively.

**Enzyme Kinetics**

We next studied the inhibition kinetics of ubiquinol oxidase by tannic acid at various concentrations of ubiquinol-1. As shown in Fig. 3, the inhibitory pattern of ubiquinol-1 oxidase activity on the membrane was biphasic. To examine the difference between the two phases of inhibition, we studied the kinetics of the oxidase at about 20% and 80% inhibition. The kinetic pattern in the presence of 20 \(\mu\)g/ml tannic acid was similar to that in the presence of 120 \(\mu\)g/ml, showing that the \(K_m\) value did not change in the presence of the inhibitor. The results are illustrated in a double-reciprocal plot in Fig. 5, which clearly indicates that tannic acid is a noncompetitive inhibitor of ubiquinol-1 oxidase activity. We then performed similar experiments on the ubiquinol-1 oxidase activity of purified cytochrome b560-d complex (Fig. 6). The inhibition constant was 69 \(\mu\)g/ml, which was close to the ID\(_{50}\) value estimated from the dose-response curve. The \(K_m\) value did not change in the presence of the inhibitor.

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**Fig. 7. Double-Reciprocal Plot of the Effect of Tannic Acid 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, 50 mM Tris–HCl, 5 mM ascorbate, various concentrations of TMPD, and/or tannic acid. (○), no addition; (●), in the presence of 100 \(\mu\)g/ml tannic acid.

**Fig. 8. Double-Reciprocal Plot of the Effect on TMPD Oxidase Activity of Purified Cytochrome b560-d Complex of *P. phosphoreum* in the Presence of Ascorbate**

The conditions of assay were the same as in the legend to Fig. 7. (○), no addition; (●), in the presence of 40 \(\mu\)g/ml tannic acid; (△), 100 \(\mu\)g/ml tannic acid.
The effect of tannic acid on TMPD + ascorbate oxidase activity was also examined (Fig. 3). The kinetics of inhibition by tannic acid of TMPD + ascorbate oxidase activity in sonicated membrane was uncompetitive, as shown in Fig. 7, and this was different from the case of ubiquinol-1 oxidase. A similar result was obtained with purified cytochrome b560-d complex (Fig. 8).

**Discussion**

We showed that tannic acid inhibits the growth of *P. phosphoreum*. One of the inhibitory sites is probably the respiratory chain, based on the results of glucose oxidase assay on whole cells and NADH oxidase assay on sonicated membrane (Fig. 3). The inhibition of glucose oxidase activity was observed only when preincubation with inhibitor was carried out, and the uptake of inhibitor by the cells was probably a limiting step. NADH dehydrogenase activity was also sensitive to the inhibitor, but no further study was carried out at this time. Experiments on the dehydrogenase activity are in progress. Tannic acid also inhibited the ubiquinol-1 oxidase activity and TMPD oxidase activity in the presence of ascorbate, indicating that the terminal oxidase was one of the sites of action of tannic acid.

In *E. coli*, it was reported that the only terminal oxidase present in the early exponential phase of growth was cytochrome b562-o complex and that cytochrome b558-d complex was also synthesized in the late exponential phase or early stationary phase. The $K_m$ and $V_{max}$ values of ubiquinol oxidase activity in membrane vesicles at the early exponential phase were the same as those of the purified oxidase, cytochrome b562-o complex, but the values of the oxidase activity in the membrane at the late exponential phase were between those of purified cytochrome b562-o and cytochrome b558-d. The cytochrome b560-d of *P. phosphoreum* was probably the only terminal oxidase over the growth phase, because the $K_m$ and $V_{max}$ values of the oxidase activity were the same on the membrane and in a purified sample.

It is interesting that the inhibition pattern of ubiquinol oxidase activity on membrane vesicles is biphasic. Both phases were examined kinetically, and only $V_{max}$ varied in the presence of the inhibitor, indicating that this inhibition is noncompetitive. In the presence of low concentrations of inhibitor, the pattern of inhibition of ubiquinol-1 oxidase was the same as that of TMPD + ascorbate oxidase. Inhibition of the ubiquinol-1 oxidase activity of the purified enzyme at relatively low concentrations was monophasic. The TMPD + ascorbate oxidase activities of sonicated membrane and purified cytochrome b560-d were also decreased by the addition of the inhibitor, and uncompetitive-type inhibition was observed. It is possible that this inhibitor does not interact with the free enzyme (E) or substrate (S), but interacts with ES complex. If this is correct, the inhibitor may bind to substrate-reduced cytochrome b560-d complex.

Various lots of tannic acid were used (see Materials and Methods), and their inhibitory activities on growth and on oxidase activity on membrane vesicles were almost the same (data not shown). These results indicate that the major compound(s) contained in all the tannic acid preparations exhibit the activities.

Commercial tannic acid is a very complex and non-uniform mixture. We therefore attempted the isolation and purification of components of commercial tannic acid. Several partially purified compounds were obtained. However, gallic acid, digallic acid, and minor components of higher molecular weight did not have inhibitory activity. The other components which were partially purified have almost the same activity as the commercial preparation (data not shown). Complete purification and characterization are in progress in our laboratory.
References