Inhibitory Effects of Pentagalloylglucose on Reduced Nicotinamide Adenine Dinucleotide Dehydrogenase of *Photobacterium phosphoreum*

Hirokazu ADACHI,*[,a] Kiyoshi KONISHI,[b] and Isamu HORIKOSHI[a]

Department of Hospital Pharmacy,[a] and Department of Biochemistry, Faculty of Medicine,[b] Toyama Medical and Pharmaceutical University, Sugitani, Toyama 930-01, Japan. *Received September 9, 1991*

Inhibitory effects of pure pentagalloylglucose (1,2,3,4,6-penta-*O*-galloyl-β-D-glucose) on purified nicotinamide adenine dinucleotide (NADH) dehydrogenase of the respiratory chain of *Photobacterium phosphoreum* were investigated. Pentagalloylglucose inhibited the NADH-ubiquinone-1, NADH-menadione, and NADH-2,6-dichlorophenolindophenol oxidoreductase activities with less than 150 nM of a 50% inhibition concentration (IC_{50}), but hardly inhibited NADH-ferricyanide and NADH-cytochrome c oxidoreductase activities with at least less than 0.5 μM of pentagalloylglucose.

Pentagalloylglucose inhibited noncompetitively the NADH dehydrogenase of *P. phosphoreum*, which belongs to NADH dehydrogenase II (NADH dh II), and its inhibitor constant (K_i) to NADH-ubiquinone-1 oxidoreductase activity was estimated as 30 nM of pentagalloylglucose. Therefore, pentagalloylglucose is the potent inhibitor of NADH dh II, more than flavone (IC_{50} = 150 μM for NADH-ubiquinone-1 oxidoreductase activity) which has previously been reported as an inhibitor for NADH dh II.

Keywords pentagalloylglucose; respiratory chain; nicotinamide adenine dinucleotide dehydrogenase; *Photobacterium phosphoreum*

Introduction

Tannic acids contained in Chinese nutgall and Turkish nutgall are polyphenols, being composed of glucose and gallic acid.[1] Commercial tannic acid is a very complex and nonuniform mixture,[2,3] and has a wide variety of biological activities.[1,4-6] We have previously observed that 1,2,3,4,6-penta-*O*-galloyl-β-D-glucose (pentagalloylglucose) purified from tannic acid inhibited the activity of nicotinamide adenine dinucleotide (NADH) dehydrogenase and terminal oxidase in sonicated membrane vesicles of *Photobacterium phosphoreum*.[7] Although we examined the fine inhibitory kinetics of pentagalloylglucose by use of purified terminal oxidase which contains cytochrome b and d subunits, we have not yet carried out detailed studies on NADH dehydrogenase. Therefore, we decided to use the purified enzyme in order to elucidate the effect of the inhibitor. NADH dehydrogenases are divided into two groups depending on their properties.[8-11] One of the NADH dehydrogenases (NADH dh I or NDH-1) has the coupling site for oxidative phosphorylation, and is significantly more sensitive to rotenone, capsaicin, *N,N'*-dicyclohexylcarbodiimide (DCCD), 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and piericidin A.[9-12] The other dehydrogenases (NADH dh II or NDH-2) do not bear the coupling site and are not inhibited by the NADH dh I inhibitors.[10,11] Other large differences are the subunit structure, and the prosthetic groups. The mitochondrial NADH dh I is composed of more than 25 unlike polypeptides,[13,14] and the bacterial counterpart has more than 10 subunits.[8,15] These enzyme have several nonheme irons and flavin mononucleotide (FMN) as prosthetic groups. On the other hand, many bacterial NADH dh II have only one subunit, and contain flavin adenine dinucleotide (FAD) as a prosthetic group.[10,11]

We have recently purified and characterized the NADH dehydrogenase of *P. phosphoreum*.[16] This enzyme is a single polypeptide with a molecular weight of 49000, and contains one FAD per mol enzyme. The purified NADH dehydrogenase was not susceptive to the NADH dh I inhibitors.

To date, only a NADH dh II inhibitor, flavone, has been reported in the NADH dh II of plant and yeast mitochondria.[17,18] However, there was no inhibitor of the bacterial NADH dh II. In this paper, we report that pentagalloylglucose inhibited the activity of the purified NADH dehydrogenase of *P. phosphoreum* as a potent NADH dh II inhibitor.

Materials and Methods

Preparation of NADH Dehydrogenase of *P. phosphoreum* The procedure for purification of the NADH dehydrogenase of *P. phosphoreum* was as described previously.[16]

Assay of NADH Dehydrogenase Activity The enzyme assays were carried out at 25 °C in 50 mM Tris–HCl buffer, pH 7.4, using a Hitachi Spectrophotometer U-3410 when ubiquinone-1 (100 μM), menadione (100 μM) or dichlorophenolindophenol (DCIP) were the electron acceptors used. When potassium ferricyanide (1 mM) or horse heart cytochrome c (50 μM) were used as electron acceptors, the enzyme assays were carried out in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethansulfonic acid (HEPES) buffer, pH 7.0, 1 mM disodium ethylenediaminetetraacetate (EDTA), and 0.03% Triton X-100. NADH (150 μM) was used as an electron donor. NADH–ferricyanide, NADH–cytochrome c, and NADH–DCIP oxidoreductase activities were measured by following the change in the absorption of the electron acceptors at 420 nm (ε_{mM} = 1.0), 550 nm (ε_{mM} = 18.5), and 600 nm (ε_{mM} = 20.6) for ferricyanide, cytochrome c, and DCIP, respectively. NADH–ubiquinone-1 and NADH–menadione oxidoreductase activities were measured by following the decrease of absorption of NADH at 340 nm (ε_{mM} = 6.81 and 6.22, respectively).

Determination of Protein Concentration Protein concentration was determined by the method of Lowry *et al.*[19] with bovine serum albumin as a standard.

Chemicals Pure pentagalloylglucose isolated form Chinese nutgall was a generous gift from Drs. I. Nishioka and G. Nonaka (Kyushu University). This pentagalloylglucose showed a single peak in reverse phase high performance liquid chromatography. Ubiquinone-1 was a generous gift from Eisai Co., Ltd. Other reagents used were of the highest grade commercially available.

Results

The Effects of Pentagalloylglucose on NADH Dehydrogenase Activity We previously reported that pentagalloylglucose inhibited the NADH–menadione oxidoreductase activity of sonicated membrane vesicles of *P. phosphoreum*.[7]

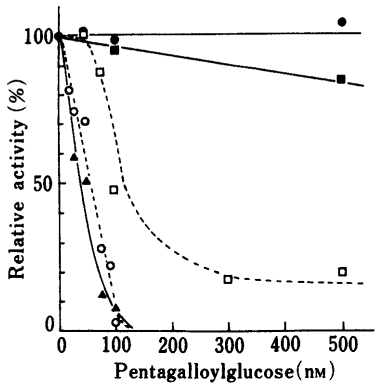


Fig. 1. Effects of Pentagalloylglucose on NADH Dehydrogenase Activity

The assay medium (2 ml) described in Materials and Methods contained NADH dehydrogenase, 150 μM NADH, various concentrations of pentagalloylglucose and electron acceptors. Electron acceptors were described as ○, 100 μM ubiquinone-1; ▲, 100 μM menadione; □, 50 μM DCIP; ■, 50 μM cytochrome c; and ●, 1 mM potassium ferricyanide. The control activities were 32, 46, 19, 160, 396 μmol/min/mg of protein, respectively.

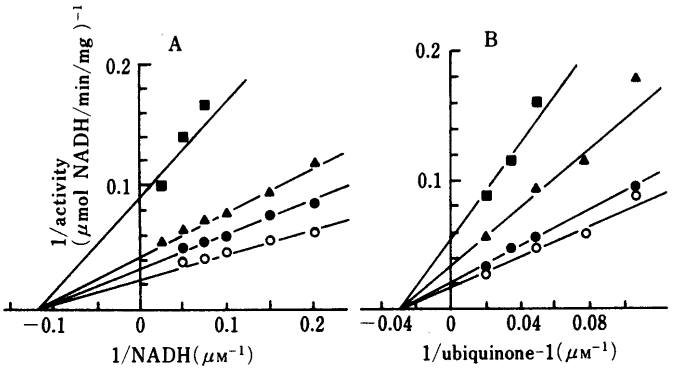


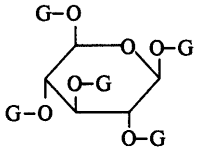
Fig. 2. Double-Reciprocal Plots of the Effects of Pentagalloylglucose on NADH–Ubiquinone-1 Oxidoreductase Activity of Purified Enzyme

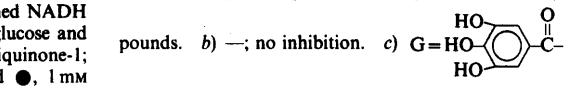
(A) The assay mixture (2 ml) contained NADH dehydrogenase, 50 mM Tris–HCl, pH 7.4, various concentrations of NADH, 100 μM ubiquinone-1 and/or pentagalloylglucose. ○, no addition; ●, 5 nM; ▲, 20 nM; ■, 80 nM inhibitor. (B) The assay mixture (2 ml) contained NADH dehydrogenase, 50 mM Tris–HCl, pH 7.4, various concentrations of ubiquinone-1, 150 μM NADH, and/or pentagalloylglucose. ○, no addition; ●, 10 nM; ▲, 30 nM; ■, 70 nM inhibitor.

In this paper, we used the following electron acceptors to the purified enzyme: ubiquinone-1, menadione, potassium ferricyanide, cytochrome c and DCIP. Fig. 1 shows that substrate-specificity exists in the inhibitory effects of pentagalloylglucose. The pentagalloylglucose strongly inhibited NADH–menadione and NADH–ubiquinone-1 oxidoreductase activity, and their 50% inhibition concentrations (IC_{50}) are 45 nM and 60 nM, respectively. NADH–DCIP oxidoreductase activity was also inhibited by pentagalloylglucose, but its effect (IC_{50} = 120 nM) was somewhat lower than that of the former. However, pentagalloylglucose hardly inhibited NADH–cytochrome c and NADH–ferricyanide oxidoreductase activity with at least less than 500 nM pentagalloylglucose.

Enzyme Kinetics We studied the kinetics of inhibition by pentagalloylglucose at various concentrations of substrates, ubiquinone-1 and NADH on the purified NADH dehydrogenase. The results are illustrated in a double-reciprocal plot in Fig. 2, indicating that pentagalloylglucose is a noncompetitive inhibitor of NADH–ubiquinone-1 oxidoreductase. From these experiments, the inhibitor constants (K_i) by estimating the changing of the concentrations of NADH and ubiquinone-1 were estimated as 32 and 28 μM, respectively.

TABLE I. Effects of Gallic Acid-Related Compounds on NADH Dehydrogenase[a]

Compound	IC_{50}	Structure[c]
Gallic acid	—[b]	G–OH
Ethylgallate	—[b]	G–OC_2H_5
Propylgallate	—[b]	G–OC_3H_7
Laurylgallate	10 μM	G–$OC_{12}H_{25}$
Pentagalloylglucose	60 nM	

a) The NADH–ubiquinone oxidoreductase activities were assayed in the presence of 100 μM ubiquinone-1, 150 μM NADH and/or gallic acid-related compounds. b) —; no inhibition. c) 

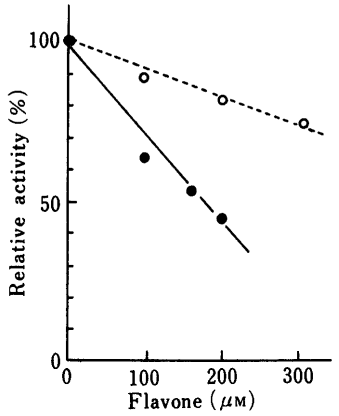


Fig. 3. Inhibitory Effects of Flavone on the NADH Dehydrogenase Activity

The concentrations of ferricyanide (○) and ubiquinone-1 (●) as electron acceptors were 1 mM and 100 μM, respectively.

The Effects of Gallic Acid-Related Compounds and Other Inhibitors We examined the relationships between the structure and function of gallic acid-related compounds. We used gallic acid, ethylgallate, propylgallate, and laurylgallate as gallic acid-related compounds (Table I). Gallic acid is a component of pentagalloylglucose, but does not inhibit the NADH–ubiquinone-1 oxidoreductase activity of NADH dehydrogenase. Laurylgallate, which is a lipophilic gallate ester, inhibits the enzyme activity with 10 μM of IC_{50}. The other compounds hardly inhibit the enzyme activity with at least less than 100 μM.

Flavone is the only NADH dh II inhibitor that has ever been reported in plant and yeast mitochondrial dehydrogenase. We therefore examined the effect of flavone on purified NADH dehydrogenase. Figure 3 shows that flavone is a weak inhibitor of bacterial enzyme, and inhibits the NADH–ubiquinone-1 oxidoreductase activity more than the NADH–ferricyanide oxidoreductase activity. The NADH dehydrogenase of *P. phosphoreum* is hardly inhibited by HQNO, capsaicin, and rotenone which are the NADH dh I inhibitors (data not shown).

Discussion

We previously reported that pure pentagalloylglucose,

which is the lowest-molecular weight compound contained in Chinese gallotannin, inhibits the NADH dehydrogenase and the terminal oxidase of sonicated membrane vesicles of *P. phosphoreum*.[7] Later, we reported inhibitory effects of pentagalloylglucose on purified terminal oxidase (cytochrome bd complex) to examine in detail the inhibitory effects.[20] However, we have not studied the NADH dehydrogenase site of the respiratory chain of *P. phosphoreum* in detail. In this paper, we examined how strongly the pure pentagalloylglucose inhibited the purified NADH dehydrogenase of *P. phosphoreum*.

The results that the inhibitory effects of pentagalloylglucose on NADH dehydrogenase are dependent on electron acceptors suggest that at least two electron flow pathways to the acceptors are present in NADH dehydrogenase of *P. phosphoreum*. One is the pentagalloylglucose-susceptive pathway from which the electron is transported to ubiquinone-1, menadione, and DCIP, and the other is the inhibitor-independent pathway from which the electron is transported to ferricyanide, and cytochrome c. Flavone, which is the NADH dh II inhibitor of plant and yeast mitochondria, also inhibited NADH–ubiquinone-1 oxidoreductase activity more than NADH–ferricyanide oxidoreductase (Fig. 3). Pentagalloylglucose noncompetitively inhibited the NADH–ubiquinone-1 oxidoreductase activity. NADH dehydrogenase of *P. phosphoreum* is not inhibited by HQNO, capsaicin, DCCD, and rotenone, but is inhibited by flavone. These results are consistent with recent reports[16] that this NADH dehydrogenase belongs to NADH dh II.

Previously, Unemoto's group reported that *Vibrio alginolyticus*, a marine bacterium, has a sodium ion-dependent NADH–ubiquinone oxidoreductase which is composed of three subunits (α, β and γ),[21] and seems to belong to NADH dh I.[10] This enzyme also has Na^+-pumping activity. Although some property of subunit β which has NADH dehydrogenase activity, resembles the purified enzyme of *P. phosphoreum*, there is a large difference in susceptibility to the inhibitor between the two enzymes in the membrane. The NADH ubiquinone oxidoreductase activity of the enzyme complex of *V. alginolyticus* is strongly inhibited by HQNO, but that of *P. phosphoreum* in membrane vesicles is completely resistant to a high concentration of HQNO (unpublished observation). The possibility that the NADH dehydrogenase of *P. phosphoreum* is coupled with a sodium ion pump, is in progress in our laboratory.

We previously reported that pentagalloylglucose is a potent and specific inhibitor to NADH dh I and dh II of *Escherichia coli*, and that NADH dh II is more sensitive to the inhibitor than NADH dh I.[22] In addition, it is confirmed in this report that pentagalloylglucose is a potent inhibitor to NADH dh II of *P. phosphoreum*. We examined the relationship between the structure and function of gallic acid-related compounds. Gallic acid does not inhibit the NADH dehydrogenase. On the other hand, only laurylgallate, which is a very hydrophobic compound, weakly inhibited the NADH dehydrogenase activity. The inhibitory effects of pentagalloylglucose disappeared in a high pH, more than pH 8.5, because in alkaline conditions pentagalloylglucose is hydrolyzed to gallic acid and glucose, or the hydroxyl group of gallate ($pK_a = 7.8$) is deprotonated. These results suggest that the whole structure of galloylglucose, consisting of an ester form of gallate and glucose, is necessary for the strong inhibitory activity.

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