Effects of Various Hydroxyl Compounds on Stability of Protocatechuate 4,5-Dioxygenase.

Kiyofumi Maruyama

Laboratory of Chemistry, Faculty of General Educution,
Gifu University

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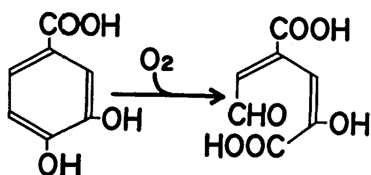
Effects of various hydroxyl compounds on the stability of protocatechuate 4,5-dioxygenase have deen studied. Most hydroxyl compounds more or less stabilized the enzyme. The effectivenss of hydroxyl compounds increased in the following order; monohydroxyl compound, dihydroxyl compound, trihydroxyl compound, pentose, hexose, disaccharide, suggesting that the more effective stabilizers were the compounds containing the more hydroxyl groups in their chemical structures. The coexistance of the hydroxyl compounds in the reaction mixture of protocatechuate 4,5-dioxygenase resulted in inhibition of the enzyme activity. The kinetic examinations suggested that the stabilizing effect of hydroxyl compounds was not due to the binding of these compounds to the active sites but due to the binding to other sites on the enzyme.

Protocatechuate 4,5-dioxygenase (protocatechuate: oxygen 4,5-oxidoreductase, EC 1.13.1.18), one of the non-heme iron containing oxygenases, catalyzes the incorporation of two atoms of molecular oxygen to protocatechuic acid (1,2). The benzene ring is ruptured with the formation of α-hydroxy-γ-carboxymuconic ε-semialdehyde.



The enyzme was found to be extremely unstable (3). Neither the anaerobic conditions nor the addition of the reducing reagents, such as fe-rous ion and cysteine, prevented the inactivation of the enzyme (3). Ono *et al.* found that ethanol stabilized the enzyme and they attempted the purification of the enzyme from the soil bacteria (3). However, the stabilizing effect of ethanol was incomplete as demonstrated in the present paper. Therefore. it seems to be valuable to find some other compounds which protect the enyzme from inactivation.

Based on these considerations, the effects of various compounds on the stability of the enzyme have been studied. In the present study, the appropriate stabilizers were searched among the various hydroxyl compounds. These compounds have either a part or the whole of their chemical structures in common with a part of the protocatechuate molecule and might therefore be expected to more or less bind to the enzyme. It is likely that the binding of some compounds to the enzyme induces the conformational change of the enzyme protein so as to be more stable against inactivation.

Materials and Methods

Chemicals …… All chemicals were reagent grade chemicals from commercial sources and used without further purifications.

Isolation and Growth of Microorganism …… The microorganism used throughout the present study was isolated from soil in Gifu University. It was selected by its ability to grow in a medium containing phthalate as sole carbon source, and purified by selection of single colonies from phthalate-agar. The selected microorganism was identified as *Pseudomonas ochraceae*. For large-scale preparation this bacteria was grown at 28°C for 22 h with vigorous aeration in a 10-liter medium containing 30 g phthalic acid, 40 g $(NH_4)_2SO_4$, 15 g K_2HPO_4, 5 g KH_2PO_4, 2 g $MgCl_2 \cdot 6H_2O$, 8 g yeast extract. The pH of the medium was adjusted to 7.5 with KOH. The cells were harvested by centrifugation, washed once with 0.05 M potassium phosphate buffer, pH 7.0, and stored at −20°C until needed.

Preparation of Crude Enzyme …… Washed cells (about 5 g) were resuspended in 15 ml of 0.05 M potassium phosphate buffer, pH 7.0. The suspension was then treated at 0°C for 10 min with a Tomy ultrasonic disintegrator UR 150P at maximum power. Then, the cell debris was removed by centrifugation for 15 min at 25,000 x g at 4°C. The resulting supernatant was referred to as "crude enyzyme." Each experiment was carried out with newly prepared crude enzyme. The enzyme activity in the crude enzyme did not decrease significantly at least for 3 h at 0°C.

Determination of Enzyme Activity …… The activity of protocatechuate 4,5-dioxygenase was spectrophotometrically determined by measuring the increase in absorbance at 410 nm with Hitachi recording spectrophotometer model 200-10, since the reaction product α-hydroxy-γ-carboxymuconic ε-semialdehyde showed a yellow color with an absorption maximum at 410 nm in an alkaline solution (1). The molecular extinction coefficient of the reaction product at pH 8.0 is 2,600 M^{-1} cm^{-1}. The reaction mixture (3.0 ml) contained 150 μmoles of potassium phosphate buffer, pH 8.0, 0.5 μmole of protocatechuic acid, and the crude enzyme. The reactions were carried out in the rectangular cells (light path 1 cm), which were thermostated at 24° C by circulating the temperature-controlled water through the cell holder.

Protein concentration was determined by the method of Lowry *et al.* using bovine serum albumin as a standard (4).

Results

Effects of Various Hydroxyl Compounds on Enzyme Stability …… Protocatechuate 4,5-dioxygenase is unstable and easily inactivated (3). When the enzyme was preincubated at 24°C under the aerobic conditions, the enzyme was almost completely inactivated for about 30 min as shown in Fig. 1. The rate of inactivation apparently followed first order kinetics. In the presence of ethanol at indicated concentra-

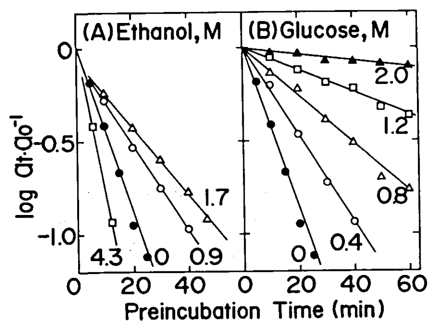


Fig. 1. Effect of ethanol and glucose on the stability of protocatechuate 4,5-dioxygenase. The preincubation was carried out at 24° C in 1 ml of 0.05 M potassium phosphate buffer, pH 7.0, containing the crude enzyme (2.85mg) and ethanol (A) or glucose (B) as indicated concentrations. At indicated time, an aliquot (30 μl) was removed and transferred into the assay mixture. The residual enzyme activity was spectrophotometrically determined as described under "Methods." The ordinate shows the logarithm of the ratio of the enzyme activity at various time (a_t) to that at zero time (a_0).

tions, this inactivation of the enzyme occurred at slower rate (Fig. 1A). However, the enzyme activity rapidly decreased, by about 15 %, in initial few minutes. Similar initial inactivation was observed with all of the organic solvents tested and was attributable to the thermal liberation upon mixing of these organic solvents with aqueous solution of the enzyme. The enyme was also stabilized in the coexistance of glucose (Fig. 1B) With glucose and other sugars examined, the initial rapid inactivation found with ethanol was not significant. Such stabilizing effect was markedly dependent on the concentrations of the hydroxyl compounds. The quantitative treatment of the results of Fig. 1 was shown in Fig. 2, wherein the slope (k) of each curve shown in Fig. 1 was replotted against the concentrations of hydroxyl compounds. As the concentrations of ethanol and glucose were raised, the value of k gradually decreased. k-Values were

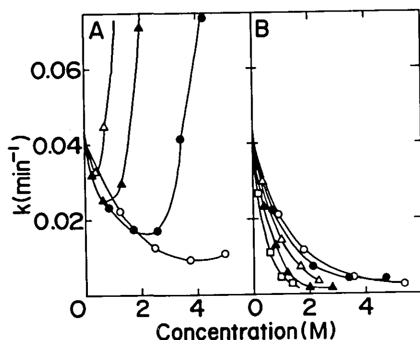


Fig. 2. Effect of various compounds on the stability of protocatechuate 4, 5-dioxygenase. The experiments described in Fig. 1 were carried out with various compounds. The slope (k) of each curve was replotted against concentrations of hydroxyl compounds. A, monohydroxyl compounds: methanol (○), ethanol (●), n-propanol (△), iso-propanol (▲); B, polyhydroxyl compounds: ethylene glycol (○), glycerol (●), xylose (△), glucose (▲), saccharose (□).

1/2.6 with 2.6 M ethanol and 1/26 with 2 M glucose, respectively, compared with that in the absence of the hydroxyl compounds.

The effects of other hydroxyl compounds on the enzyme stability were also examined and shown in Fig. 2 and Table I. Methanol was the most effective stabilizer among the monohydroxyl compounds tested. n-propanol and iso-propanol slightly stabilized the enzyme. All the monohydroxyl compounds caused the significant denaturation of the enzyme in their high concentrations. Allyl alcohol and n-butanol did not serve as stabilizers even if in their low concentrations and only caused the denaturation of the enzyme. Glycerol, ethylene glycol and propylene glycol stabilized the enzyme in essentially same manner as glucose. In addition to glucose, the enzyme was well stabilized with various sugars.

Effects of Various Hydroxyl Compound on Enzyme Reaction ······ All of the hydroxyl compounds examined, which served more or less as stabilizers as described above, exerted inhibition when they were present in the reaction mixture (Fig. 3).

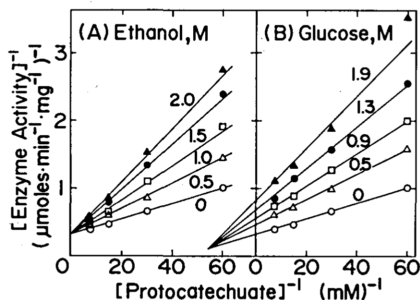


Fig. 3. Inhibition of protocatechuate 4,5-dioxygenase by ethanol and glucose. The enzyme activity was determined as described under "Methods" in the presence of indicated concentrations of ethanol (A) or glucose (B).

With ethanol, the inhibition was found to be competitive with respect to protocatechuate as described previously by Ono *et al.* (3) (Fig. 3A). Thus, the double reciprocal plots for protocatechuate intersected on the ordinate. The inhibition constant (Ki) evaluated by the method of Dixon (5) was 0.75 M. Methanol, iso-propanol and propylene glycol also showed the competitive-type inhibition. On the other hand, the double reciprocal plots with glucose intersected above the abscissa, indicating that the inhibition was mixed-type, namely, mixed inhibition of competitive- and non-competitive-type inhibition (Fig. 3B). Such mixed-type inhibition was also observed with glycerol, ethylene glycol, and all the sugar compounds tested. A summary of these experimental results is shown in Table I.

Table I Summary of stabilizing effect and type of inhibition of various hydroxyl compounds. Minimum k values were estimated from the experiments as shown in Fig. 2. In the absence of hydroxyl compounds, k-values were 0.0392-0.0435 min^{-1}.

Compounds (concn.)	Minimum k	Type of inhibition (Ki)
(M)	min^{-1}	(M)
Methanol (3.8)	0.0092	competitive (4.6)
Ethanol (2.6)	0.0168	competitive (0.75)
n-Propanol (0.3)	0.0327	competitive (0.30)
iso-Propanol (0.7)	0.0255	competitive (0.53)
Ethylene glycol (5.4)	0.0023	mixed
Propylene glycol (3.4)	0.0040	competitive (0.78)
Glycerol (3.4)	0.0045	mixed
Glucose (2.0)	0.0017	mixed
Galactose (2.0)	0.0021	mixed
Xylose (2.3)	0.0035	mixed
Sorbitol (1.3)	0.0029	mixed
Fructose (2.0)	0.0013	mixed
Methylglycoside (1.7)	0.0043	mixed
Saccharose (1.4)	0.0033	mixed

Discussion

The oxidative metabolism of protocatechuate by microorganisms is initiated with aromatic ring fission catalyzed by either protocatechuate 3,4-dioxygenase or protocatechuate 4,5-dioxygenase (1). The former enyzme was stable and purified previously by Fujisawa and Hayaishi (6). In contrast, the purification of the latter enzyme is greatly hampered by the fact that the enzyme is too unstable to be extensively purified (2,3). Therefore, the effects of various hydroxyl compounds on the stability of the enzyme was examined.

Following conclusions could be drawn from the experimental results. First, with monohydroxyl compounds the enzyme was more stabilized with shorter carbon chain compounds, such as methanol and ethanol, presumably because of their low tendency of denaturating the proteins (Fig. 2A). However, the stabilizing effect of these compounds was still incomplete. Longer carbon chain compounds were less effective and

showed the significant denaturating effect. Secondly, the enzyme was generally much more stabilized with polyhydroxyl compounds compared with the monohydroxyl compounds. No protein denaturation was detected with the polyhydroxyl compounds. As expectedly, the stabilizing effect was non-specific and all the polyhydroxyl compounds tested were almost equally effective in their high concentrations. However, the compounds which had more hydroxyl groups in their chemical structures showed a higher stabilizing effect as demonstrated in Fig. 2B. Thus, the effectiveness of hydroxyl compounds increased in the following order; monohydroxyl compound, dihydroxyl compound, trihydroxyl compound, pentose, hexose and its glycoside, disaccharide. Thirdly, with all the hydroxyl compounds tested there was no correlation between the effectiveness of stabilizing the enzyme and the affinity to the active sites of the enzyme. All the monohydroxyl compounds tested exhibited the competitive-type inhibition with respect to protocatechuate. With longer carbon chain compounds the value of Ki was smaller, in other words, the affinity of each compounds to the active sites of the enzyme was more strong. This is contrary to the above observation that the enzyme was more stabilized with shorter carbon chain compounds (Table I). Most polyhydroxyl compounds except propylene glycol exhibited the mixed-type inhibition in contrast with the competitive-type one with the monohydroxyl compounds. These results suggest that the stabilizing action of various hydroxyl compounds is mainly attributable to the binding of these compounds to the sites different from the active sites on the enzyme molecules.

Although various sugars were effective stabilizers, the enzyme purification in the presence of high concentrations of sugars was difficult by reason of possible microbial contamination even if at lower temperature. Therefore, the enzyme purification was carried out at 4° C in the presence of 20 % (about 3.6 M) ethylene glycol. Under these conditions, the enzyme was easily purified from the crude enzyme without significant enzyme inactivation by the usual purification procedures, such as ammonium sulfate fractionation, Sephadex G-200 column chromatography and DEAE-cellulose column chromatography. These procedures resulted in 30-fold purification with about 20 % yield. When the purified enzyme preparation was stored at −20° C, no appreciable loss of enzyme activity was not observed at least for 4 months. The purification and some properties of protocatechuate 4,5-dioxygenase will be published elsewhere.

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