Growth-retarding Effect of Degradation Solution of Dehydroascorbic Acid on *Escherichia coli*

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Many studies on the physiological function of dehydroascorbic acid (DHA) have been done in correlation with the redox system, but there are also papers showing that DHA reacts with cell components in the mode of action different from the redox reaction. We have had a concept that DHA possibly takes part in the control of cell growth through working directly on the cell components such as DNA. To clarify this point, we studied the effect of DHA on the growth of mouse tumor cells or callus cells and the action on DNA. However, it is difficult to fix the real active principles since DHA is unstable in aqueous solution and is decomposed into various unstable intermediates. In order to make the effect clearer, it is desirable first of all to isolate the active compounds from the mixture of degradation products in the solution of DHA. This paper reports the bacterial growth-retarding potencies of the degradation solution of DHA and its fractions separated by ion-exchange chromatography.

1. Materials and Methods

Reagents

DHA was prepared by Von Euler's method and crystallized in acetone by a modification of the method of Kurata et al. 2,3-Diketogulonic acid (DKG) was prepared by the method of Kagawa et al. The purities of DHA and DKG were 99 and 90%, respectively, according to the ion-exchange chromatographic procedure. Other chemicals were of reagent grade.

Fractionation of degradation solution of DHA

The procedure described previously was modified as follows. Ten ml of a 3% aqueous solution of DHA was incubated at 30°C for 10 days. This solution was applied on a Dowex 1-X8 column (formate type, 3.2 × 25 cm), and the column was eluted first with a linear gradient from 0 to 1.0 M of formic acid (1200 ml) and then with a linear gradient from 2.5 to 3.5 M of the same acid (1200 ml) at a flow rate of 450 ml/h. Twenty ml fractions were collected, and degradation products in them were monitored by measuring the absorbance at 295 nm and/or by measuring the absorbance at 520 nm after formation of the corresponding 2, 4-dinitrophenylhydrazones.

Determination of the growth of *E. coli*

The growth medium contained the following compounds (g per liter): K2HPO4, 7.0; KH2PO4, 3.0; sodium citrate dihydrate, 0.5; MgSO4·7H2O, 0.1; (NH4)2SO4, 0.1; glucose, 5.0; polypeptone, 2.0; and thiamine hydrochloride, 0.005. The pH of the medium was adjusted to 7.2. *Escherichia coli* IFO 3301 obtained from the Institute for Fermentation, Osaka, was precultured in a test tube containing 10 ml of the medium for 24 h at 30°C on a reciprocating shaker. This preculture was diluted with the medium to the concentration of 0.3 unit in the turbidity at 610 nm on an ANA-1 photometer (Tokyo Photoelectric Co., Ltd.) in a 18 mm diameter test tube. As a routine work, 7.5 ml of the fresh medium was mixed with 2.0 ml of the test solution which had been neutralized with a NaOH solution and sterilized through a GS Millipore filter, and 0.5 ml of the diluted preculture of *E. coli* was added, and the turbidity of the mixture was measured periodically while the incubation was continued under the same conditions as in the preculture.

2. Results and Discussion

Fig. 1 shows that the DHA solution incubated at 30°C for 10 days (10 day-incubated solution) retarded the growth of *E. coli*. The solution incubated for 3 days (3 day-incubated solution) also exerted the retarding effect. The effect, however, was not so noticeable as that of the 10 day-incubated solution. Ascorbic acid (ASA) did not retard the growth of *E. coli* at the equivalent concentration. DHA or DKG was more or less inhibitory to the growth of
E. coli. Cupric ion (0.1 mM) enhanced the growth-retarding potency of the 3 or 10 day-incubated solution, whereas it hardly enhanced that of ASA, DHA or DKG. In the case of the 10 day-incubated solution, the lowest concentration necessary for growth retardation was 0.2% in the absence of Cu²⁺, and 0.05% in the presence of Cu²⁺ (0.1 mM) as a total amount of DHA and its degradation products.

Fig. 1 Effect of degradation solution of DHA on the growth of E. coli

Ten ml of a 3% DHA solution in a 30 × 45 mm weighing bottle was incubated at 30°C for 3 or 10 days. This solution was neutralized with 1 N NaOH and diluted with water to 2.5%. The 210 mM solution of ASA, DHA or DKG was neutralized with 1 N NaOH, if necessary, and diluted with water to 140 mM. Two ml of these neutral solutions was added to 8.0 ml (a) or 7.5 ml (b) of the mixture of a medium and a preculture of E. coli. In the case of (b), 0.5 ml of 2 mM CuSO₄ dissolved in the medium was added. The final concentrations of test materials were as follows: ASA, DHA or DKG, 28 mM; DHA solution incubated for 10 days, 0.5% (as solid), this was equivalent to 28 mM of DHA; Cu²⁺, 0.1 mM. Growth was determined by the method described in the text. (a) in the absence of Cu²⁺. (b) in the presence of Cu²⁺. (a) --- O, ASA; (b) ---, DHA; (b) --- O, DKG; (b) ---, DHA solution incubated for 3 days; (b) --- O, DHA solution incubated for 10 days; (b) ---, control.

Fig. 2 Effect of concentration of DHA solution incubated for 10 days on the growth of E. coli

The DHA solution prepared as described in the legend to Fig. 1 was diluted to 1.0, 0.5, 0.2 and 0.1%, and 2.0 ml of these dilute solutions as well as the 2.5% solution was used as test solutions. Growth was determined by the method described in the text. (a) in the absence of Cu²⁺. (b) in the presence of Cu²⁺. The final concentrations of the DHA solution incubated for 10 days were as follows: (a) --- O, 0.5%; (b) --- O, 0.2%; (b) --- O, 0.1%

The pH of a 3% DHA solution prepared for growth test was below 2.5 throughout the period of incubation at 30°C. In this pH range, it is estimated from the half-life reported by TERADA et al.⁸) that more than 99% of DHA degrade within 4 days at 37°C. In fact, little amount of DHA was found in the solution incubated for 10 days at 30°C by ion-exchange chromatography. Therefore, the growth-retarding effect of the incubated solution is not attributable to the presence of DHA. On the other hand, the 3 or 10 day-incubated solution possessed the reducing power and
Fig. 3 Effect of concentration of Cu\(^{2+}\) on the growth of E. coli in cooperation with DHA solution incubated for 10 days

The final concentration of the DHA solution incubated for 10 days was 0.02%. Concentrations of Cu\(^{2+}\): ○—○, 300 μM; •—•, 100 μM; ○—○, 50 μM; •—•, 10 μM; ○—○, 0 μM; •—•, control. Growth was determined by the method described in the text.

Fig. 4 Elution pattern of degradation solution of DHA on Dowex 1-X8 ion-exchange chromatography

Chromatographic conditions: See the description in the text. ○—○ A\(_{320}\); •—•, A\(_{295}\)

gave a few spots which were detectable by reduction of 2,6-dichlorophenol indophenol on a thin-layer chromatoplate. This suggests the presence of reductones in the solutions.

Reductones such as triose reductone\(^{9-11}\) and ASA\(^{12}\) are known to retard the growth of microorganisms. Moreover, SHINOHARA et al.\(^{12}\) observed that Cu\(^{2+}\) enhanced the growth-retarding potency of triose reductone or ASA. Similar effect of Cu\(^{2+}\) was observed in the incubated solutions of DHA. Consequently, the fractionation of the 10 day-incubated solution was attempted by ion-exchange chromatography. A typical elution pattern of the solution is shown in Fig. 4. Fraction B (fraction No. 35~40) exhibited its absorption maximum at 295 nm in a strongly acidic solution and
reduced 2,6-dichlorophenol indophenol. Fraction E (fraction No. 78~89) reacted with 2,4-dinitrophenyl-hydrazine. The growth-retarding effect of each fraction is shown in Fig. 5. Fractions B and E retarded the growth of E. coli, and the other fractions did not affect the growth. Both fractions, B and E, collected from 10 ml of the 3% DHA solution incubated for 10 days, inhibited completely the growth of E. coli in the presence of 0.1 mM Cu²⁺. These data do not indicate the relative growth-retarding potencies of the compounds in each fraction. However, the results of the present study imply that the growth-retarding effect of the 10 day-incubated solution is mainly due to the action of the degradation products of DHA contained in the fractions B and E.

3. Summary

The dehydroascorbic acid solution which had been incubated at 30°C for 10 days retarded the growth of E. coli, and the effect was enhanced by Cu²⁺. The lowest concentration of the solution exerting the growth-retarding effect was 0.2% in the absence of Cu²⁺, and it was 0.05% in the presence of Cu²⁺. Dehydroascorbic acid, 2,3-diketogulonic acid or the dehydroascorbic acid solution incubated at 30°C for 3 days also retarded the growth of E. coli, but the effects were less than that of the dehydroascorbic acid solution incubated at 30°C for 10 days. The fractionation of the solution incubated at 30°C for 10 days by ion-exchange chromatography on Dowex 1-X8 revealed that two fractions had high potency of growth retardation. One fraction exhibited the absorption maximum at 295 nm in an acidic solution, and the other reacted with 2,4-dinitrophenylhydrazine.

References


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