Rapid Paper

Pyrroloquinoline Quinone as an Essential Growth Factor for a Poly(vinyl alcohol)-degrading Symbiont, Pseudomonas sp. VM15C

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From a supernatant of a mixed continuous culture of Pseudomonas sp. VM15C and Pseudomonas putida VM15A, of which the former produced a poly(vinyl alcohol) (PVA)-degrading enzyme and the latter an essential growth factor (trivial name, factor A) for PVA utilization by the former, purified factor A showing homogeneity on high performance liquid chromatography was obtained. From spectral and other evidence, factor A was identified as pyrroloquinoline quinone (PQQ). PQQ was effective in not only causing growth of the PVA-degrading bacterium on PVA but also in enhancing the growth rate and the cell yield at concentrations of 0.05 to 4 ng/ml. Thus, a novel coenzyme, PQQ, was reported as a bacterial growth factor for the first time.

In the preceding studies,1~4) we reported poly(vinyl alcohol) (PVA) utilization by mixed cultures of pairs of bacterial symbionts. Pseudomonas sp. VM15C and Pseudomonas putida VM15A were such a pair which formed a potent PVA-utilizing mixed culture. The former produced a PVA-degrading enzyme and the latter a growth factor (trivial name, factor A) essential for PVA utilization by the former. In this work, we succeeded in purification of factor A, and determined that factor A is pyrroloquinoline quinone (PQQ). PQQ is a novel coenzyme which was discovered in methylotrophic bacteria.5) Since determination of the structure in 1979,6) several types of dehydrogenases of oxidative bacteria have been found to be quinoproteins which have PQQ as the prosthetic group.7~9) Furthermore, recently some mammalian enzymes were suggested to be quinoproteins.10) Thus, PQQ is a significant coenzyme which is assumed to be distributed widely from procaryotes to mammals, however, there has been no report concerning its physiological effect. This paper reports PQQ as a bacterial growth factor for the first time.

MATERIALS AND METHODS

Materials. Authentic PQQ was purified from a culture supernatant of a methylotrophic bacterium.10) PVA (average mol. wt, 2,000; Aldrich Chemical. Co., Inc.) was saponified in 0.8 n NaOH at 30°C at 8% concentration (degree of saponification, 99.9%).

Microorganisms and media. A d-glucose dehydrogenase activity-less mutant of Acinetobacter calcoaceticus was obtained by a similar method with acriflavine to that described previously.7) P. putida VM15A, PVA-degrading Pseudomonas sp. strains, VM15C, VM10B, VM6B, VA3B and VT1B, and the basal and PVA media were the same as described previously.1~3) PVA-ethanol medium consisted of 0.25% of the saponified PVA and 0.05% ethanol in the basal medium. Glucose-BTB agar was composed of 1% glucose, 1% peptone, 0.04% bromothymol blue and 1.5% agar, pH 7.3.

Isolation of factor A. A mixed continuous culture of the bacterial symbionts was established in a chemostat (Bioflo C32, New Brunswick Scientific Co., Inc.) with PVA-ethanol medium at a dilution rate of 0.064/hr at 30°C. Ten liters of the supernatant harvested from the steady-state culture was adjusted to pH 3.5 with conc. HCl and passed through a DEAE-Toyopearl 650M column (2 x 16 cm) previously equilibrated with 20 mM potassium phosphate buffer (pH 7.0). After washing with 500 ml of the same buffer, factor A was eluted with a linear concentration gradient of KCl in the buffer. The active fractions were combined and adjusted to pH 2.0 with dilute HCl and then passed through a Sep-pak C18 cartridge to remove the inorganic salts. After washing the cartridge with 20 ml of dilute HCl (pH 2.0), factor A was eluted with 10 ml of methanol/water (7/3, v/v).

High-performance liquid chromatography (HPLC). HPLC was performed on a µBondapak C18 column (0.38 x 30 cm) with 12.5 mm K2HPO4 (adjusted to pH 2.0 with conc. H2PO4)/methanol (8/3, v/v) at a flow rate of 1.0 ml/min in a HPLC system (Waters Associates Inc.), according to Duine and Frank.11) The effluent was moni-
Biological assay. The factor A content was determined from the activity which caused axenic growth of VM15C in PVA medium. The strain was cultivated in 5 ml of PVA medium supplemented with a sample in a test tube at 30°C for 3 to 4 days with reciprocal shaking. The growth was calibrated from the linear relationship between it and the added amount of a standard sample. A culture supernatant of VM15A grown for 4 days in basal medium containing 0.5% glucose and stored at -20°C was used as the standard. One unit of the activity was taken as the amount that caused growth corresponding to 1 ml of the standard.

The PQQ content was assayed by a paper disk method with the mutant of A. calcoaceticus, which was unable to form PQQ but produced apo-glucose dehydrogenase requiring PQQ as a coenzyme in its membrane. Paper disks (6mm0) immersed in sample and authentic PQQ solutions were placed on the culture grown on a glucose-BTB agar plate, and the plate was incubated for 16 hr at 30°C. The diameters of the yellow circles which resulted from the pH decrease due to the formation of gluconic acid around the disks were calibrated from the linear relationship between them and the log PQQ concentration in the authentic solution.

RESULTS AND DISCUSSION

Purification of factor A

To find a good source for isolation of factor A, production was studied in various axenic cultures of VM15A and mixed cultures with VM15G. In the course of the study, it was found that rather abundant factor A activity was produced by the mixed chemostat culture described in Materials and Methods. Isolation of factor A was achieved with the supernatant. On DEAE-Toyopearl 650M column chromatography, factor A was eluted as a peak superimposed on that of absorbance at 249 nm and a low shoulder, as shown in Fig. 1. The active fractions (numbers 37~40) which showed a pink color were combined and dried under reduced pressure after the Sep-pak C_{18} treatment. About 1.5 mg of purified factor A was obtained as a reddish solid material. The purified factor A was found to be homogeneous and eluted as a single peak on reversed-phase HPLC as described in Materials and Methods.

Identification of factor A

The purified factor A showed characteristic absorption and fluorescence spectra as indicated in Fig. 2. In the absorption spectrum, maxima at 248 and 330 nm, a shoulder at 270 nm, and a low broad peak at 400 to 600 nm were observed. As to the fluorescence spectrum, excitation at 365 nm resulted in an emission peak with a maximum at 485 nm, and emission at 465 nm gave an excitation spectrum with a maximum at 380 nm and shoulders at 260 and 330 nm. These spectra were consistent with those reported for PQQ. On HPLC, the purified factor A showed the same retention volume as the authentic PQQ. Furthermore, as shown in Fig. 3, the biological activity of a solution of the purified factor A on the mutant of A. calcoaceticus was the same as that of a solution of the authentic PQQ on the basis of the PQQ concentration determined with a molar absorption coefficient of 16,037 M^{-1} cm^{-1} at 259 nm. It was also confirmed with the biological assay that elution of factor A was superimposed on that of PQQ in the ion exchange chromatography (Fig. 1). From these results, factor A was identified as PQQ.

PQQ as a growth factor

The purified PQQ (factor A) was a very effective growth factor essential for PVA utilization by VM15C. As shown in Fig. 4, even at a PQQ concentration of 0.05 ng/ml, slight
Growth Factor, PQQ

Fig. 2. Absorption Spectrum (A) and Fluorescence Spectrum (B) of the Purified Factor A in Water at pH 7.0.

A solution of the purified factor A was adjusted to pH 7.0 with dilute NaOH. The absorption spectrum was obtained with a Shimadzu model UV-300 spectrophotometer. The fluorescence spectrum was determined with a Hitachi model 650-10S fluorescence spectrophotometer.

Fig. 3. Biological Activity of the Purified Factor A as PQQ.

The purified factor A (○) and authentic PQQ (●) solutions were assayed by the paper disk method described in MATERIALS AND METHODS. Diameters of yellow circles around paper disks from the solutions are shown as a function of the PQQ concentration determined with the molar absorption coefficient as described in the text.

growth on PVA occurred, and the growth rate and the growth yield were enhanced with increasing concentration to 4 ng/ml. One milligram of the purified PQQ was calculated to exhibit 480,000 units of factor A activity. Authentic PQQ showed the same effect (480,000 units of factor A activity per mg) on growth as the purified PQQ. PQQ was also required as an essential growth factor for PVA utilization by PVA-degrading Pseudomonas sp. strains, VT1B, VA3B, VM6B and VM10B, as well as VM15C. On the other hand, when carbon sources (D-glucose, D-mannose, L-alanine, L-glutamate, etc.) other than PVA were utilized by VM15C, PQQ affected the growth to only a slight or
negligible extent. These facts indicated the possibility that a PQQ-dependent enzyme may catalyze a specific and significant reaction in the bacterial PVA metabolism.

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REFERENCES