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DETECTION OF CARBON-PHOSPHORUS LYASE ACTIVITY IN CELL FREE EXTRACTS OF *ENTEROBACTER AEROGENES*

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SUMMARY: The bacterium *Enterobacter aerogenes* could grow on a medium containing alkylphosphonic acid as a phosphorus source. The extracts prepared from the cells grown on phosphonoacetic acid as a sole source of phosphorus showed an activity of carbon-phosphorus lyase and hydrolyzed methylphosphonic acid, phosphonoacetic acid and phenylphosphonic acid with a liberation of inorganic phosphates.

Alkylphosphonic acids, compounds with a direct carbon-phosphorus (C-P) bond, are widely distributed in biological systems either free or combined form with lipids, polysaccharides and other cellular constituents. Insecticides, herbicides, fungicides, nerve gases, flame retardants and several other important synthetic chemicals also contain C-P bond in their structures and have been used in large quantities. Since the C-P bond is highly resistant to chemical hydrolysis and thermal decomposition, the breakdown of alkylphosphonic acids by biological systems, especially by microbial cells, is important to protect an overaccumulation of these acids in nature.

From this standpoint, many efforts have been made to select microbial cells having a capability of hydrolyzing

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the C-P bond in alkylphosphonic acids (1). Some of gram negative bacteria were found to utilize alkylphosphonic acids as a sole source of phosphorus (Pi). However, to date, no intrinsic activity of the enzyme responsible for the C-P bond cleavage (equation 1) has not been obtained in cell free systems despite intensive efforts (2, 3, 4, 5).

(1) $R\text{-}PO_3^{2-} + H_2O \longrightarrow R\text{-}H + Pi$

In order to clarify physiological significance of alkylphosphonic acids in living systems and to apply this unique enzyme to processes of organic chemistry as a biomimetic model, we screened C-P bond cleavage enzyme (C-P lyase) in microorganims, and, for the first time, we detected the enzyme activity in cell extracts of *Enterobacter aerogenes*.

MATERIALS AND METHODS

Chemicals Phosphonoacetic acid was purchased from Nakarai Chemicals Co., Ltd., Kyoto. Methylphosphonic acid, phenylphosphonic acid and 2-aminoethylphosphonic acid were from Aldrich Chem. Co., Milw.

Screening of microorganisms Microorganisms were aerobically grown in 2.5 ml of Medium A or Medium B at 30 °C for 24 h. Medium A contains 0.5 % glucose, 0.2 % yeast extract (Pi-free) and 0.4 % phosphonoacetic acid (pH 7.2). Medium B contains 0.5 % glucose, 0.1 % $(NH_4)_2SO_4$, 0.01 % $MgSO_4 \cdot 7H_2O$, 0.02 % yeast extract (Pi-free) and 0.4 % alkylphosphonic acid (pH 7.2). Yeast extract (Pi-free) was prepared by magnesia treatment (6), and contained no detectable Pi, when the concentration was determined by the method of Fiske and Subbarow (7). Alkylphosphonic acids were used after millipore-filtration and glasswear was rinsed with nitric acid before use.

Assay for C-P lyase Cells of *Enterobacter aerogenes* IFO 12010 were aerobically grown in 100 ml of Medium B containing 0.4 % phosphonoacetic acid at 30 °C for 24 h. The cells were collected, washed once in 10 mM Tris/HCl buffer (pH 7.5), resuspended in the same buffer, and then disrupted ultrasonically at 90 kHz, 0 °C for 15 min. The homogenate was centrifuged at 25,000 x *g* for 30 min at 0 °C and resulting supernatant was used as an enzyme source after dialysis against 10 mM Tris/HCl buffer (pH 7.5) at 4 °C overnight. C-P lyase was assayed in a mixture (0.5 ml) consisting of 50 mM alkylphosphonic acid, 20 mM $MgCl_2$ and 50 mM Tris/HCl buffer (pH 7.5). The reaction was initiated by the addition of enzyme (about 3 mg/ml as protein) and terminated by adding 0.5 ml of 25 % trichloroacetic acid. Pi liberated in the reaction mixture was determined as above and protein was determined according to the method of Lowry *et al.* (8).

RESULTS AND DISCUSSION

Screening of C-P lyase activity among bacteria

Thirty-six bacterial strains [*Escherichia* (2), *Bacillus* (10), *Brevibacterium* (2), *Micrococcus* (3), *Alcaligenes* (1), *Arthrobacter* (2), *Sarcina* (2), *Corynebacterium* (1), *Pseudomonas* (1), *Flavobacterium* (4), *Staphylococcus* (2), *Agrobacterium* (3), *Rhizobium* (1), and *Enterobacter* (2)] were grown on Medium A with or without phosphonoacetic acid. All the strains showed a sufficient growth on Medium A in the absence of phosphonoacetic acid, since cells of these strains could utilize organophosphate esters in yeast extract. However, in the presence of phosphonoacetic acid, only 11 strains [*Agrobacterium tumefaciens* IFO 3085, *Bacillus roseus* IFO 3525, *Brevibacterium ammoniagnes* AKU 0642, *Brevibacterium incertum* IFO 12145, *Corynebacterium equi* IAM 1038, *Enterobacter aerogenes* IFO 12010, *Enterobacter aerogenes* IFO 3319, *Escherichia coli K-12*, *Pseudomonas iodinum* IFO 3568] were resistant to phosphonoacetic acid and showed the growth comparable with that in the absence of the acid. The growth of other strains was significantly repressed by phosphonoacetic acid. Pi concentrations in the culture fluids of phosphonoacetic acid resistant strains were measured and the cultures of *Bacillus roseus* AKU 0208 and *Enterobacter aerogenes* IFO 12010 were found to contain substantial amount of Pi (0.2 mM and 1.1 mM, respectively). Other phosphonoacetic acid-resistant strains showed no detectable accumulation of Pi. The accumulation of Pi by above two strains seemed to be due to the direct cleavage of C-P bond in phosphonoacetic acid, and *Enterobacter aerogenes* IFO 12010 was used in further studies on alkylphosphonic acids utilization and detection of C-P lyase activity in cell free extracts.

Table 1. Utilization of alkylphosphonic acids by *Enterobacter aerogenes* IFO 12010

Phosphorus source ($R PO_3^{2-}$, R=)		Growth
None		0.0
Orthophosphate (HO-)	0.2 % 0.4 %	2.2 2.5
Methylphosphonic acid (CH_3-)	0.2 % 0.4 %	0.6 2.4
Phosphonoacetic acid (HOOC-CH_2-)	0.2 % 0.4 %	1.9 2.1
2-Aminoethylphosphonic acid (H_2N-CH_2-CH_2-)	0.2 % 0.4 %	1.7 2.2
Phenylphosphonic acid (C_6H_5-)	0.2 % 0.4 %	0.6 1.3

Cells of *E. aerogenes* were grown on Medium B containing various alkylphosphonic acids at 0.2 % or 0.4 %. Cultivation was carried out as described in text. Growth was determined by measuring the turbidity of the culture (O.D. at 610 nm).

Utilization of alkylphosphonic acids by E. aerogenes

The cells of *E. aerogenes* IFO 12010 were cultured in Medium B with or without alkylphosphonic acids (Table 1). In the absence of phosphorus source, the cells could not grow on Medium B, thus suggesting that yeast extract (Pi-free) supports no growth of the bacterium at 0.02 %. Methylphosphonic acid, phosphonoacetic acid, 2-aminoethylphosphonic acid and phenylphosphonic acid were utilized as a source of phosphorus. The result clearly indicated that the cells contained an enzyme (C-P lyase) catalyzing the hydrolysis of a direct C-P bond, although an alternative enzymatic route (equation 2) has been postulated as to the decomposition of 2-amino-

ethylphosphonic acid (9). This route (equation 2) has not been confirmed in the cells of *E. aerogenes* IFO 12010.

(2) 2-Aminoethylphosphonic acid $\xrightarrow{\text{Transaminase}}$ Phosphonoacetaldehyde

Phosphonoacetaldehyde $\xrightarrow{\text{Phosphonatase}}$ Acetaldehyde + Pi

C-P lyase activity in cell free extracts of E. aerogenes

To detect the enzyme responsible for the C-P bond cleavage, *E. aerogenes* IFO 12010 cells were cultured in Medium B containing phosphonoacetic acid as a sole source of phosphorus. The cell extracts were incubated with methylphosphonic acid, phosphonoacetic acid and phenylphosphonic acid in the presence of Mg ion (Fig. 1). The liberation of Pi from these alkylphosphonic acids proceeded linearly with the reaction time and protein concentration (data not shown). No liberation of Pi was observed in the absence of cell extracts or when boiled extract was used.

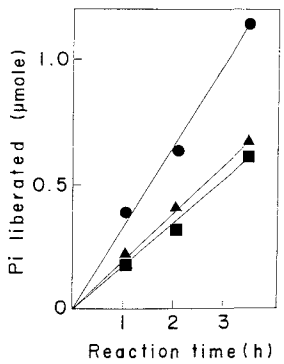

FIG. 1. Liberation of inorganic phosphates from alkylphosphonic acids by cell free extracts of *E. aerogenes* IFO 12010. Reaction was carried out as described in text and Pi liberated from alkylphosphonic acids was determined periodically. -▲-, Methylphosphonic acid; -●-, Phosphonoacetic acid; -■-, Phenylphosphonic acid

Thus, we detected the C-P lyase activity in *Enterobacter aerogenes* IFO 12010. This is the first report that presented the C-P lyase activity in cell free system. The detection of the enzyme activity in cell free extracts will facilitate the studies on the physiological significance of alkylphosphonic acids in living cells and the application of the enzyme to biomimetic organochemical processes. We are now engaged in the isolation of the enzyme and its gene from *Enterobacter aerogenes* IFO 12010.

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