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Purification and Characterization of a Tetrachloroethylene (PCE) Dehalogenase from *Clostridium bifermentans* DPH-1

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C. bifermentans DPH-1 균주로 부터 정제한 테트라클로로에틸렌 (PCE) 분해효소의 제성질

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국문요약

DEAE-Toyopearl 650S, Superdex pg-75, Poros HQ, Superdex HR200의 각종 칼럼크로마토그래피를 이용하여 *C. bifermentans* DPH-1균주로 부터 테트라클로로에틸렌(PCE) 분해 효소를 정제했다. 이 PCE 분해효소(PCE dehalogenase)는 PCE를 환원적 탈염소화 반응에 의해 시스디클로로에틸렌(*cis*-1,2-dichloroethylene)에 전환 가능하며, 이 때의 V_{max} 및 K_m 値는 각각 73 nmol/h · mg protein, 12 μM이었다. 본 PCE dehalogenase의 겔여과 분자량 Maker Kit를 이용한 분석결과(70 kDa)는 SDS-PAGE에 나타난 분자량(35 kDa)의 약 2배인 것으로 확인되었다. 따라서 본 효소는 분자량 70 kDa의 二量體(Homo dimer)인 것으로 추정되었다. 본 효소의 최적 온도 및 pH는 각각 35°C 및 8.0이었다. 또한 본 효소는 PCE외의 트리클로로에틸렌, 디클로로에틸렌 이성체, 1,2-디클로로에텐, 1,2-디클로로프로판, 1,1,2-트리클로로에탄에 대하여도 활성을 나타내었다. N-말단 아미노산 분석결과에서도 본 효소는 현재 알려진 PCE dehalogenase와 그 배열이 전혀 다른 것으로 나타나 각종 유기염소 화합물의 분해능을 보유한 신종의 PCE 분해효소인 것이 확인되었다.

Keywords : Tetrachloroethylene, Trichloroethylene, Chlorinated Aliphatic compounds, Corrinoid

I. Introduction

Chlorinated aliphatic compouns such as tetrachloroethylene (PCE) and trichloroethylene (TCE), isomers of dichloroethylene (*cis*-1,2-DCE, *trans*-DCE, and 1,1-DCE), vinyl chloride (VC) and other chloroaliphatics occur in significant concentrations in the environment due to industrial usage. Tetrachloroethylene and trichloroethylene (TCE), in particular, are excellent solvents, widely used in dry cleaning and in the textile industry, and in the scouring of machines, in the extraction of fats (1). These chemicals pose serious public health problems, therefore, remediation of contaminated sites and water courses is now an urgent issue worldwide.

PCE is an important model for the study of biodegradation of chlorinated aliphatic compounds because of its high halogen content and toxicity. PCE is recalcitrant under aerobic condition but can be reductively dechlorinated by a few microbes under anaerobic conditions (2-4). Members of the Gram-positive genus *Desulfitobacterium*, namely PCE1 (2) and strain PCE-S (3), and only one strain of the genus *Clostridium*, namely strain DPH-1 (4) are able to reductively dehalogenate a variety of chlorinated hydrocarbons. In addition, enzyme-catalyzed PCE reductive dechlorination in pure cultures has been described for the Gram-negative bacteria *Dehalospirillum multivorans* (1, 5,

6), *Desufomonile tiedjei* (7), strain MS-1 (8), and strain TT4B (9) as well as for the gram-positive bacteria *Dehalobacter restrictus* (10, 11) and phylogenetically unrealated *Dehalococcoides ethenogenes* strain 195 (12).

Cell-free extracts of *Clostridium bifermentans* DPH-1, catalyzed the degradation of PCE and other chlorinated aliphatic compounds to a significant extent (13). In cell extracts, enzymatic activity was inhibited by cyanide and the addition of propyl-iodide caused a light reversible inhibition of PCE degradation, suggesting the involvement of a corrinoid cofactor (14, 15).

Recently, for the first time a PCE reductive dehalogenase has been purified from the cytoplasmic fraction of the strictly anaerobic, gram-negative *D. multivorans* (16). The protein catalyzes the reductive dehalogenation of PCE and TCE to *cis*-1,2-dichloroethylene (cDCE) with methyl viologen as the artificial electron donor and contains a corrinoid as well as iron and acid-labile sulfur as prosthetic groups. In addition, a membrane-associated PCE reductive dehalogenase of strain PCE-S has been isolated (17). The enzyme was not similar to the enzyme of *D. multivorans* with respect to the molecular mass and cofactor content. Here we report on the purification and characterization of a membrane-associated PCE dehalogenase from the strictly anaerobic, gram-positive strain DPH-1.

II. Materials and Methods

Chemicals PCE was obtained from the Kanto (Tokyo, Japan). TCE, cDCE, *trans*-DCE, 1,1-DCE, dichloromethane (DM), 1,3-dichloropropene (1,3-DP), 1,2-dichloropropane (1,2-DP), 1,1,1-trichloroethane (1,1,1-TE), 1,1,2-trichloroethane (TE) and VC were obtained from GL Sciences Inc. (Tokyo, Japan). All other chemicals were of analytical quality and purchased from the Wako Chemical Co., Inc (Kyoto, Japan).

Microorganism and culture condition *Clostridium bifermentans* DPH-1 was isolated from ditch sludge, in Gifu prefecture, Japan (18). The culture and PCE degrading activity was maintained by weekly subculture on MY medium of the following composition (grams per liter): K_2HPO_4, 7.0; KH_2PO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; sodium citrate, 0.5; yeast extract, 2.0. The pH of the medium was 7.2. After autoclaving 10 m*l* of the medium in 26 m*l* serum bottles, 0.1 m*l* of filter sterilised vitamin solution (1 g of p-aminobenzoic acid, 1 mg of biotin.per liter) and 0.1 m*l* $FeSO_4 \cdot 7H_2O$ (2.0 g/*l*) was added. Headspaces were flushed with oxygen free nitrogen (OFN) and sealed with Teflon-lined rubber septa and aluminum crimp caps. Thereafter, PCE was added to a final concentration of 12 μM and incubated. For enzyme preparation, cells were grown in 120 m*l* serum bottles containing 100 m*l* MY medium at 30°C until the peak of PCE degradation (46 to 56 h). *C. bifermentans* cells were then harvested by centrifugation (8,000 rpm, 15 min) after adding dithiothreitol (DTT) to a final concentration of 2 mM and stored at -30°C.

Enzyme preparation *C. bifermentans* cells (0.8 or 1.6 g) resuspended in 6 m*l* of 20 mM Tris-chloride pH 8.2 containing 2 mM DTT and 5% glycerol, was lysed using a TOMY ultrasonic disrupter UD-201 at 30 seconds flash for 5 min. Unbroken cells and debris were separated by centrifugation (14,000 rpm, 20 min, 4°C) and the extraction procedure repeated with the unbroken cells and debris. The two supernatant fractions were combined and passed through a 0.22 μ millipore filter. The filtrate served as the enzyme extract.

Enzyme assay Dehalogenase assay was adapted from the methods of Neuman *et al.* (16) and Magnuson *et al.* (19). Assay was performed in 26-m*l* serum bottles sealed with Teflon rubber stopper and aluminium crimp cap. Four milliliters of the assay mixture was composed of 100 mM Tris-chloride pH 7.5, 1.5 mM methyl viologen, 1.5 mM titanium (III) citrate, 1.0 m*l* enzyme, and 12 μM PCE. Headspaces of all solutions were purged with OFN for 5 min. Incubation was at 30°C for 30 min. In some cases reaction was terminated by the addition of 0.4 m*l* of 5 M H_2SO_4. Headspace sample (10 μ*l*) was subjected to gas chromatography analysis. One unit of enzyme activity was defined as nmol of products (TCE+cDCE) produced under the assay condition.

Gas-chromatography analysis of chlorinated aliphatic compounds PCE, TCE, cDCE, *trans*-DCE, 1,1-DCE, DE, 1,1-DE, DM, DP, 1,2-DP, TE, and 1,1,1-TE in a 10-μ*l* headspace sample were quantified by gas-chromatography using a model GC-14B gas chromatograph (Shimadzu Co., Japan) equipped with an electron capture detector (ECD) system and a 3.2 mm×2.1 m glass column (Silicone DC-550 20% Chromosorb W [AWDMCS] 80/100). Injection, detection, and column temperatures were

set at 200, 300, and 80°C respectively. The carrier gas was OFN at a flow rate of 50 m*l*/min. High concentration of PCE was determined gas chromatographically by a model GC-9A gas chromatograph (Shimadzu Co., Japan) equipped with a flame ionization detector (FID) and a glass column (i.d. 3.2×2.1 m; Silicone DC-550 20% Chromosorb W [AWDMCS] 80/100). The carrier gas was OFN.

Enzyme purification Enzyme extract was passed through a 50 kDa nominal molecular weight limit ultrafiltration membrane (Centriprep YM-50, Millipore) and concentrated to approximately 1.5 m*l*. The concentrate was applied to DEAE-Toyopearl anion-exchange column (12×1.5 cm), previously equilibrated with 20 mM Tris-chloride buffer pH 8.2 to which 2 mM DTT was added. Proteins were eluted using a linear gradient of 0-0.5M NaCl in 20 mM Tris-chloride pH 8.2, at a flow rate of 1 m*l*/min. Forty-five 5 m*l* fractions were collected at 4°C and assessed for dehalogenase activity. Active fractions were pooled and subjected to size exclusion chromatography as follows. Pooled fractions were concentrated to about 1.5 m*l* using the YM-50 ultrafiltration membrane. The concentrate was applied to Superdex pg-75 column (70×1.5 cm) previously equilibriated with elution buffer (150 mM KCl in 20 mM potassium phosphate buffer, pH 7.5). Protein elution was with the same buffer at a flow rate of 0.36 m*l*/min. Fifty 2.7 m*l* fractions were collected at 4°C and subjected to dehalogenase assay. Further purification by ion-exchange high performance liquid chromatography (IEX-HPLC) was carried out on Poros HQ anion-exchange colum (4.6 ×100 mm), using a Shimadzu LC-7A Bio liquid chromatograph equipped with a Shimadzu SPD-7AV UV/VIS spectrophotometric detector, a Shimadzu SCL-6B system controller, a Shimadzu CR4A computerized data analyzer and 1 m*l* injection loop. Elution (1 m*l*/min) was initially isocratic (10 min) with 20 mM Tris-chloride buffer pH 7.5 and thereafter by linear gradient (20 min) using 0-1 M NaCl in 20 mM Tris-chloride buffer pH 8.2. Column was sequentially washed with 1 M NaCl in 20 mM Tris-chloride buffer pH 8.2 (10 min) and 20 mM Tris-chloride buffer pH 8.2 (10 min). Size-exclusion high-performance liquid chromatography (SE-HPLC) was performed using the same HPLC system, equipped with 500 μ*l* injection loop. The colum was a Superdex 200 HR 10/30 (1×30 cm). Isocratic elution was performed using 150 mM NaCl in 20 mM Tris-chloride buffer pH 7.5 at a flow rate of 0.5 m*l*/min.

Molecular weight determination The molecular weight of the native enzyme was determined by SE-HPLC on Superdex 200 HR 10/30. The column was calibrated using a molecular weight kit from Sigma (MW-GF-200-KIT) according to the manufacturers instructions. The purity and molecular weight of protein sample were examined by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) using 10 % or 12% polyacrylamide (Laemmli 1970). Molecular weight markers ranging from 17,201 to 200,000 ("DAIICHI".II, Daiichi pure chemicals co., Japan), were used. Gels were stained with Coomasie brilliant blue G-250 dye.

Time course of PCE degradation and product formation This was performed using the standard assay mixture. Initial concentration of PCE was 12 μM. PCE biotransformation and product formation (TCE and cDCE) were continuously monitored, using the gas chromatograph equipped with ECD.

Determination of basic enzyme properties All assays were performed using the standard assay mixture and conditions described above. Temperature activity profile of the dehalogenase was determined by incubating at 20, 25, 30, 35, and 40°C. For pH activity profile, this was examined using either Tris-chloride (pH 7.5, 8.0, 9.0) and phosphate buffer (pH 6.0, 7.0). pH stability was determined by incubating the enzyme at each pH value for 1 h, at room temperature, using the same buffers. The effects of metal ions (Co^{2+}, Fe^{2+}, Mg^{2+}, Na^{2+}, Mn^{2+}, Hg^{2+}), NADH and cyanocobalamin were examined at a final concentration of 5 mM. Effect of substrate concentration was determined using different concentrations of PCE (3-30 mM).

Effect of propyl iodide on PCE dehalogenase activity PCE dehalogenase (1 m*l*; 0.122 mg protein ml^{-1}) was added to 3 m*l* 0.1 M Tris-HCl (pH 7.5) in glass bottle wrapped with aluminum foil. The activity of the dehalogenase was measured in the test system described above except that titanium (III) citrate (2 mM) was used instead of DTT. Propyl iodide, 1-iodopropane (PI; 0.5 mM) was added at the times indicated by arrows in Fig. 6. The cell extract was illuminated (250-W lamp) for 5 min at the time indicated by the arrow (Fig. 6).

Comparative degradation of PCE and other substances Stock solutions of all the chlorinated aliphatic compounds were prepared in methanol. Appropriate volumes of each compound was injected into the standard assay mixture to give a final concentration of 12 μM. After incubation at 30°C for 1 h, residual substrates were determined by analyzing 10-μ*l* headspace sample in a Shimadzu model GC-9A gas chromatograph (Shimadzu Co., Japan), equipped with a flame ionization detector (FID) and a 3.2 mm×2.1 m glass column (Silicone DC-550 20% Chromosorb W [AWDMCS] 80/100). Column, injection and determination temperatures were 80, 200 and 250°C respectively. The carrier gas (nitrogen), air and hydrogen flow rate were 50 m*l*/min.

N-terminal sequencing After SDS-PAGE of protein sample, the protein was electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-PSQ, Millipore Corporation, Bedford, USA) using Bio-Rad electroblotting apparatus. Relevant band was excised and analyzed by automated Edman degradation performed using an Applied Biosystems 491 protein sequencer; equipped with a PROCISETM 1.1a data analysis software.

III. Results and Discussion

Purification of PCE dehalogenase The PCE reductive dehalogenase was purified from ethanol- and yeast extract-grown cells of strain DPH-1. The purification scheme is presented in Table 1. The specific activity of the purified enzyme was determined with methyl viologen (1.5 mM) as electron donor to be approximately 59.46 (U mg protein^{-1}) with a yield of nearly 5% throughout the purification procedure.

Gel filtration on Superdex HR 200 indicated an apparent molecular mass for the PCE dehalogenase of approximately 70 kDa (Fig. 1). SDS-PAGE revealed a single protein band (Fig. 2), indicating that the dehalogenase consists of one

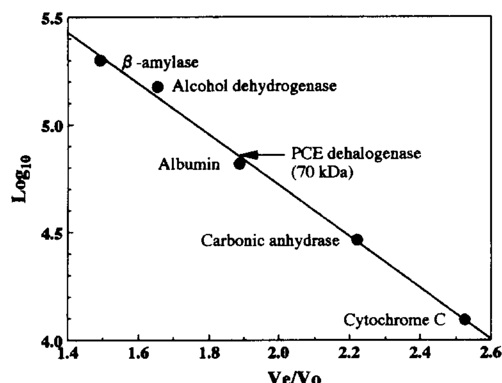

Fig. 1. Molecular weight determination of the native enzyme by SE-HPLC on Superdex 200 HR 10/30.

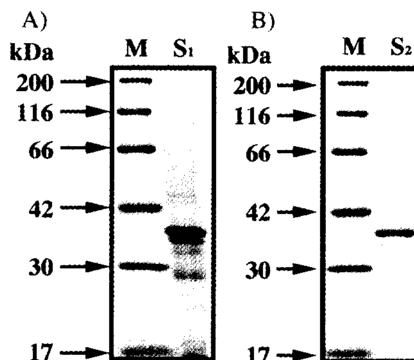

Fig. 2. SDS-PAGE analysis of PCE dehalogenase after CBB staining. M: Protein marker, S_1: Enzyme sample after Superdex pg-75 (gel filtration), S_2: PCE dehalogenase after Superdex HR 200 (gel filtration).

Table 1. Purification of PCE dehalogenase of *Clostridium bifermentans* DPH-1

Purification step	Total activity (U)	Yield (%)	Specific activity (U mg protein^{-1})	Purification (fold)
Cell extract	154.75	100.00	6.53	1.00
DEAE-Toyopearl 650 ion-exchange	102.50	66.21	30.15	4.62
Superdex pg-75 gel filtration	65.40	42.24	86.05	13.18
Poros HQ ion-exchange	10.92	7.05	37.66	NC
Superdex HR 200 gel filtration	7.73	4.96	59.46	NC

One unit(U) is equivalent to nmol of total product(TCE+cDCE) produced under the assay condition. NC: not calculated because there was no further increase in specific activity after superdex pg-75 gel filtration.

subunit. The apparent molecular mass of the subunit was calculated to be 35 kDa (Fig. 2), indicating that the enzyme consists of two polypeptide. From the results, it was assumed that the enzyme would be a homodimer.

Characterization of PCE dehalogenase The kinetics of dechlorination of PCE and product formation were studied in time course experiments. PCE was rapidly converted to cDCE without apparent accumulation of TCE (Fig. 3). It indicates that transformation of PCE, via TCE to cDCE occurred very rapid (Fig. 3). The kinetic parameters

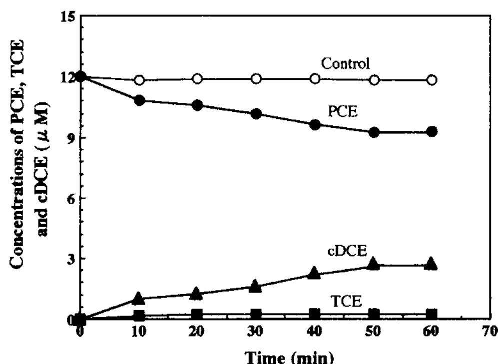

Fig. 3. Time course of PCE dechlorination. Initial protein concentration of PCE dehalogenase was 0.122 mg/m*l*.

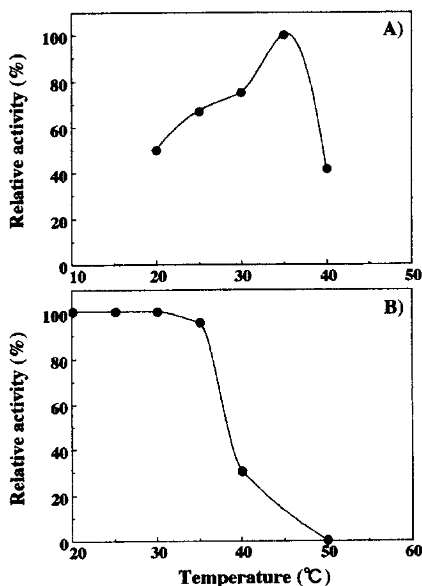

Fig. 4. Effect of temperature (A) and temperature stability (B) on PCE dehalogenase activity. 100% activity = 55 U mg protein^{-1}.

for PCE dechlorination was computed from the Lineweaver-Burk transformation (1/V versus 1/S) of the Michaelis-Menten equation. The V_{max} and K_m for PCE were estimated as 73 nmol/h·mg protein and 12 μM, respectively. The PCE dehalogenase from strain DPH-1 exhibited a lower specific activity than those reported for dehalogenase from other anaerobic PCE degrading organisms; *Dehalospirillum multivorans* (16) and *Desulfitobacterium* sp. PCE-S (17). However, the specific activity is comparable to, reported for *Desulfitobacterium* sp. Y-51 (20).

The PCE dehalogenase dechlorinated PCE in the temperature range 20 to 40°C, with an optimum at 35°C (Fig. 4A). The temperature stability was recorded in the temperature range 20 to 35°C (Fig. 4B). PCE degradation was recorded in the pH range 5 to 10, with an optimum at 8 (Fig. 5A). The enzyme was most stable at pH range 7.5 to 8 (Fig. 5B). Addition of metal ions and other cofactors had no significant effect on PCE degradation (Table 2). No inhibition of enzyme activity by EDTA was observed with the purified enzyme. This is possibly due to the fact that Co^{2+} being a prosthetic cofactor of the enzyme, cannot be chelated by EDTA (21). Moreover, PCE corrinoid dehalogenases have been postulated to effect PCE breakdown in the superreduced state (Co^{+}) and thus cannot be chelated by EDTA (16).

Effect of propyl iodide on PCE degradation The

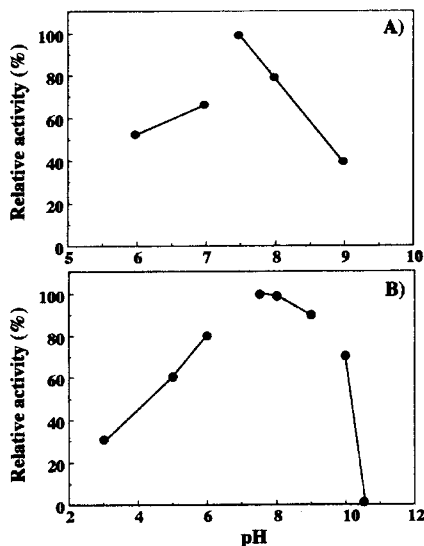

Fig. 5. Effect of pH (A) and pH stability (B) on PCE dehalogenase activity. 100% activity = 55 U mg protein^{-1}.

Table 2. Effect of metal ion or cofactor on PCE dechlorination

Metal ion or Cofactor	PCE dehalogenase activity(U)	Relative activity (%)
Control*	2.58	100
$FeSO_4 \cdot 7H_2O$	2.63	102
$CoCl_2 \cdot 6H_2O$	2.58	100
$MnCl_2 \cdot 6H_2O$	2.63	102
$ZnCl_2$	2.58	100
$HgCl_2$	2.53	98
NaCl	2.58	100
$MgSO_4 \cdot 7H_2O$	2.63	102
EDTA-2Na · $7H_2O$	2.63	102
NADH	2.53	98
Vitamin B_{12}	2.63	102

*Control experiments was conducted with enzyme without metal ion or cofactor. Initial protein concentration of PCE dehalogenase was 0.04 mg/m*l*.

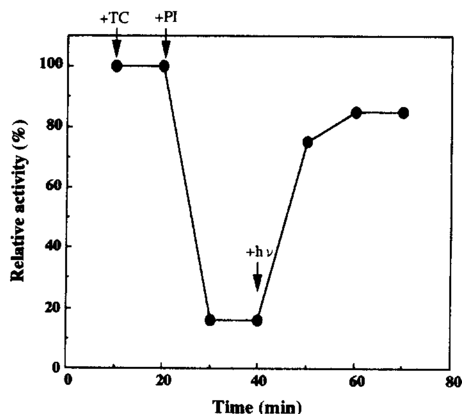

Fig. 6. Inactivation of PCE dehalogenase by propyl iodide and reactivation by light. 100% activity = 55 U mg protein-1. Titanium (III) citrate (TC: arrow), Propyl iodide (PI), The light (hv, 250-W).

effect of propyl iodide and titanium citrate on PCE degradation by the cell free enzyme extract is presented in Fig. 6. The enzyme lost 66% of its PCE degrading activity upon incubation with propyl iodide and titanium (III) citrate in the dark. Subsequent exposure to light restored enzyme activity. Titanium citrate in the absence of propyl iodide did not have any inhibitory effect. Protein-bound cobalamin from a number of anaerobic organisms e.g. *Dehalospirillium multivorans* (21), *Sporomusa ovata* (22) and *Methanosarcina thermophila* (23) degrade PCE. It has been postulated that, the enzyme bound cobalamin is in

AEVYNKDANKLDLYGKVHAQHYFSNDT- *Clostridium bifermentans* DPH-1

MEKKKKPELSRRDFGKLIIGGGAAATIAPF- *Dehalospirillum multivorans*

ADIVAPITETSEFPYKVDAY- *Desulfitobacterium* sp. strain PCE-S

ADIVAPITQTSQFPYKVDAE- *Desulfitobacterium* sp. strain Y-51

Fig. 7. N-terminal amino acid sequence of known PCE dehalogenases, including PCE dehalogenase of strain DPH-1.

the superreduced state [cob(I)alamin]. In this state, the alkyl residue of an alkyl halide can bind to the cobalt atom (24). The same mechanism applies to the binding of the propyl chain of propyl iodide to cobalt, thus inactivating the enzyme (25). In this communication, we have clearly showed that the mechanism of PCE degradation by *C. bifermentans* DPH-1 cell-free extract is mediated by a corrinoid protein since the dehalogenase was inactivated by propyl iodide exclusively after reduction by titanium citrate.

Degradation of other halogenated aliphatic compounds The rate of degradation of other halogenated aliphatic compounds by the PCE dehalogenase was investigated. The degradation of PCE, TCE, cDCE, 1,1-DCE, 1,1-dichloroethane, 1,2-dichloroethane, 1,2-dichlor-opropane, and 1,1,2-trichloroethane were determined to be 25, 16, 6, 4, 8, 8, 9, and 8% of added compounds concentration, respectively.

Only a few studies had described the anaerobic transformation of a variety of chlorinated hydrocarbons (6, 16, 26) by pure organism. *C. bifermentans* dehalogenase is unique in that it represents the first dehalogenase acting on a variety of chloroaliphatics. Surprisingly, cDCE was also degraded when added as the initial compound for degradation. The product(S) of cDCE degradation and the reasons why cDCE as an intermediate product is recalcitrant, are not clear. This aspect requres indepth studies to understand the mechanism and product(S).

N-terminal amino acid sequencing of the purified PCE dehalogenase using Edman degradation revealed the following sequence: AEVYNKDANKLDLYGKVHAQH-YFSNDT. No significant sequence similarities to the deduced amino acid sequence from the PCE dehalogenase genes of *D. multivorans* and *Desulfitobacterium* sp. PCE-S were detected (Fig. 7).

In conclusion, our results indicate that the PCE dehalogenase from *C. bifermentans* DPH-1 could play

some important role in the initial breakdown of PCE and particularly, a variety of other chlorinated aliphatic compounds, in sites contaminated with mixtures of halogenated substances. Moreover, an anaerobic enrichment microbial consortia from which *C. bifermentans* was isolated could degrade PCE to CO_2 and ionic chloride (27). Indepth investigation of each of the organisms involved in this mixed culture system would elucidate their respective roles. Molecular characterization of the PCE dehalogenase from *C. bifermentans* DPH-1 is currently under study in our laboratory.

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