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Haloalkylphosphorus Hydrolases Purified from *Sphingomonas* sp. Strain TDK1 and *Sphingobium* sp. Strain TCM1

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Phosphotriesterases catalyze the first step of organophosphorus triester degradation. The bacterial phosphotriesterases purified and characterized to date hydrolyze mainly aryl dialkyl phosphates, such as parathion, paraoxon, and chlorpyrifos. In this study, we purified and cloned two novel phosphotriesterases from *Sphingomonas* sp. strain TDK1 and *Sphingobium* sp. strain TCM1 that hydrolyze tri(haloalkyl)phosphates, and we named these enzymes haloalkylphosphorus hydrolases (TDK-HAD and TCM-HAD, respectively). Both HADs are monomeric proteins with molecular masses of 59.6 (TDK-HAD) and 58.4 kDa (TCM-HAD). The enzyme activities were affected by the addition of divalent cations, and inductively coupled plasma mass spectrometry analysis suggested that zinc is a native cofactor for HADs. These enzymes hydrolyzed not only chlorinated organophosphates but also a brominated organophosphate [tris(2,3-dibromopropyl) phosphate], as well as triaryl phosphates (tricresyl and triphenyl phosphates). Paraoxon-methyl and paraoxon were efficiently degraded by TCM-HAD, whereas TDK-HAD showed weak activity toward these substrates. Dichlorvos was degraded only by TCM-HAD. The enzymes displayed weak or no activity against trialkyl phosphates and organophosphorothioates. The TCM-HAD and TDK-HAD genes were cloned and found to encode proteins of 583 and 574 amino acid residues, respectively. The primary structures of TCM-HAD and TDK-HAD were very similar, and the enzymes also shared sequence similarity with fenitrothion hydrolase (FedA) of *Burkholderia* sp. strain NF100 and organophosphorus hydrolase (OphB) of *Burkholderia* sp. strain JBA3. However, the substrate specificities and quaternary structures of the HADs were largely different from those of FedA and OphB. These results show that HADs from sphingomonads are novel members of the bacterial phosphotriesterase family.

Tri(haloalkyl)phosphates have been used as flame retardants and plasticizers in a wide variety of applications. However, halogenated organophosphorus flame retardants, such as tris(1,3-dichloro-2-propyl) phosphate (TDCPP) and tris(2-chloroethyl) phosphate (TCEP), have received little attention with regard to human exposure and potential adverse effects in comparison with polybrominated diphenyl ethers.

Widespread use of these flame retardants has led to their accumulation in various environments at concentrations ranging from nanograms to several micrograms per liter (1–5). Although these compounds are considerably less toxic than organophosphorus pesticides and chemical warfare agents, many studies have shown their diverse toxic effects (6). TCEP is known to adversely affect brain, liver, and kidney and the fertility of male rats and mice (7). In addition, it is a suspected carcinogen (8) and has been shown to inhibit the expression of cell cycle regulatory proteins, DNA synthesis, and cell proliferation (9), although there are contradictory results regarding its carcinogenicity (10). TDCPP has exhibited genotoxicity in several *in vitro* assays conducted in prokaryotic and eukaryotic cells (11), and there are some indications that it is carcinogenic (7). These findings have prompted recognition of the potential ecological and human health concerns with these compounds.

Recently, we published the first reports of microorganisms capable of degrading tri(haloalkyl)phosphates (12, 13). In these studies, *Sphingomonas* sp. strain TDK1 and *Sphingobium* sp. strain TCM1 were isolated by growth on TDCPP or TCEP, respectively, as a sole phosphorus source. Both strains were also able to grow on another tri(haloalkyl)phosphate, tris(2,3-dibromopropyl) phosphate, and on two triaryl phosphates, tricresyl phosphate and triphenyl phosphate.

Phosphotriesterases catalyze the first step in the degradation of organophosphorus triesters, and many bacterial phosphotriesterases have been purified and characterized for the detoxification of sites contaminated with these compounds (14). Based on similarities in primary structure, well-characterized bacterial phosphotriesterases are mainly classified into three groups: organophosphorus hydrolases (OPHs), organophosphorus acid anhydrolases (OPAAs), and methyl parathion hydrolases (MPHs) (14). All of these enzymes mainly degrade aryl dialkyl phosphates, such as parathion, paraoxon, chlorpyrifos, diazinon, and fenitrothion. Because *Sphingomonas* sp. strain TDK1 and *Sphingobium* sp. strain TCM1 degrade tri(haloalkyl)phosphates (TDCPP and TCEP), we considered it likely that they have phosphotriesterases that do not belong to any of the three groups described above. It is important to clarify the enzymatic properties and primary structure of the phosphotriesterases of these two strains to understand the biodegradation of halogenated organophosphorus flame retardants.

The present paper describes the characterization and cloning of the TDCPP-degrading enzyme of *Sphingomonas* sp. strain TDK1 (TDK-HAD) and the TCEP-degrading enzyme of *Sphingobium* sp. strain TCM1 (TCM-HAD). We named these enzymes

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haloalkylphosphorus hydrolases (HADs) because the enzymes hydrolyzed not only chlorinated organophosphates but also a brominated organophosphate.

MATERIALS AND METHODS

Materials. TDCPP, TBPP, triethyl phosphate, tris(2-butoxyethyl) phosphate, tris(2-ethylhexyl) phosphate, parathion-methyl, parathion, and dichlorvos were purchased from Wako Pure Chemical Industries (Osaka, Japan). TCEP was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Paraoxon-methyl and paraoxon were from Sigma-Aldrich (St. Louis, MO). Chlorpyrifos was from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Trimethyl phosphate, tributyl phosphate, triphenyl phosphate, and tricresyl phosphate were from Nacalai Tesque (Kyoto, Japan). Restriction enzymes and kits for genetic manipulation were from TaKaRa Shuzo (Kyoto, Japan), Toyobo (Osaka, Japan), New England BioLabs (Beverly, MA), and Qiagen (Valencia, CA). All other reagents were of analytical grade and were obtained from Wako Pure Chemical Industries and Nacalai Tesque. The fast protein liquid chromatography (FPLC) apparatus and the Phenyl Sepharose 6 Fast Flow, SP Sepharose, and Sephacryl S-200 columns were from GE Healthcare UK Ltd. (Buckinghamshire, United Kingdom). The LC-10Ai Bio-Inert high-performance liquid chromatography (HPLC) system was from Shimadzu (Kyoto, Japan).

Purification of TDK-HAD from *Sphingomonas* sp. strain TDK1. All procedures were carried out at 4°C. The standard buffer used throughout column chromatography was 20 mM sodium phosphate (pH 7.0) containing 10% (vol/vol) glycerol. An Amicon stirred cell apparatus with a YM-10 membrane (Millipore, Billerica, MA) and an Amicon Ultra-15 centrifugal filter unit (10-kDa cutoff; Millipore) were used for concentration and buffer exchange. *Sphingomonas* sp. strain TDK1 was aerobically cultivated in MAY medium containing 200 μM TDCPP as a sole phosphorus source at 30°C for 30 h (15), and the cells were harvested by centrifugation (5,000 × *g* for 10 min). After two washes with 20 mM sodium phosphate buffer (pH 7.0), the wet cells were suspended in the same buffer and disrupted by sonication, and the lysate was centrifuged at 24,000 × *g* for 80 min. Ammonium sulfate was dissolved in the supernatant of the lysate to make a 30% saturated solution, which was kept for 12 h and then centrifuged (24,000 × *g* for 80 min). The supernatant was applied to a butyl-Sepharose FPLC column (5.0 by 7.1 cm) equilibrated with the standard buffer containing ammonium sulfate at 30% saturation. The enzyme was eluted with a linear gradient of ammonium sulfate from 30% saturation to 0% in the buffer (flow rate, 1 ml/min). The active fractions were collected, concentrated, and dialyzed against the standard buffer. The dialyzed solution was applied to a Bio-Inert HPLC system equipped with an SP-Sepharose column (2.6 by 11.3 cm) equilibrated with the standard buffer. The enzyme was eluted with a linear gradient of 0 to 500 mM NaCl in the buffer (flow rate, 1 ml/min). The enzyme fractions were collected and concentrated. The concentrate was applied to a Bio-Inert HPLC system equipped with a Sephacryl S-200 column (1.6 by 60 cm) equilibrated with the standard buffer containing 0.15 M NaCl, and the enzyme was eluted with the same buffer at a flow rate of 0.3 ml/min. The active fractions were collected and concentrated. The final preparation of the enzyme was stored at −80°C until use.

Purification of TCM-HAD from *Sphingobium* sp. strain TCM1. *Sphingobium* sp. strain TCM1 was aerobically cultivated in MAY medium containing 20 μM TCEP as a sole phosphorus source at 30°C for 48 h. The cells were harvested by centrifugation (5,000 × *g* for 10 min). After two washes with 50 mM morpholinepropanesulfonic acid (MOPS) buffer (pH 7.4), the wet cells were suspended in the same buffer and disrupted by sonication, and the lysate was centrifuged at 24,000 × *g* for 30 min. The enzyme fraction was precipitated from the supernatant between 30% and 60% saturation with ammonium sulfate. The precipitate was dissolved and dialyzed against the standard buffer of 20 mM sodium phosphate (pH 7.0) containing 10% (vol/vol) glycerol. The dialyzed solution was applied to an FPLC apparatus equipped with an SP-Sepharose column equilibrated with the standard buffer. The enzyme was eluted with a linear gradient of 0 to 250 mM NaCl in the buffer (flow rate, 1.5 ml/min). The active fractions were collected and concentrated, and ammonium sulfate was dissolved in the concentrate to make a 30% saturated solution. The enzyme solution was applied to a Bio-Inert HPLC system with a phenyl-Sepharose HP column (10 by 26 cm) equilibrated with the standard buffer containing ammonium sulfate at 30% saturation. The enzyme was eluted with a linear gradient of ammonium sulfate from 30% saturation to 0% in the buffer (flow rate, 0.7 ml/min). The active fractions were collected and concentrated. The final preparation of the enzyme was stored at −80°C until use.

Enzyme assays. The activities of TDK-HAD and TCM-HAD were assayed by measuring the decrease in the amount of TDCPP and TCEP, respectively. The triesters were analyzed using a GC-17A gas chromatograph equipped with a flame photometric detector with a phosphorus optical filter (Shimadzu) as described in a previous report (13). The assay mixture (2 ml) for TDK-HAD, containing 50 mM Tris-HCl buffer (pH 8.0), 50 μM TDCPP, and 100 μl of enzyme sample, was incubated for 30 min at 30°C. The assay mixture (2 ml) for TCM-HAD, containing 50 mM Tris-HCl buffer (pH 9.0), 1 mM TCEP, and 4 μl of enzyme sample, was incubated for 30 min at 25°C. A mixture without enzyme sample was used as a blank. A total of 200 μl of the reaction mixture was removed after 0, 15, and 30 min, and 20 μl of 1 M HCl was added to the aliquots to stop the reaction. TDCPP and TCEP in the mixture were extracted with 1 ml of ethyl acetate and analyzed as described above.

Concentrations of 2-chloroethanol and 1,3-dichloro-2-propanol, which are hydrolysis products of TCEP and TDCPP, respectively, were determined with a GCMS-QP2010 gas chromatograph mass spectrometer (Shimadzu) as described in a previous report (13).

Kinetic parameters of TDK-HAD were estimated by the statistical method of Wilkinson (16) and expressed as means ± standard errors (SEs). The final concentration of TDK-HAD in the assay mixture was 0.41 μg/ml.

Substrate specificity of HADs. The degradation activities toward parathion, parathion-methyl, paraoxon, and paraoxon-methyl were measured at 30°C by monitoring the liberation of *p*-nitrophenol from each compound at 405 nm with a UV-2500PC spectrophotometer (Shimadzu). The assay mixture (1 ml) contained 50 mM Tris-HCl buffer (pH 8.0), 1 mM substrate, and 0.41 μg/ml of TDK-HAD or 2.8 μg/ml of TCM-HAD.

Other assays were carried out by measuring the decrease in the amount of substrate by gas chromatography as described above. A standard reaction mixture contained 50 mM Tris-HCl buffer (pH 8.0), 5 μM substrate, and 0.41 μg/ml of TDK-HAD or 2.8 μg/ml of TCM-HAD.

ICP-MS. The purified enzyme preparations (0.35 mg of TDK-HAD or 0.7 to 0.86 mg of TCM-HAD) were hydrolyzed in 3 ml of 5% (vol/vol) HNO_3 for 12 h at room temperature to release the metal ions and then diluted with 5 volumes of water. The metal ion contents of the diluted solutions were determined by inductively coupled plasma mass spectrometry (ICP-MS) using a Seiko Instruments SPQ-9000 spectrometer (Seiko, Japan). The metal content analysis of each HAD with ICP-MS was carried out using two enzyme preparations that had been purified separately.

Molecular mass determination. The molecular masses of the native enzymes were determined by gel filtration with a Bio-Inert HPLC system equipped with a Sephacryl S-200 column. The column was equilibrated and operated at a flow rate of 0.3 ml/min with 20 mM sodium phosphate buffer (pH 7.0) containing 10% (vol/vol) glycerol and 0.15 M NaCl. The protein standards used were aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and RNase A (13.7 kDa) from GE Healthcare.

Internal amino acid sequencing. Purified enzyme (300 μg) was digested at 25°C for 12 h with 80 μg of lysyl endopeptidase (Wako Pure Chemical Industries) in a reaction mixture containing 50 mM Tris-HCl, pH 8.0, and 2 M guanidine hydrochloride. The resulting peptides were separated by reversed-phase HPLC on a Capcell Pak C_8 column (0.2 mm by 15 cm) (Shiseido, Tokyo, Japan) using a 2 to 80% acetonitrile gradient in 0.06% trifluoroacetic acid, and the amino acid sequences were determined with a Shimadzu PPSQ-21 protein sequencer.

TABLE 1 Primers used in this study

Application and name	Gene	Primer sequence (5′ to 3′)	Comment
Cloning of *had* genes			
TCM1_F1	TCM-*had*	AA(A/G)AT(A/T/C)GTIGGIGA(A/G)TT(C/T)AA	Degenerate PCR
TCM1_R2	TCM-*had*	TT(A/G)AA(C/T)TC(A/T/G/C)CCIAC(A/G/T)AT(C/T)TT	Degenerate PCR
TDK1_F1	TDK-*had*	AA(A/G)GGIGGIGTICA(T/C)GT(A/T/G/C)(C/T)T	Degenerate PCR
TDK1_R1	TDK-*had*	TT(A/G)AA(T/C)TCICCIAC(A/T/G/C)AC(T/C)TT	Degenerate PCR
TCMDK_Inv_F1	TCM-*had* or TDK-*had*	TCGTCGATGGGCGCTCTCAAC	Inverse PCR
TCMDK_Inv_R1	TCM-*had* or TDK-*had*	TGTAGACGCTGCCGTTCTCCAC	Inverse PCR
TCMDK_Inv_F2	TCM-*had* or TDK-*had*	CAACGGAGTTCCACACCTGG	Nested PCR
TCM_Inv_R2	TCM-*had* or TDK-*had*	AGTCCGGGCACGACTTCTGT	Nested PCR
Construction of expression vector			
TCM_Exp_F2	TCM-*had*	GGGAATTGCATATGTTGCGGATGTTAAGCGAAGC	NdeI site is underlined
TCM_Exp_R1	TCM-*had*	TCCGCTCGAGTCACTTCACGGTCGGGAAGC	XhoI site is underlined
TDK_Exp_F1	TDK-*had*	GGGAATTGCATATGTTGCGGATGTTAAGCGAAG	NdeI site is underlined
TDK_Exp_R1	TDK-*had*	TCCGCTCGAGTCACTTCACGGTCGGGAAGC	XhoI site is underlined
Sequencing			
T7 promoter		TAATACGACTCACTATAGGG	
SP6 promoter		CATACGATTTAGGTGACACTATAG	
TCM_InvSeq_F1	TCM-*had*	ATCTGGTTCGCCTGCGTCAC	
TCM_InvSeq_R1	TCM-*had*	CCATCCGCTACACAGACAGG	
TCM_InvSeq_R2	TCM-*had*	TAAGCTGGACGGCGCTTCTC	
TDK_InvSeq_F1	TDK-*had*	GATAACCTGTGTCGGCCGCTG	
TDK_InvSeq_R1	TDK-*had*	GGCCTGTCTGCTTGAAGGTGG	
TDK_InvSeq_F2	TDK-*had*	TTTGCTCGAGGCGGCAATCG	
TDK_InvSeq_R2	TDK-*had*	GCGGGCAAATCATCGGCAAG	

Gene cloning. The amino acid sequences obtained from internal peptides of the HADs and the primer sequences used for gene cloning are listed in Table S1 in the supplemental material and Table 1, respectively. Degenerate primers, TCM1_F1, TCM1_R2, TDK1_F1, and TDK1_R1, were designed based on the internal amino acid sequences WPANIHM (a part of peptide C4), KIVGEFK (peptide C2), KGGVHVL (a part of peptide D4), and KVVGEFK (peptide D2), respectively.

Parts of the HAD genes were amplified by degenerate PCR with each primer set, the genomic DNA of *Sphingobium* sp. strain TCM1 or *Sphingomonas* sp. strain TDK1, and platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) under the following conditions: initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 45°C for 30 s, and 68°C for 1 min, with a final extension at 72°C for 10 min. The 3′ and 5′ regions of the HAD genes were obtained by inverse PCR. Inverse PCR was performed using the primers and self-ligated XhoI-digested genomic DNA as a template with the following conditions: initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 62°C for 30 s, and 68°C for 1 min, with a final extension at 72°C for 10 min. The first amplification products were subjected to a nested PCR. The amplification products were gel purified, ligated into the pGEM-T Easy vector (Promega, Madison, WI), and sequenced.

Expression of HADs in *Escherichia coli*. The putative open reading frames of the HAD genes were amplified by PCR using the primers listed in Table 1, genomic DNA of *Sphingobium* sp. strain TCM1 or *Sphingomonas* sp. strain TDK1 as a template, and platinum *Pfx* DNA polymerase (Invitrogen) under the following conditions: initial denaturation at 94°C for 2 min, followed by 40 cycles at 94°C for 30 s, 56°C for 30 s, and 68°C for 1 min, with a final extension at 68°C for 10 min. The resulting PCR products were double digested with NdeI and BamHI and ligated into the same sites of pET25b. The plasmids thus obtained for expression of the proteins were introduced into *E. coli* BL21(DE3) (Novagen, Madison, WI). The transformant cells were grown in LB medium containing 100 μg/ml ampicillin at 30°C for 4 h. When the absorbance at 600 nm reached 0.5, isopropyl-1-thio-β-D-galactopyranoside was added to the culture medium to a final concentration of 1 mM. After cultivation for 6 h at 37°C, the cells were harvested, resuspended in 20 mM sodium phosphate buffer (pH 7.0), and disrupted by sonication.

Other analytical methods. Protein concentration was determined by the Bradford method (17) using a protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. The purity and the molecular mass of the subunit of the enzymes were analyzed by SDS-PAGE under reducing conditions (18).

Nucleotide sequence accession numbers. The nucleotide sequences of the HAD genes from *Sphingomonas* sp. strain TDK1 and *Sphingobium* sp. strain TCM1 have been deposited in the DDBJ/EMBL/GenBank database under accession numbers AB897515 and AB897516, respectively.

RESULTS

Purification of HADs. As summarized in Table 2, TDK-HAD and TCM-HAD were purified 25.6-fold with a specific activity of 2.44 μmol min^{-1} mg^{-1} and 14.0-fold with a specific activity of 6.40 μmol min^{-1} mg^{-1} from the cell extracts of strains TDK1 and TCM1, respectively. The purified TDK-HAD and TCM-HAD each had a single band with a molecular mass of 59.6 and 58.4 kDa, respectively, on SDS-PAGE (Fig. 1). The molecular masses of the native TDK-HAD and TCM-HAD were estimated to be 65.3 and 58.6 kDa by gel filtration, respectively. These results suggest that these enzymes are monomers.

Gas chromatography-mass spectrometry (GC-MS) analysis showed that one of the hydrolysis products of TDCPP by TDK-HAD is 1,3-dichloro-2-propanol and one of the hydrolysis products of TCEP by TCM-HAD is 2-chloroethanol, and the stoichiometric changes were observed between decreased amounts of both triesters and produced amounts of the corresponding halo alcohols (data not shown). This result suggested that TDCPP and TCEP are hydrolyzed to bis(1,3-dichloro-2-propyl) phosphate

TABLE 2 Purification of HADs from *Sphingomonas* sp. strain TDK1 and *Sphingobium* sp. strain TCM1

Step	Total activity[c] (μmol · min^{-1})	Total protein (mg)	Sp act (μmol · min^{-1} · mg^{-1})	Yield (%)	Purification (fold)
TDK-HAD[a]					
Crude extract	32.6	343	0.0950	100	1.00
Ammonium sulfate fractionation	26.7	304	0.0877	81.8	0.924
Butyl Sepharose	4.67	21.1	0.222	14.3	2.33
SP Sepharose	1.36	0.686	1.98	4.16	20.8
Sephacryl S-200	0.461	0.189	2.44	1.41	25.6
TCM-HAD[b]					
Crude extract	219	469	0.470	100	1.00
Ammonium sulfate fractionation	267	389	0.690	122	1.50
SP Sepharose	66.0	9.95	6.60	30.0	14.0
Phenyl Sepharose	27.0	4.28	6.40	12.5	13.6

[a] Starting material was 40.0 g of wet cells.
[b] Starting material was 36.7 g of wet cells.
[c] The activity of TDK-HAD and TCM-HAD was assayed using TDCPP and TCEP as a substrate, respectively.

and 1,3-dichloro-2-propanol and to bis(2-chloroethyl) phosphate and 2-chloroethanol by TDK-HAD and TCM-HAD, respectively (see Fig. S1 in the supplemental material).

Effects of pH and temperature on enzyme activity and kinetic parameters. TDK-HAD had maximum activity at pH 8.0 in 50 mM Tris-HCl buffer with TDCPP (Fig. 2A). The optimal temperature for the activity was 35°C (Fig. 2B). The enzyme activity followed typical Michaelis-Menten kinetics in the range of 0 to 200 μM TDCPP (the maximum solubility of TDCPP in water is about 200 μM). The K_m and V_{max} values for TDCPP were 50.7 ± 6.7 μM and 4.0 ± 0.2 μmol min^{-1} mg^{-1} protein (n = 6), respectively.

TCM-HAD had maximum activity at pH 8.5 in 50 mM Tris-HCl buffer with TCEP (Fig. 2C). The optimal temperature for the activity was 25°C (Fig. 2D). As shown in Fig. 3, TCM-HAD did not follow Michaelis-Menten kinetics in the range of TCEP concentrations examined. The activity increased to a maximum at 1 mM and decreased with further increases in substrate concentration.

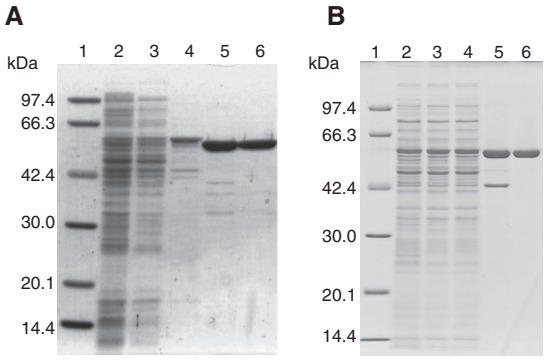

FIG 1 SDS-polyacrylamide gel electrophoresis of HADs from *Sphingomonas* sp. strain TDK1 (A) and *Sphingobium* sp. strain TCM1 (B). Protein samples were separated on a 12.5% SDS-polyacrylamide gel and stained with Coomassie brilliant blue R-250. (A) Lane 1, marker proteins; lane 2, crude extract (10 μg protein); lane 3, ammonium sulfate fractionation (10 μg); lane 4, butyl-Sepharose fraction (2 μg); lane 5, SP-Sepharose fraction (2 μg); lane 6, Sephacryl S-200 fraction (2 μg). (B) Lane 1, marker proteins; lane 2, crude extract (10 μg protein); lane 3, ammonium sulfate fractionation (10 μg); lane 4, dialyzed solution applied to SP-Sepharose (10 μg); lane 5, SP-Sepharose fraction (4 μg); lane 6, phenyl-Sepharose fraction (4 μg).

Effects of various metal ions and compounds on enzyme activity and metal content. Table 3 shows the effects of various metal salts, chelating agents, and thiol-modifying reagents on the activity of the purified HADs. All divalent metal ions increased the TDK-HAD activity, whereas chelating agents, such as EDTA and EGTA, did not affect the enzyme. TDK-HAD was markedly inhibited by dithiothreitol, moderately inhibited by 2-mercaptoetha-

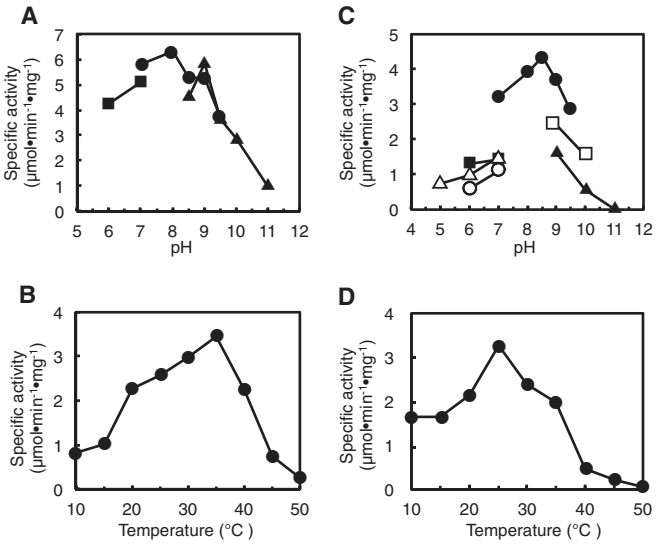

FIG 2 Effect of pH (A and C) and temperature (B and D) on TDK-HAD (A and B) and TCM-HAD (C and D) activity. (A) Enzyme activity was measured in 50 mM sodium phosphate buffer (closed squares), 50 mM Tris-HCl buffer (closed circles), and 50 mM glycine-NaOH buffer (closed triangles). The enzyme activity was assayed at 30°C for 30 min. (B) Enzyme activity was assayed in 50 mM Tris-HCl buffer (pH 8.0) at 10 to 50°C for 30 min. (C) Enzyme activity was measured in 50 mM sodium phosphate buffer (closed squares), 50 mM morpholineethanesulfonic acid buffer (open triangles), 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) buffer (open circles), 50 mM Tris-HCl buffer (closed circles), 50 mM *N*-cyclohexyl-2-aminoethanesulfonic acid buffer (open squares), and 50 mM glycine-NaOH buffer (closed triangles). The enzyme activity was assayed at 25°C for 30 min. (D) Enzyme activity was assayed in 50 mM Tris-HCl buffer (pH 9.0) at 10 to 50°C for 30 min. TDCPP (50 μM) or TCEP (1 mM) was used as the substrate for the assay of TDK-HAD (A and B) and TCM-HAD (C and D), respectively. The final concentrations of purified TDK-HAD and TCM-HAD in these assay mixtures were 0.41 μg/ml and 2.8 μg/ml, respectively.

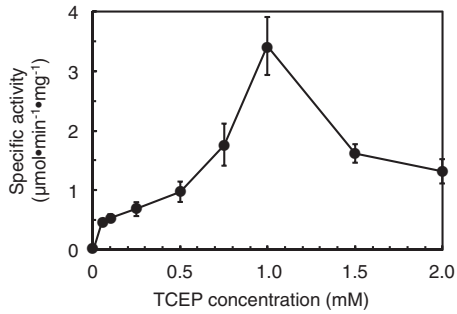

FIG 3 Effect of substrate concentration on the activity of TCM-HAD. The assay mixture contained different concentrations of TCEP, 50 mM Tris-HCl buffer (pH 9.0), and 2.8 μg/ml of TCM-HAD and was incubated at 25°C for 30 min. The data are means ± standard deviations from five independent experiments.

nol, and slightly inhibited by *N*-ethylmaleimide. TCM-HAD was moderately inhibited by Cu^{2+} and Fe^{2+} but moderately activated by Zn^{2+} and Ca^{2+}. More than a 7-fold increase in activity was observed with the addition of Co^{2+} and Mn^{2+}. The enzyme was potently inhibited by chelating agents and markedly inhibited by dithiothreitol and 2-mercaptoethanol.

The ICP-MS analysis of TDK-HAD revealed that the average Zn^{2+} and Co^{2+} contents were 1.05 mol and 0.0022 mol per mol of the enzyme, respectively, and neither Mn^{2+} nor Ca^{2+} was detected. The ICP-MS analysis of TCM-HAD showed that 1 mol of enzyme contained 0.27 mol of Zn^{2+}, 0.065 mol of Mn^{2+}, and 0.035 mol of Co^{2+}. These results suggest that both enzymes may require Zn^{2+} as a native cofactor.

TABLE 3 Effects of various metal ions and compounds on the activity of HADs from *Sphingomonas* sp. strain TDK1 and *Sphingobium* sp. strain TCM1[a]

Compound	Concn (mM)	Relative activity (%)	
		TDK-HAD	TCM-HAD
None		100	100
$FeCl_2$	0.05	170	48
$CuCl_2$	0.05	136	66
$ZnCl_2$	0.05	143	155
$CaCl_2$	0.05	126	135
$MgCl_2$	0.05	142	94
$MnCl_2$	0.05	319	766
$CoCl_2$	0.05	279	804
EDTA	0.05	91	11
	10	106	ND[b]
EGTA	0.05	92	7
	10	102	ND
Dithiothreitol	1	20	16
2-Mercaptoethanol	1	59	10
N-Ethylmaleimide	1	86	93

[a] The assay mixture contained each metal ion or compound at the final concentration indicated. The specific activities of TDK-HAD and TCM-HAD without addition of metal ions and compounds to the assay mixture were 2.56 μmol min^{-1} mg^{-1} with 50 μM TDCPP and 2.74 μmol min^{-1} mg^{-1} with 1 mM TCEP, respectively. These values were defined as 100%. The final enzyme concentration was 0.41 μg/ml for TDK-HAD and 2.8 μg/ml for TCM-HAD. Other assay conditions were the same as described in Materials and Methods.
[b] ND, not determined.

TABLE 4 Substrate specificity of HADs from *Sphingomonas* sp. strain TDK1 and *Sphingobium* sp. strain TCM1

Substrate[a]	% of degraded substrate	
	TDK-HAD[b]	TCM-HAD[c]
Tri(haloalkyl)		
Tris(1,3-dichloro-2-propyl) phosphate	65	100
Tris(2-chloroethyl) phosphate	0	31
Tris(2,3-dibromopropyl) phosphate	15	100
Triaryl		
Tricresyl phosphate	100	100
Triphenyl phosphate	100	100
Trialkyl		
Trimethyl phosphate	3	16
Triethyl phosphate	1	2
Tripropyl phosphate	0.4	0
Tributyl phosphate	0	8
Tris(2-butoxyethyl) phosphate	0	0
Tris(2-ethylhexyl) phosphate	0	8
Other organophosphorus compounds		
Parathion methyl	0[d]	0[d]
Parathion	0[d]	0[d]
Paraoxon methyl	0.002[d]	14
Paraoxon	0.02[d]	100
Chlorpyrifos	0	0
Dichlorvos	0	42

[a] The structures of organophosphates used in this experiment are shown in Fig. S2 in the supplemental material.
[b] The enzyme activity was assayed at 30°C for 30 min.
[c] The enzyme activity was assayed at 25°C for 30 min.
[d] The data were obtained by monitoring spectrophotometrically the liberation of *p*-nitrophenol from each compound at 405 nm.

Substrate specificity of HADs. Substrate specificity of the enzymes was examined with various organophosphorus compounds (Table 4). The two enzymes hydrolyzed another tri(haloalkyl)-phosphate, tris(2,3-dibromopropyl) phosphate (TBPP), and two triaryl phosphates, tricresyl phosphate and triphenyl phosphate. TDCPP and TBPP were degraded more efficiently than TCEP by TCM-HAD. TDK-HAD was not active toward TCEP. Although TDK-HAD showed only negligible activity toward paraoxon-methyl and paraoxon, TCM-HAD efficiently hydrolyzed these compounds. In addition, dichlorvos was hydrolyzed by TCM-HAD and not by TDK-HAD. The enzymes displayed no or weak degradation activities toward trialkyl phosphates, parathion-methyl, parathion, and chlorpyrifos.

Cloning and expression of HAD genes in *E. coli*. The TCM-HAD gene contained an open reading frame of 1,752 nucleotides coding for a protein of 583 amino acid residues with a molecular weight of 62,460, and the TDK-HAD gene contained an open reading frame of 1,725 nucleotides coding for a protein of 574 amino acid residues with a molecular weight of 61,824. The internal peptide sequences of HADs given in Table S1 in the supplemental material were found in the deduced amino acid sequences of HADs, suggesting that the genes cloned were those of the HADs. Putative Shine-Dalgarno sequences were found 5 bp upstream of the start codons of the TCM-HAD and TDK-HAD

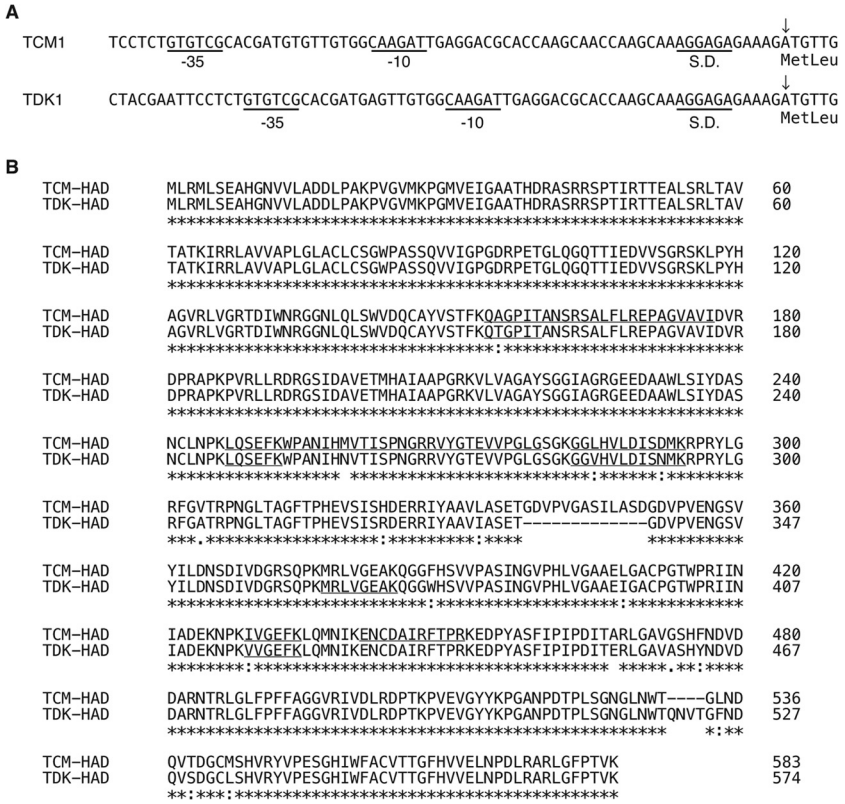

FIG 4 Putative −35, −10, and Shine-Dalgarno sequences (A) and amino acid sequence alignment (B) of TCM-HAD and TDK-HAD genes. (A) Putative −35, −10, and Shine-Dalgarno (S.D.) sequences are underlined. The arrow indicates a translation initiation site. (B) Amino acid sequence alignment was performed using ClustalW version 2.1 (http://clustalw.ddbj.nig.ac.jp). The numbers on the right side are the residue numbers for each amino acid sequence. Identical residues and amino acid substitutions with low and high similarities are indicated by asterisks and by dots and double dots, respectively. Amino acid sequences identical to those of internal peptides (see Table S1 in the supplemental material) are underlined.

genes. Possible −35 and −10 sequences were found in the upstream regions of these genes (Fig. 4A). Significant amino acid sequence identity (94% identity) was found between these enzymes (Fig. 4B), and the HADs shared sequence similarity with FedA of *Burkholderia* sp. strain NF100 (23.2% and 21.9% identity with TCM-HAD and TDK-HAD, respectively) and OphB of *Burkholderia* sp. strain JBA3 (23.0% and 21.7% identity with TCM-HAD and TDK-HAD, respectively) in experimentally characterized proteins. Motif analysis of the HADs with the MOTIF program (GenomeNet; http://www.genome.jp/tools/motif/) revealed no obvious conserved motifs.

The HAD genes of strains TCM1 and TDK1 were expressed in *E. coli* BL21(DE3) cells under the control of a T7 promoter. The crude extracts of *E. coli* cells harboring pET25-cHAD (TCM1-HAD) and pET25-dHAD (TDK1-HAD) showed hydrolase activities of 0.088 μmol min^{-1} mg^{-1} for TCEP and 0.046 μmol min^{-1} mg^{-1} for TDCPP, respectively, whereas no activity was detected in the extract of *E. coli* cells harboring pET25b.

DISCUSSION

The first step in the degradation of organophosphorus triesters is catalyzed by phosphotriesterases. Many bacterial phosphotriesterases have been purified and characterized. In the present study, HADs from *Sphingomonas* sp. strain TDK1 and *Sphingobium* sp. strain TCM1 were purified and cloned. Both enzymes are monomeric proteins with subunit molecular masses of 59.6 kDa (TDK1) and 58.4 kDa (TCM1), in contrast to the dimeric bacterial phosphotriesterases of *Brevundimonas diminuta* (19), *Agrobacterium radiobacter* (20), and *Pseudomonas* sp. strain WBC-3 (21).

The HADs of *Sphingomonas* sp. strain TDK1 and *Sphingobium* sp. strain TCM1 are the first examples of phosphotriesterases capable of degrading the tri(haloalkyl)phosphates TDCPP and TCEP. TDK-HAD displayed Michaelis-Menten kinetics with TDCPP as a substrate, but TCM-HAD did not show Michaelis-Menten kinetics with TCEP. Owing to the considerably lower solubility of TDCPP than of TCEP, it was impossible to analyze TDK-HAD activity with TDCPP as a substrate at the higher concentration range where the activity of TCM-HAD both increased and decreased with increasing TCEP concentration. Further study is required to elucidate the unique kinetic behavior of TCM-HAD with TCEP.

Both HADs hydrolyzed a brominated organophosphate and triaryl phosphates, but they were inactive or weakly active toward trialkyl phosphates (Table 4). Both enzymes had substrate specificities distinct from those of other bacterial phosphotriesterases reported to date, which hydrolyze only the aryl group of organophosphorus triesters.

The substrate specificity of TDK-HAD was in good agreement with the growth characteristics of strain TDK1 (13), except that no degradation of TCEP by TDK-HAD was detected under the present assay conditions. TDK1 was able to grow on TCEP as a sole phosphorus source, but the degradation of TCEP was much

slower than that of TDCPP (13). TCEP may be a relatively poor substrate for the enzyme. TCM-HAD showed no or only weak activity toward some trialkyl phosphates that were degraded by strain TCM1. Although we cannot eliminate the possibility that another phosphotriesterase capable of degrading these compounds is present in strain TCM1, trialkyl phosphates may be a poor substrate for TCM-HAD, because strain TCM1 grew only slightly on these compounds. Because the HADs degraded paraoxon but not parathion, it is suggested that both enzymes degrade only organophosphates and not organothiophosphates. Similar trends were observed for *Sso*Pox of *Sulfolobus solfataricus* and OpdA of *A. radiobacter*, and this phenomenon is known as the "thiono effect" (22, 23).

It has been reported that an OPH of *B. diminuta* (19) and an MPH of *Pseudomonas* sp. strain WBC-3 (21) contain two Zn^{2+} per subunit as a native cofactor. In this study, ICP-MS analysis suggested that the HADs contain one Zn^{2+} per subunit. Both purified enzyme preparations were activated by the addition of several metal salts. The dissociation of a metal ion(s) (mostly Zn^{2+}) from the enzymes during purification may convert the HAD holoenzymes to inactive apoenzymes, and the apoenzymes may be partially reactivated by the addition of metal salts. The present enzyme preparations had the highest activity upon the addition of Mn^{2+} and Co^{2+}. Similar effects of metal salts were reported in a previous study of OPH from *B. diminuta* (24), in which the Co^{2+}-containing enzyme showed the highest specific activity.

Aspartate, glutamate, and histidine residues of the active sites of an OPH (25), an OPAA (26), and an MPH (21) are involved in metal ion coordination. Sequence alignment of the HADs with well-characterized fenitrothion hydrolases showed that the HADs contain several conserved aspartate, glutamate, and histidine residues (see Fig. S3 in the supplemental material), and some of them appear to be located in the relatively conserved regions (see Fig. S3, arrow). These conserved residues may be involved in coordination with metal ions. However, further studies, such as mutational analysis and three-dimensional structural analysis of the enzymes, are necessary to clarify the function of the conserved residues.

Of interest was that 10 mM EDTA as well as EGTA had no effect on the activity of TDK-HAD. A significantly higher concentration of EDTA may be required to inactivate TDK-HAD, as reported for an OPH (24). In contrast, despite the high sequence similarity between TCM-HAD and TDK-HAD, TCM-HAD was highly sensitive to the chelating agents. The metal cofactor(s) seems to be more tightly associated with TDK-HAD than with TCM-HAD. Two notable differences between the amino acid sequences were observed at Gly338-Asp350 in TCM-HAD and Gln520-Thr523 in TDK-HAD (Fig. 4B). It seems that these regions might affect the binding of metals by these enzymes.

It has been reported that the OPH of *B. diminuta* is competitively inhibited by dithiothreitol, dithioerythritol, and 2-mercaptoethanol (27). In the present study, the HADs were also inhibited by dithiothreitol and 2-mercaptoethanol, although the mechanism of inhibition was not elucidated.

The HADs showed no sequence similarity with well-characterized bacterial phosphotriesterases such as OPHs, OPAAs, and MPH, but they shared sequence similarity with two enzymes classified in the OphB group, FedA from *Burkholderia* sp. strain NF100 and OphB from *Burkholderia* sp. strain JBA3 (14). Although little is known about the enzymatic properties of FedA and OphB, both enzymes can efficiently hydrolyze organophosphorothioates such as fenitrothion and parathion-methyl (28, 29). It has also been reported that FedA requires a putative membrane protein, FedB, for enzymatic activity, and the FedA/FedB complex is found in the cell membrane fraction (30). On the other hand, the HADs hydrolyzed only organophosphates and not organothiophosphates, and they did not require other components for their activity.

In the present study, we have clearly demonstrated that HADs of *Sphingomonas* sp. strain TDK1 and *Sphingobium* sp. strain TCM1 are novel phosphotriesterases capable of degrading tri(haloalkyl)phosphates. The substrate specificities and primary structures of both HADs were distinct from those of other phosphotriesterases reported to date.

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