Identification, Characterization, and Cloning of a Phosphonate Monoester Hydrolase from *Burkholderia caryophilli* PG2982*

(Received for publication, June 19, 1996, and in revised form, July 23, 1996)

Stanton B. Dotson‡§, Christine E. Smith¶, Cliff S. Ling¶, Gerard F. Barry**,**, and Ganesh M. Kishore**

From the ‡Glycobiology Group, G. D. Searle, St. Louis, Missouri 63167, the ¶Protein Biochemistry Group, G. D. Searle, Chesterfield, Missouri 63198, the ¶Analytical Sciences Center, Monsanto Corporate Research, Saint Louis, Missouri 63167, and **Ceregen, A Unit of Monsanto Company, Chesterfield, Missouri 63198

The glyphosate-degrading bacterium, *Burkholderia caryophilli* PG2982, was observed to utilize glycercy glyphosate as a sole phosphorus source. The hydrolysis of glycercy glyphosate to glyphosate by a phosphonate ester hydrolase (PEH) was identified as the first metabolic step in the mineralization pathway. This observation provides the first biological role for a phosphonate ester hydrolase activity. Purified PEH enzyme hydrolyzed several phosphonate esters including *p*-nitrophenyl phenylphosphonate, *β*-napthyl phenylphosphonate, and 5-bromo-4-chloro-3-indolyl phenylphosphonate. The purified PEH also hydrolyzed some phosphodiesters including *p*-nitrophenyl 5′-thymidine monophosphate and *p*-nitrophenyl phosphorylcholine. The most catalytically efficient substrate identified was bis-*p*-nitrophenyl phosphate with a *Kₐ* of 0.9 mM and a *Kₐ* of 6.2 × 10⁻⁴ min⁻¹, suggesting that the enzyme may also function in *vivo* as a phosphodiesterase. The native enzyme was a homotetramer of 58-kDa subunits and exhibited a pI of 4.2. The enzyme activity had a pH activity optimum of 9.0 and was stimulated 14-fold by Mn²⁺ ions, but a metal cofactor was not essential for activity. N-terminal and tryptic fragment amino acid sequences were obtained from the purified PEH protein and used to clone the *B. caryophilli* PG2982 gene, designated *pehA*. The unique substrate specificity of the enzyme and potential use as a novel conditional lethal gene in plants are discussed.

Phosphonate monoester hydrolases capable of hydrolyzing *p*-nitrophenyl phenylphosphonate are widespread in nature (1), even though phosphonate monoesters are generally considered as xenobiotics. The phosphonate monoester hydrolase activities typically arise from the nonspecific nature of 5′-phosphodiesterases (2, 3), 5′-nucleotidases (4), DNases (5), and cyclic nucleotide phosphodiesterases (6). No physiological role typically arises from the nonspecific nature of 5′-nucleotidases (2, 3), 5′-nucleotidases (4), DNases (5), and cyclic nucleotide phosphodiesterases (6).

Radionuclide experiments confirmed that glycercy glyphosate was adsorbed, translocated and relatively stable in plants.1 Similarly, *Escherichia coli* cells, which were growth-inhibited by 0.5 mM glyphosate, did not display any growth inhibition in the presence of 5 mM glycercy glyphosate. These preliminary results suggested that glycercy glyphosate is not a substrate for the previously described, ubiquitous phosphonate monoester hydrolases (1). An enzyme capable of hydrolyzing the phosphonate ester bond of glycercy glyphosate would likely be unique among currently described enzymes in this class. As described herein, the glyphosate-degrading bacteria *Burkholderia caryophilli* PG2982 (8) was observed to utilize glycercy glyphosate as a sole phosphorus source. The hydrolysis of glycercy glyphosate to glyphosate by a phosphonate ester hydrolase was identified as the first metabolic step in the pathway. The PEH* enzyme from PG2982 was more fully characterized, and the gene has been cloned. The purified enzyme exhibited a broad substrate specificity for phosphonate phosphonate monoesters and phosphodiesters.

**MATERIALS AND METHODS**

Reagents—All buffer components and 5-bromo-4-chloro-3-indolyl phenylphosphonate were from Research Organics. Enzymes for coupled assays and DNA modifying enzymes were from Boehringer Mannheim. All bacteriological media components were obtained from Difco. Oligonucleotides were obtained by custom synthesis from Midland Scientific. Unless otherwise indicated, all other reagents were the highest quality available from Sigma. The *p*-nitrophenyl phenylphosphonate was purified before use by extraction of *p*-nitrophenol into hexane at pH 5.0 (5) and then a stock solution standardized using an extinction coefficient for *p*-nitrophenol of 18,320 cm⁻¹ · M⁻¹. Unless otherwise specified, all cationic and anionic buffers were prepared as the CI⁻ and Na⁺ salts, respectively.

1 S. B. Dotson and G. M. Kishore, unpublished results.

2 The abbreviations used are: PEH, phosphonate ester hydrolase; MOPS, 3-(N-morpholino)propanesulfonic acid; Bio-Tris propane, 1,3-bis{[tris(hydroxymethyl)methylamino]propane} (pH 7.0); pNPP, *p*-nitrophenyl phosphonate; XPP, 5-bromo-4-chloro-3-indolyl phenylphosphonate; HPLC, high performance liquid chromatography; RP-HPLC, reverse phase HPLC; PAGE, polyacrylamide gel electrophoresis; PTH, phenylthiohydantoin; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); DF, Dworkin-Foster; TAPS, 3-[tris(hydroxymethyl)methylamino]propanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid.
Synthesis of Glyceryl Glyphosate—Glyceryl glyphosate was prepared as described previously by provided by P. Dirig of the Shonhondo Agricultural Group. The preparation was termed to be >99% pure by glyophosphate analysis and by 31P NMR. The crystalline compound and 100 mM solutions at neutral pH were stable for greater than 1 year. Radioactive glyceryl glyphosate was obtained from DuPont NEN at a specific activity of 52.3 mCi/mmol. The radioactive compound was unstable over 3–4 months and was routinely purified by anion exchange on a MonoQ HR10/10 (Pharmacia) equilibrated in H2O and then eluted with a 1600-ml gradient of 0–500 mM triethylammonium bicarbonate, pH 7.5. Fractions containing glyceryl glyphosate were identified using an analytical HPLC method (described for enzymatic assays below). The pool of glyceryl glyophosphate (still contaminated with glyphosate) was fractionated on the same column a second time with an 80-mM buffer of 0–100 mM triethylamine acetate, pH 5.5. The purified radioactively compound and the stable analog were shown to be identical and >99% pure by 31P NMR, by anion exchange chromatography, and by ion-pair chromatography under several conditions.

Enzyme Purification from PG2982—Five 10-liter fermentations of PG2982 cells were carried out in DF minimal medium to obtain PEH enzyme for purification. Cells were collected by centrifugation and stored at −20°C until used. All procedures were carried out at 4°C. During the purification, activity assays were based on the release of glycerol from glyceryl glyophosphate, except during the final purification step. The assay was first screened using a colorimetric assay. The cells (200 g) were resuspended in 1 liter of 100 mM Tris, pH 8.0, 100 mM KCl, 2 mM dithiothreitol (Buffer I) and lysed by passing through a 2.0 × 1.0 cm Sepharose Fast Flow anion exchange column (4 × 40 cm) equilibrated in dialysis buffer. The PEH protein eluted toward the end of a 2-liter, 0–500 mM KCl gradient in the same buffer. The fractions containing the highest activity were pooled and PEH precipitated with 50% [NH4]2SO4 as before. The [NH4]2SO4 pellet was then loaded onto a 2.0 × 1.0 cm Sepharose S-200 gel filtration column eluted in 10 mM Tris, pH 8.0, 50 mM KCl, and 5-ml fractions were collected. The PEH activity eluted just after the void volume, and the fractions containing most of the activity were pooled and loaded onto a FPLC MonoQ anion exchange column. The PEH activity was eluted with a 30-mL 0–500 mM KCl gradient in 10 mM Tris, pH 8.5. The fractions containing the highest activity were pooled and loaded onto a MonoQ column using a 0–500 mM KCl gradient in 60 mL of 10 mM Tris, pH 8.5. Fractions containing the highest activity were again pooled and further purified using native PAGE as described above but with a preparative 4 mm × 8 cm resolving gel. The PEH activity was assayed by overlaying a solution of 4 nM nPFP in 100 mM Tris, pH 8.5, 100 mM KCl. The assay was stopped by rinsing the gel with H2O as soon as a yellow band appeared. The band displaying the activity was excised, and the PEH protein was electroeluted using a Bio-Rad Mini Protean II electroeluter in 10 mM Tris, 20 mM glycine, pH 8.5. The PEH protein was then brought to 40% ammonium sulfate using a saturated stock solution, and the precipitated protein was collected by centrifugation at 10,000 × g for 20 min. The [NH4]2SO4 pellet was resuspended in 100 mL of Buffer I and dialyzed overnight against two 4-liter changes of 50 mM Tris, pH 8.0, 2 mM dithiothreitol (dialysis buffer). The dialyzed enzyme was loaded on a Q-Sepharose fast flow anion exchange column (4 × 40 cm) equilibrated in dialysis buffer. The PEH protein eluted toward the end of a 2-liter, 0–500 mM KCl gradient in the same buffer. The fractions containing the highest activity were pooled and PEH precipitated with 50% [NH4]2SO4 as before. The [NH4]2SO4 pellet was then loaded onto a 2.0 × 1.0 cm Sepharose S-200 gel filtration column eluted in 10 mM Tris·Cl, pH 8.0, 50 mM KCl, and 5-ml fractions were collected. The PEH activity eluted just after the void volume, and the fractions containing most of the activity were pooled and loaded onto a FPLC MonoQ anion exchange column. The PEH activity was eluted with a 30-mL 0–500 mM KCl gradient in 10 mM Tris, pH 8.5. The fractions containing the highest activity were pooled and loaded onto a MonoQ column using a 0–500 mM KCl gradient in 60 mL of 10 mM Tris, pH 8.5. Fractions containing the highest activity were again pooled and further purified using native PAGE as described above but with a preparative 4 mm × 8 cm resolving gel. The PEH activity was assayed by overlaying a solution of 4 nM nPFP in 100 mM Tris, pH 8.5, 100 mM KCl. The assay was stopped by rinsing the gel with H2O as soon as a yellow band appeared. The band displaying the activity was excised, and the PEH protein was electroeluted using a Bio-Rad Mini Protean II electroeluter in 10 mM Tris, 20 mM glycine, pH 8.5. The PEH protein was then brought to 40% ammonium sulfate using a saturated stock solution and loaded on a FPLC alkyl-Superose HR5/5 column equilibrated in 10 mM TAPS, pH 9.0, 40% [NH4]2SO4. The PEH activity eluted in the middle of a 25-mL gradient of 40%–0% [NH4]2SO4.

Tryptic Mapping—Tryptic maps were made for both the 66- and 59-kDa polypeptides to compare their similarity and to obtain tryptic fragments for protein sequence analysis. 600 μg of purified PEH protein was subjected to full reduction and alkylation with iodoacetic acid (18). The 66- and 59-kDa polypeptides were then separated from each other by electrophoresis on a 3–17% acrylamide gradient SDS-PAGE minigel (Jule, Inc.) run at 30 mA. The two polypeptides were visualized by a brief staining (15 min) in 0.3% Coomassie Blue R-250 in H2O, excited, and electroeluted into 25 mM Tris, 192 mM glycine, and 0.1% SDS (Bio-Rad MiniPROTEIN II electroelution chamber). The eluted polypeptides were dialyzed against H2O for 4 h, precipitated with five volumes of 1.0 M ammonium sulfate, and then desalted into 0.1 mM ammonium bicarbonate, pH 8.1 using a Sephadex G-25 column. The polypeptides were digested with trypsin (1.25 wt/wt) overnight at 37°C, and the tryptic peptides were separated by RP-HPLC, using a Brownlee RP-300 Aqueous C8 column developed with a 0–70% acetonitrile gradient in 0.1% trifluoroacetic acid over 60 min.
Amino Acid Sequencing—Automated Edman degradation chemistry was used to determine the peptide sequence of the recombinant phosphonate monoester hydrolase (pehA) gene product. G. F. Barry and M. Weldon, unpublished data.

The culture was induced for 2 h with 50 to 5% ammonium sulfate and loaded on a 5-cm phenyl-Sepharose column (20). A portion of the recovered protein (34 g; the remaining 40 g was frozen) was centrifuged. 

E. coli—Purification from E. coli—The plasmid pMON9428 was transformed into E. coli W3110 and grown in a 10-liter fermenter in LB broth at 30 °C. 

Enzyme Activity pH Optimum—The optimum pH for activity was determined using p-nitrophenyl phenylphosphate as a substrate. A three-buffer system of constant ionic strength (20) was used, which consisted of 0.052 M MES, 0.052 M HEPES, and 0.1 M diethanolamine. The actual pH values after dilution of the buffer with substrate were 9 and 9.3, respectively. Genomic DNA from PG2982 was prepared as described previously (22) and used as a PCR template. The results were used to partially construct the restriction map in Fig. 3. The 3-kb Ncol and 2.2-kb HindIII fragments containing the pepA gene were ligated into a pUC118-derived vector (modified to contain a Ncol site in the polylinker) and Bluescript pSK (Clontech), respectively. The Ncol and HindIII fragments were mapped using common restriction enzymes and DNA sequencing. As DNA sequence became available, new primers were synthesized until a complete set of primers were available every 250 bp for both strands, allowing the gene to be completely sequenced on both strands.

Metal Ion Analysis—The primer GTGCTCTGAGCTCATAATTGTCG was used to insert a SacI site just 3′ to the predicted stop codon. To facilitate further cloning, the primers GAAGCCGGATCTTCTTGGACAGAT, ATGACGAAGCTTXTCCGATTTGTA, CAGCTCTTTCAATTCAAAAGACAGCGCCGGTC were used to remove internal SacI and BamHI fragments from the starting parent. A third primer, A′ Ncol-HindIII fragment of the pepA gene was ligated with a BamHI to SalI′ fragment into an E. coli expression vector containing the recA promoter and T7 phase gene 10 leader (26) and the resulting plasmid designated pMON9428.

Miscellaneous Procedures—All nucleic acid sequences were analyzed on a VAX using the GCG sequence analysis programs (27). Data base searches were performed using the BLAST algorithm against the non-redundant sequence data bases at the National Center for Biotechnology Information (28). Enzyme kinetic data were analyzed using the ENZFITTER program (29).

RESULTS

Identification of Glyceryl Glyphosate Phosphonate Ester Hydrolyase Activity—B. carophylli PG2982 has been previously characterized for its ability to utilize glyphosate as a sole phosphorus source (8). In this study, PG2982 was observed to utilize glyceryl glyphosate as a sole phosphorus source, suggesting the presence of a phosphonate monoester hydrolase activity. The glyceryl glyphosate phosphonate esterase activity was confirmed by directly demonstrating the conversion of glyceryl glyphosate to glyphosate using in vivo and in vitro radioactive assays. Intact PG2982 cells and crude extracts were incubated with glyceryl [3-14C]glyphosate and the products identified by HPLC analysis. The only radioactively labeled species formed from glyceryl [3-14C]glyphosate was glyphosate, thereby confirming that hydrolysis of the ester was the first step in the mineralization of glyceryl glyphosate. The PG2982 PEH appeared to be expressed constitutively and was unaffected by growth in DF medium with 0.2 mM phosphate, in L-broth, or in M9 medium with 100 mM phosphate.
The PEH reaction products were further authenticated in crude extracts of PG2982 incubated with 10 mM cold glyceryl glyphosate for 4 h at 30 °C. The enzyme-dependent formation of glycerol was verified by coupling the reaction to glycerol dehydrogenase, and the appearance of glyphosate was verified using a HPLC assay. The purified PG2982 PEH (see below) was also incubated with 10 mM glyceryl glyphosate in 30% D₂O, and the time-dependent formation of glyphosate was confirmed with ³¹P NMR.

An *E. coli* phosphonate ester hydrolase activity has been described previously (1) and was evaluated for activity against glyceryl glyphosate, in order to investigate whether the glyceryl glyphosate phosphonate ester hydrolase activity was unique to PG2982. The growth of *E. coli* was expected to be similarly inhibited by glyphosate and glyceryl glyphosate, if the *E. coli* phosphonate ester hydrolase activity was able to hydrolyze the phosphonate monoester bond of glyceryl glyphosate. However, glyceryl glyphosphate was observed to be at least 50-fold less inhibitory than glyphosate to *E. coli* JM101 when plated on minimal medium (data not shown). Consistent with these results, no hydrolysis of glyceryl [³¹C]glyphosphate was observed when *E. coli* strains JM101 and MM294 were grown in the presence of the radioactive compound for 48 h in DF or MOPS minimal medium with either 0.2 mM or 0.01 mM (limiting) phosphate or in LB medium. The washed *E. coli* cell pellets contained a substantial amount of intact radioactive substrate, indicating that the lack of hydrolysis was not due to poor uptake (data not shown). The recovery of intact glyceryl glyphosphate in these experiments implied that the putative *E. coli* phosphonate esterases were unable to hydrolyze the phosphonate ester bond. Several commercial enzyme preparations capable of hydrolyzing β-nitrophenyl phenylphosphonate were tested for their ability to hydrolyze glyceryl glyphosphate. Phosphodiesterase I (Sigma P6903; 0.14 μmol/min) and 5′ nucleotidase (Sigma N-4005 from *Crotalus adamanteus* venom; 2.5 μmol/min) were incubated with glyceryl [³¹C]glyphosphate (8 mM) in 100 mM diethanolamine, pH 9.0, for 60 min at 30 °C. No hydrolysis of glyceryl glyphosphate was observed (<0.1 nmol/min).

In summary, the PG2982 PEH activity appeared to be novel because other known phosphonate ester hydrolases were unable to hydrolyze glyceryl glyphosphate.

**Characterization and Partial Sequence of the PG2982 Phosphonate Monoester Hydrolase**—To aid in further characterization of the PEH enzyme, the corresponding gene was cloned. The cloning strategy began with the purification of the enzyme from PG2982 in order to obtain amino acid sequence information. Purification was assisted by the development of a qualitative colorimetric assay, which measured the release of glycerol from glyceryl glyphosphate. At the end of the purification, the PG2982 PEH activity appeared homogeneous, as evidenced by a single silver-stained band after native PAGE. A band of phosphonate ester hydrolase activity, which corresponded to the single silver-stained protein, was evident when the gel was incubated with β-naphthyl phenylphosphonate or when gel slices were incubated with glyceryl [³¹C]glyphosphate. These results demonstrated that the silver-stained protein was the PEH enzyme and, importantly, that the enzyme possessed a broad substrate specificity beyond glyceryl glyphosphate. Separation of a isoelectric focusing gel (range of pH 4–6) revealed a single band with a pI of 4.2 that stained in situ with β-naphthyl phenylphosphonate. During purification, the enzyme eluted as a single peak on Sephadex S200 with an apparent native molecular mass of 240 kDa.

The purified PEH enzyme, which migrated as a single band by native PAGE, was resolved by SDS-PAGE revealing 66- and 59-kDa polypeptides (Fig. 1). Protein sequence and tryptic map analyses were employed to decide if the presence of the two polypeptides resulted from partial proteolysis or if they represented heteromeric subunits of the phosphonate monoester hydrolase. Initially, a single N-terminal sequence was obtained from the mixture of both polypeptides (Table I). Following purification of each polypeptide using preparative SDS-PAGE (Fig. 2), the individual N-terminal sequences were found to be identical and confirmed the sequence obtained from the mixture (Table I). Furthermore, tryptic profiles of the two polypeptides appeared nearly identical (Fig. 2). The similar tryptic profiles and identical N-terminal sequences suggested that the two polypeptides were encoded by the same gene and probably resulted from either post-translational modification or limited proteolysis during enzyme isolation. The possibility of alternate translation start sites was ruled out since the two polypeptides had identical N-terminal sequences. In addition to the N terminus, sequences were obtained for two tryptic fragments of the 66-kDa polypeptide, T20 and T37, which were subsequently used to clone the *pehA* gene (Table I).

**Cloning the Phosphonate Monoester Hydrolase *pehA* Gene—**Probes for the PG2982 *pehA* gene were obtained by PCR using degenerate primers designed from the tryptic fragment sequences described above. A 450-bp product was amplified using primers designed from the T20 and T37 tryptic peptide sequences, and a 880-bp fragment was amplified using primers designed from the N terminus and T37 tryptic peptide sequences. The T20-T37 450-bp PCR product was used as a probe to obtain a full-length *pehA* gene from a PG2982 cosmid library. Three cosmid clones were identified from screening 1800 colonies and the *pehA* gene mapped to a 3.2-kb *SalI* fragment (Fig. 3). Starting with degenerate sequencing primers designed from the N-terminal and tryptic fragment sequences, the entire gene was sequenced on both strands (Fig. 4). The starting methionine was identified as the only in-frame methionine between the encoded N-terminal amino acid sequence and an upstream in-frame stop codon. The N-terminal and tryptic fragment sequences (T20, T32, and T37) obtained from the purified PEH protein were identified in the deduced amino acid sequence, confirming the intended gene had been cloned (Fig. 4). The predicted size of the protein encoded by the *pehA* open reading frame was 58.2 kDa, which was 12% smaller than the 66-kDa polypeptide previously observed by SDS-PAGE for the purified PG2982 PEH (Fig. 1), indicating the protein migrated somewhat anomalously on SDS-PAGE. The predicted pI was 5.8 and varied significantly from the observed pI of 4.2, likely reflecting intramolecular interactions of ionizable groups within the PEH polypeptide.

The complete deduced amino acid sequence of the *pehA* gene...
A single N-terminal sequence was determined from a purified PEH preparation, which contained both 66- and 59-kDa polypeptides. N-terminal sequences were also obtained for the individual 66- and 59-kDa PEH polypeptides after they were purified from each other using preparative SDS-PAGE. Tryptic peptides were isolated by RP-HPLC after trypsin digestion of the 66-kD polypeptide. Unequivocal sequence was obtained for sequences were also obtained for the individual 66- and 59-kDa PEH polypeptides after they were purified from each other using preparative SDS-PAGE. A portion of each preparation appeared to be composed substantially of a single polypeptide. Each polypeptide was then reductively alkylated and subjected to trypsin digestion. Tryptic maps were obtained by separating tryptic peptides on a Brownlee C8 reverse phase column and subjected to trypsin digestion. Tryptic maps were obtained by

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**Table I**

*Table of amino acid sequencing of the 66- and 59-kDa PEH polypeptides.*

| Polypeptide | Fragment | Sequence |
|-------------|----------|----------|
| Mixed       | N terminus | XRNKYLLL/NVQAD/TFIPHMRAEGERPFLXPN |
| 66 kDa      | N terminus | XRNKYLLL/NVQAD/TXKNVLLL |
| 59 kDa      | T20      | XNXAVF2A/PN |
| 66 kDa      | T32      | AVLDFTQQ |
| 66 kDa      | T37      | AVLDFTQQ |

*The numbers indicate the RP-HPLC fraction number of the purified fragment.*

*Parentheses indicate a tenuous determination. A slash indicates alternate sequence possibilities.*
pressed in E. coli transformed with pMON9428 and then purified 3.5-fold to homogeneity (Table I). The PEH enzyme has been shown to hydrolyze both glyceryl glyphosate and β-naphthyl phenylphosphonate. The PEH enzyme is catalyzed the hydrolysis of glycerol glyphosate at pH 9.0. Manganese ions stimulated the purified enzyme 2.5-fold, while Mg\(^{2+}\), Ca\(^{2+}\), Fe\(^{2+}\), and Fe\(^{3+}\) had little or no effect. The stimulatory effect of Mn\(^{2+}\) ions on PEH activity was further explored. PEH activity was fully stimulated by Mn\(^{2+}\) concentrations as low as 0.1 μM. The low concentration of Mn\(^{2+}\) required for full activation confirmed that the PEH enzyme had high affinity for Mn\(^{2+}\) and suggested the possibility that Mn\(^{2+}\) might be required for enzyme catalysis. To test this hypothesis, a PEH preparation, with a specific activity of 6.9 μmol/min/mg at saturating pNPP in the presence of saturating Mn\(^{2+}\), was dialyzed against EDTA and subjected to metal ion analysis. The total remaining manganese was determined to be 1.58 μM, while the protein content, determined by amino acid analysis, was 464 μM. In this dialyzed preparation, only 1.58% of the total enzyme would be catalytically active with Mn\(^{2+}\) ions. 

### Metal Ion Activation of the Phosphonate Monoester Hydrolase—

The effect of inorganic ions on PEH activity was evaluated using the purified enzyme. In general, anions such as Cl\(^{-}\), CH\(_3\)COO\(^{-}\), and SO\(_4\)\(^{2-}\) salts stimulated activity 20–40% at 100 mM. Heavy metal ions such as Cu\(^{2+}\) and Zn\(^{2+}\) were potent inhibitors. Co\(^{2+}\) appeared to stimulate activity; however, subsequent analysis indicated that the Co\(^{2+}\) per se chemically catalyzed the hydrolysis of glycerol glyphosate at pH 9.0. 

### Substrate Specificity of the Phosphonate Monoester Hydrolase—

The PEH enzyme has been shown to hydrolyze both glycerol glyphosate and β-naphthyl phenylphosphonate. 

![Fig. 5. Plasmid map of pMON9428 E. coli expression vector.](http://www.jbc.org/Downloaded from http://www.jbc.org/)
A three-buffer system with constant ionic strength of 0.1 M between pH 5 and 10 was employed. Protamine sulfate 3220 98.5 34,132 0.35 1.1 109
50% (NH₄)₂SO₄ 2170 74.6 21,700 0.29 0.83 69
Phenyl-Sepharose 110 1.67 1884 1.1 3.5 14

**Fig. 6. The pH optimum for PEH activity.** A three-buffer system was used to measure PEH activity with 4 mM p-nitrophenyl phenylphosphonate as a substrate. The actual pH values after dilution of the buffer with substrate were recorded. Enzyme was preincubated at each pH for 2 min, and then assays were run for 5 min. Assays were stopped by the addition of diethanolamine base to 0.1 M and the absorbance at 404 nm recorded. A second set assays were performed after first incubating the enzyme at various pH values for 12 min at 30 °C.

**Fig. 7. The kinetics of glyceryl glyphosate hydrolysis by purified PEH.** PEH activity was measured at increasing concentrations of glyceryl glyphosate. Reactions were performed in 20 mM diethanolamine, pH 9.0, 500 μM MnCl₂ at 30 °C. The appearance of glyphosate was assayed by fixed time points using a radioactive HPLC analysis. The appearance of glyphosate was previously observed to be linear with time and with enzyme concentration. The $K_m$ and $k_{cat}$ were determined by fitting the data to the Michaelis-Menten equation using nonlinear regression analysis (29).
The kinetic constants of PEH substrates were determined. Reactions were performed in 20 mM diethanolamine, pH 9.0, 500 µM MnCl₂ at 30 °C. For p-nitrophenyl substrates, the appearance of p-nitrophenol was measured continuously by spectrophotometric detection at 405 nm. Reaction rates were calculated by least squares analysis of the change in absorbance over time. The hydrolysis of glyceryl glyphosate was assayed at fixed time points using a radioactive HPLC analysis.

Substrate | $K_m$ (mM) | $k_{cat}$ (min⁻¹) | $k_{cat}/K_m$ (min⁻¹ m⁻¹)
---|---|---|---
Glyceryl glyphosate | 49 ± 13 | $2.8 \times 10^3 ± 5$ | $5.7 \times 10^2$
p-Nitrophenyl phenylphosphonate | 2.3 ± 0.08 | $4.0 \times 10^3 ± 5$ | $1.8 \times 10^5$
Bis-p-nitrophenyl phosphate | 0.9 ± 0.03 | $6.2 \times 10^3 ± 7$ | $7.2 \times 10^5$
p-Nitrophenyl thymidine-5'-phosphate | 4.7 ± 0.8 | $8.9 \times 10^4 ± 9$ | $1.9 \times 10^4$

As a family of compounds, alkyl and alkoxy esters, with 3 or more carbons, of glyphosate were observed to exhibit at least 10-fold less vegetative phytotoxicity than glyphosate in herbicide field trials at Monsanto (7). The differences in phytotoxicity were unlikely due to transport differences, since these compounds were similar with respect to these properties. Phosphonate esterases with broad substrate affinities are widespread in nature, including plants (1), and were expected to hydrolyze the glyphosate esters resulting in apparent phytotoxities similar to glyphosate, given phosphonate monoester hydrolysis is amenable to enzyme catalysis. However, the common plant phosphonate esterases appeared to have little activity on glyphosate esters. Likewise, $E. coli$ enzymes were not observed to hydrolyze these glyphosate esters.

The occurrence of a unique enzyme that will hydrolyze a phosphonate ester of glyphosate may find interesting uses in genetics as a conditional lethal gene. The hydrolysis of glyphosate esters may find interesting uses in genetic disruption and use as a conditionally lethal gene in $E. coli$. The PEH activity is encoded by a single polypeptide and does not require an unusual cofactor for activity, although the enzyme is stimulated by Mn²⁺ ions, which are found in plant cells. The broad pH optimum for PEH activity makes the enzyme suitable for plastid or cytosolic expression. Certainly, the unknown intracellular function of the pehA gene makes it difficult to predict what effects might be observed on plant metabolism; however, there were no discernible effects when the active protein was overexpressed within $E. coli$ cells. Current research is exploring the potential of the pehA gene as a conditional lethal gene in plant genetics (30).

Acknowledgments—We thank Marcia Weldon for supplying bacterial cultures and for the PG2982 genomic library. We are indebted to Dr. Om Dhingra for glyceroyl glyphosate, to Dr. Ron Beasley for glyphosate analysis, to Jim Zobel for amino acid analysis, and to Dr. Hideji Fujiwara for mass spectrometer analysis. We thank Ned Seigel for performing the cGMP assay. We thank Carl Mathis, Bruce Bishop, and Bob Clayton for PG2982 and $E. coli$ fermentations. We thank Dr. Joe Welplfy for critical review of this manuscript.

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Stanton B. Dotson, Christine E. Smith, Cliff S. Ling, Gerard F. Barry and Ganesh M. Kishore

*J. Biol. Chem.* 1996, 271:25754-25761.  
doi: 10.1074/jbc.271.42.25754

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