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Purification and Partial Characterization of an Acid Phosphatase from *Spirodela oligorrhiza* and Its Affinity for Selected Organophosphate Pesticides

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An acid phosphatase from the aquatic plant *Spirodela oligorrhiza* (duckweed) was isolated by fast protein liquid chromatography and partially characterized. The enzyme was purified 1871-fold with a total yield of 40%. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) of the pure acid phosphatase resolved a single protein band that migrated to approximately 60 kDa. Nondenaturing SDS−PAGE electrophoresis revealed a single protein band around 120 kDa after staining with Coomassie Brilliant blue. Quantitative gel filtration chromatography estimated a native molecular mass of this enzyme to be 120 kDa. Thus, this acid phosphatase likely functions as a homodimer, consisting of two similar 60 kDa subunits. An electrophoretic technique using the flourogenic substrate 4-methylumbelliferyl phosphate enabled visualization of an acid phosphatase activity that corresponded to the protein band at 120 kDa on a nondenaturing PAGE gel. It was determined that the acid phosphatase had a pH optimum of 6.0 at 25 °C. The enzyme activity appeared to be stable over a broad range of temperatures (10−40 °C) and in the presence of the metals Zn^{2+}, Mn^{2+}, and Mg^{2+} as well as the chelating agents ethylenedinitrilotetraacetic acid and ethylene glycol tetraacetic acid. It was shown that this acid phosphatase could hydrolyze a variety of physiological organophosphate compounds including β-glycerophosphate, phosphoserine, adenosine triphosphate, adenosine diphosphate, adenosine monphosphate, and pyrophosphate. Furthermore, analysis using capillary electrophoresis demonstrated that this hydrolytic enzyme could transform a wide array of organophosphate pesticides including *S*-2-ethylthioethyl *O*,*O*-dimethylphosphorothioate (demeton-*S*-methyl); *S*-1,2-bis(ethoxycarbonyl)ethyl *O*,*O*-dimethylphosphorodithioate (malathion); *O*,*O*-dimethyl *O*-4-nitrophenyl (paraoxon); *O*,*O*,*O*,*O*-tetraethyldithiopyrophosphate (sulfatep); *O*-2-chloro-4-nitrophenyl *O*,*O*-dimethylphosphorothioate (dicapthon); and 2,2-dichlorovinyl dimethylphosphate (dichlorvos).

KEYWORDS: Acid phosphatase; organophosphates; phytometabolism; duckweed; *Spirodela oligorrhiza*

INTRODUCTION

Acid phosphatases (EC 3.1.3.2, orthophosphoric-monoester phosphohydrolases) are considered some of the most ubiquitous and multifunctional enzymes in the natural environment (*1*, *2*). The critical role and function of these enzymes have been well-documented in many living organisms including bacteria (*3*), fungi (*4*−*6*), zooplankter (*7*), and plants (*8*−*14*). Acid phosphatases' general reaction mechanism is the hydrolysis of ester phosphate linkages of organophosphate compounds, resulting in the release of inorganic phosphate. Studies have shown that most acid phosphatases have similar pH optima, ranging between 5.0 and 6.5, and exhibit temperature profiles between 25 and 37 °C. Structural and immunological work on acid phosphatases have demonstrated that many of these enzymes are similar in physical structure, levels of glycosylation, and amino acid sequence (*8*). Another reported feature of acid phosphatases is their nonselective ability to hydrolyze a variety of organophosphate compounds (*15*).

Numerous reports have investigated the physiological function of acid phosphatases. Because acid phosphatases have been previously localized to the plasmalemma and intracellular membrane, it has been suggested that they may be involved in transporting inorganic phosphate in and out of the plant cell wall (*16*). Further studies with plant acid phosphatases have led researchers to associate these enzymes with various physiological functions, including seed germination (*17*), cellular autophagy in meiosis (*18*), and cell autolysis (*19*).

Acid phosphatases have been purified and characterized from plants such as lupin roots (*20*), potato tuber (*10*), barley roots (*12*), tomato cell culture (*11*), cotton (*13*), and peanut (*14*). Results of acid phosphatase studies suggest similar structural characteristics; they often exist as either homodimers or het-

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erodimers with apparent molecular masses between 51 and 110 kDa. In addition, reports indicate that many acid phosphatases are glycoproteins (*10*, *20*, *21*). Varying levels of glycosylation on proteins generally give rise to multiple isoforms, which was observed with the acid phosphatases isolated from both potato tuber (*10*) and lupin roots (*20*).

Studies focusing on substrate specificity of acid phosphatases indicate that many of these enzymes have a broad and nonspecific hydrolytic capability. Because some organophosphate pesticides are similar in molecular structure to known phosphatase substrates, these compounds may serve as suitable substrates for phosphatase transformation. Accordingly, this observation has been confirmed through the discovery of the phosphatase-mediated hydrolysis of methyl parathion by microalgae, cyanobacteria (*22*), and microbes present in clay suspensions (*23*). Furthermore, hydrolytic metabolism of both parathion and diazinon was demonstrated by both a bacterial isolate of *Flavobacterium* (*24*) and through bacterial cometabolism in rhizosphere soil (*25*).

Aquatic plants have been the focus of several investigations into their capability to sequester and metabolize toxic organophosphate chemicals. The aquatic macrophyte, *Elodea nuttallii*, has been shown to rapidly uptake and transform the organophosphate insecticide chlorpyrifos, which serves as the active ingredient in dursban (*26*). Further interests in plant-mediated organophosphate degradation led Gao et al. (*27*) to investigate the accumulation and metabolism of several organophosphate insecticides including demeton-*S*-methyl, crufomate, and malathion by both intact *Spirodela oligorrhiza* plants and enzyme extracts. Their results indicate that enzymatic hydrolysis may play an important role in determining the fate of organophosphate chemicals. The purpose of this study is to identify and characterize the role of acid phophatase enzyme(s) in the aquatic plant *S. oligorrhiza* responsible for the transformation of a selected group of organophosphate pesticides.

MATERIALS AND METHODS

Chemicals and Reagents. 2-*N*-Morphilinoethanesulfonic acid (MES), polyvinylpolypyrrolidone (PVPP), phenylmethylsulfonylfluoride (PMSF), polysorbitan monolaurate (Tween 20), dithiothrieotol (DTT), pyrophosphate, adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), *p*-nitrophenyl phosphate, phospho-L-serine, phospho-L-threonine, phospho-L-tyrosine, ethylenedinitrilotetraacetic acid (EDTA), polyoxyethylenesorbitan monolaurate (Tween 80), acrylamide, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and gel filtration molecular mass standards, alkaline phosphatase, wheat germ, and potato acid phosphatase, were all purchased from Sigma (St. Louis, MO). Bradford protein assay reagent was obtained from Biorad (Richmond, CA). Poly(vinylidene difluoride) membranes (Immobilon transfer 0.45 μm pore size) were obtained from Millipore (Beverly, MA). A fast protein liquid chromatography (FPLC) equipped with OS2 software and chromatography materials including high-substituted phenyl sepharose, resource S resin, and Sephacryl-400 gel filtration matrices were purchased from Pharmacia (Piscataway, NJ). The organophosphate insecticides, *S*-1,2-bis(ethoxycarbonyl)ethyl *O*,*O*-dimethylphosphorodithioate (malathion); *O*,*O*-dimethyl *O*-4-nitrophenyl (paraoxon); *O*,*O*,*O*,*O*-tetraethyldithiopyrophosphate (sulfatep); *O*-2-chloro-4-nitrophenyl *O*,*O*-dimethylphosphorothioate (dicapthon); 2,2-dichlorovinyl dimethylphosphate (dichlorvos); *S*-2-ethylthioethyl *O*,*O*-dimethylphosphorothioate (demeton-*S*-methyl); and 2,2,2-trichloro-1-hydroxyethyl]phosphonate (dylox) were purchased from ChemService (West Chester, PA).

Plants and Culture Conditions. *S. oligorrhiza* plants were collected from a small lagoon in Crawford, Georgia (35° 55.919′ N and 83° 22.233′) and brought back to the laboratory. The plants were washed and cultivated on 1/2 strength Hoagland growth media (*29*). This medium is composed of 0.78 mM $Ca(NO_3)_2$, 0.78 mM KNO_3, 0.21 mM $MgSO_4$, 0.013 mM KCl, 0.018 mM Sequestrene NaFe, 0.07 μM H_3BO_3, 0.17 μM $MnCl_2$, 0.12 μM $ZnSO_4$, 0.05 μM $CuSO_4$, 0.017 μM H_2MoO_4, and 2.5 μM KH_2PO_4. *S. oligorrhiza* plants were also grown in media under phosphate deprivation conditions. This medium was prepared in the absence of the normally added 2.5 μM KH_2PO_4. The plants were maintained under cool flourescent bulbs using a continuous photoperiod.

Extraction of Acid Phosphatase. One kilogram of *S. oligorrhiza* was extensively washed with distilled water and added to a surface sterilization solution containing 0.26% (w/v) sodium hypochlorite and 0.1% (v/v) Tween 20 for 15 min, followed by a 30 min wash with Nanopure water, and then air-dried for approximately 15 min. The plants were ground to a fine powder in liquid nitrogen using a mortar and pestle. The powdered plant debris was subsequently transferred to a 50 mM MES buffer (pH 6.0) containing 3 mM DTT, 1 mM PMSF, and 1% PVPP and was thoroughly mixed at high speed for three 15 s pulses in a Waring blender. The homogenate was collected and filtered through layered cheesecloth and centrifuged at 10000*g* (radius, 4.25 in.) for 30 min at 4 °C. The supernatant was collected and filtered through miraclothe (EMD Biosciences, San Diego, CA) and served as the crude extract for enzyme purification.

Enzyme Assays. For routine analysis of acid phosphatase activity, protein samples were monitored before and after purification steps by adding aliquots (100 μL) of protein extract to 900 μL of 50 mM MES buffer (pH 6.0) containing 3 mM DTT and 200 mg/L *p*-nitrophenylphosphate and methylparaoxon for a final reaction volume of 1 mL. Enzyme assays began with the addition of the enzyme; after 30 min of incubation at 25 °C, the formation of 4-nitrophenol was spectrophotometrically monitored using a Varian DMS 1100 spectrophotometer at A_{410} similar to the method of Brightwell and Tappel (*29*). To quantitate enzyme activity, one unit of acid phosphatase activity was defined as the amount of enzyme required to produce 1 μmol of 4-nitrophenol/min at 25 °C. Calculation of acid phosphatase activity was performed using a 4-nitrophenol standard curve, ranging in concentrations from 0.02 to 2.0 μM. All enzyme assays were performed in triplicate, whereas the controls were prepared by using heat-inactivated enzyme in the reaction.

The spectrophotometric method of Eibl and Lands (*30*) was used to detect and quantify the release of inorganic phosphate from organophosphate substrates such as β-glycerophosphate, phosphoserine, pyrophosphate, ATP, ADP, and AMP. Acid phosphatase extract (100 μL) was added to 700 μL of 50 mM MES buffer (pH 6.0) containing 4 mM $MgCl_2$ and incubated at 25 °C for 6 min. The enzyme reactions were stopped by the addition of 50 μL of 3 M H_2SO_4 containing 20 mM ammonium molybdate and 10 μL of 1% (v/v) Triton X-100. The samples were measured on a spectrophotometer at 660 nm at 25 °C. Calculation of enzyme activities was performed using a phosphate standard curve, ranging in concentrations from 0.02 to 2.0 μM. All enzyme assays were performed in triplicate, and the controls were prepared by adding 3 M H_2SO_4 containing 20 mM ammonium molybdate and 10 μL of 1% (v/v) Triton X-100 before adding the enzyme to the reaction. A unit of enzyme activity was defined as the amount of enzyme required to produce 1 μM inorganic phosphate/min. Determination of total protein in the enzyme extracts was carried out using the Bradford method (*31*).

Salt Precipitation. Solid ammonium sulfate was slowly added to the crude extract until 30% saturation (176 g/L) was achieved and stirred overnight at 4 °C. The crude extract (approximately 2.3 L) was centrifuged at 13000*g* (radius, 5.25 in.) for 30 min. The supernatant was collected and filtered through Whatman 1 filter paper (Whatman, Clifton, NJ) using a 1000 mL vacuum flask. The crude filtrate was then subjected to ultrafiltration using a 100 kDa cassette (Filtron, Beverly, MA) attached to a peristaltic pump. Buffer exchanges were performed using 50 mM MES buffer (pH 6.0) containing 3 mM DTT and 30% saturated ammonium sulfate (buffer A). The enzyme extract was concentrated to 100 mL using a 100 kDa MWCO membrane in a pressurized stirred-cell concentrator (Amicon).

High-Substituted Phenyl Sepharose Chromatography. The protein extract (50 mL) was next loaded on a prepacked high-substituted phenyl sepharose column (1.2 cm × 12.3) preequilibrated with buffer A. After the addition of the protein extract, the column was washed with buffer

A until a A_{280} baseline absorbance of 0.01 was established by the FPLC UV detector. Proteins were eluted from the column using an increasing step gradient of 50 mM MES buffer (pH 6.0) with 3 mM DTT (buffer B). The void volume and all eluant fractions (5 mL) were assayed with *ρ*-nitrophenylphosphate and methylparaoxon to detect acid phosphatase activity. Fractions containing acid phosphatase activity were then pooled, and the sample was stored at 4 °C.

Resource-S Cation Exchange. Pooled acid phosphatase from the previous step was desalted using prepacked PD-10 columns containing G-25 beads (Pharmacia) preequilibrated with Nanopure water. The sample was then transferred into 50 mM sodium citrate buffer (pH 5.0) with 3 mM DTT (buffer C). Using a 10 mL syringe, the acid phosphatase sample was added to a Resource-S column (1 mL of resin, Pharmacia) preequilibrated with buffer C. The column was then subsequently washed with buffer C, and acid phosphatase was eluted by applying a linear gradient of 50 mM sodium citrate buffer (pH 5.0) containing 1 M KCl and 3 mM DTT. The void volume and all fractions (2 mL) were assayed with *p*-nitrophenylphosphate and methylparaoxon for acid phosphatase activity. Acid phosphatase fractions were then pooled and immediately dialyzed against 50 mM MES buffer (pH 6.0) containing 150 mM KCl and 3 mM DTT.

Sephacryl S-400. To determine the functional mass of the acid phosphatase, the pooled sample from the previous step was subjected to gel filtration chromatography using Sephacryl S-400. The acid phosphatase sample was concentrated to 1 mL using a centricon with a 100 kDa MWCO (Filtron) and added to a Sephacryl S-400 column (1 cm × 120 cm) at a flow rate of 0.4 mL/min. The molecular mass of the functional enzyme was estimated using a linear graph plotting relative mobility vs logMW based on a variety of gel filtration standard proteins including cytochrome *c* (124 kDa), carbonic anhydrase (29.0 kDa), bovine serum albumin (66 kDa), alcoholdehydrogenase (150 kDa), and *β*-amylase (200 kDa). After this column was run, fractions with acid phosphatase activity were collected and stored at 4 °C in buffer A.

Gel Electrophoresis of Acid Phosphatase. Denaturing SDS−PAGE electrophoresis of the acid phosphatase was carried out using a mini-gel apparatus (Hoefer) with a discontinuous buffer system by the method of Laemmli (*32*). The gel slabs were prepared 0.75 mm thick, containing 7.5% acrylamide in the resolving gel and 3% acrylamide in the stacking gel. The resolving gel was separately polymerized prior to the addition of the stacking gel. To test the purity of the enzyme fraction, 5 Fg was added to denaturing electrophoresis stock sample buffer (25 Fl) containing 0.06 M Tris-HCl (pH 6.8), 10% (v/v) glycerol, 5% (v/v) *β*-mercaptoethanol, 2% (w/v) SDS, and 0.025% (v/v) bromophenol blue. These protein samples were boiled for 90 s in a hot water bath, cooled, and either used or stored in the freezer at −20 °C. Acid phosphatase sample 10 Fl was loaded on the gel and run at 120 V for approximately 1 h. After SDS−PAGE was complete, the gels were briefly rinsed with Nanopure water and fixed in 20% (v/v) trichloroacetic acid for 10 min. The gel was washed for 5 min in Nanopure water and stained with Coomassie Brilliant blue (R-250) to visualize the purity and mass of the enzyme. Destaining the gel was performed using a solution containing 50% (v/v) methanol and 10% (v/v) glacial acetic acid. SDS−PAGE of the purified acid phosphatase was compared to an SDS−PAGE broad range of standard proteins with known molecular masses including rabbit skeletal muscle myosin (200 kDa), *Escherichia coli* *β*-galactosidase (116 kDa), rabbit muscle phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45 kDa), and hen egg white lysozyme (14.4 kDa).

Nondenaturing SDS−PAGE was carried out to approximate the functional molecular mass of the enzyme. The isolated fraction of acid phosphatase (10 *μ*L) was added to 40 *μ*L of sample buffer containing 0.06 M Tris-HCl (pH 6.8), 0.2% (w/v) SDS, 10% (v/v) glycerol, and 0.025% (w/v) bromophenol blue. The sample buffer did not contain reducing agent, and the enzyme sample was not boiled. Purified acid phosphatase (5 *μ*g) was loaded on the gel and run at 120 V for 1 h. When electrophoresis was complete, the gel was briefly washed with Nanopure water for 2−5 min to remove any residual SDS on the gel. The gel was stained with Coomassie Brilliant blue (R-250) to visualize the purity and functional mass of the enzyme. Destaining the gels was performed using a solution containing 50% (v/v) methanol and 10% (v/v) glacial acetic acid.

Acid Phosphatase Activity Stain. After nondenaturing SDS−PAGE was run, acid phosphatase activities were visualized on the gel using a fluorogenic technique described by Kishi et al. (*33*). Nitrocellulose triacetate membrane was presoaked in 400 mM sodium citrate buffer (pH 5.0) containing 3 mM 4-methylumbelliferyl phosphate. The gel was sandwiched between two pieces of presoaked membrane and incubated in a closed box for 10 min at 37 °C. The membrane was removed, and the gel was placed in a variable wavelength light box equipped with a Polaroid camera, where it was subjected to 350 nm. Visualization of acid phosphatase activity was observed by the presence of a flourescent band.

pH Optimum Determination. The ability of the enzyme isolate to hydrolyze *p*-nitrophenyl phosphate was tested over a pH range from two to nine at 25 °C. Acid phosphatase (10 *μ*g) was added to 950 Fl of 3 mM *ρ*-nitrophenylphosphate in each of the following buffers: pH 2 (50 mM maleic acid buffer), pH 3 (50 mM citric acid buffer), pH 4 (50 mM sodium citrate buffer), pH 5 (50 mM sodium acetate buffer), pH 6 (50 mM potassium phosphate buffer), pH 7 (50 mM MOPS buffer), pH 8 (50 mM Tris-HCl buffer), and pH 9 (50 mM ethanolamine buffer). Enzyme samples were incubated for 30 min at 25 °C before the enzyme activity was measured spectrophotometrically at 410 nm.

Temperature Stability Determination. To study temperature stability, 10 *μ*g of acid phosphatase was incubated for 1 h at various temperatures ranging from 10 to 80 °C. Aliquots of temperature-treated enzyme (50 *μ*L) were then added to 950 *μ*L of 50 mM MES buffer (pH 6.0) containing 3 mM *p*-nitrophenylphosphate and incubated for 30 min at 25 °C. The remaining enzyme activity was measured spectrophotometrically at 410 nm.

Organophosphate Substrate Studies. The ability of the *S. oligorrhiza* acid phosphatase to hydrolyze organophosphate substrates was studied. Using the substrates, *p*-nitrophenylphosphate and dimethylparaoxon, enzyme activity was measured by the formation of 4-nitrophenol at 410 nm, while the enzyme activity of the other substrates including *β*-glycerophosphate, phosphoserine, pyrophosphate, ATP, ADP, and AMP was determined by measuring the release of inorganic phosphate at 660 nm. The degradation of commercially available insecticides including malathion, demeton-*S*-methyl, dichlorvos, dicapthon, dylox, and sulfatep (**Figure 1**) was investigated with the *S. oligorrhiza* acid phosphatase. Stock solutions of organophosphates, each 1000 mg/L, were prepared in 50 mM MES buffer (pH 6.0) and stored at 4 °C. Because of the low water solubility of some of these compounds, methanol was added to the stock solution not exceeding 3% (v/v). Isolated acid phosphatase (10 *μ*g) was added to a reaction vial containing 100 mg/L organophosphate in 50 mM MES buffer (pH 6.0). The enzyme samples were incubated for 30 min at 25 °C and analyzed by micellar electrokinetic capillary electrophoresis (MECE).

MECE. Hydrolysis of malathion, demeton-*S*-methyl, dichlorvos, dicapthon, dylox, and sulfatep by acid phosphatase was determined by MECE using an HP-91 CE instrument (Agilent Technology, New Castle, DE) equipped with a fused silica capillary column (25 *μ*m i.d. × 72 cm length) and a diode array detector. Samples (100 *μ*L) were injected on the column under 50 mbar of injection pressure in 20 mM borate buffer (pH 8.6) containing 50 mM SDS at 23 °C. For each sample analyzed, two controls were prepared. The first control consisted of 100 mg/L organophosphate substrate in 50 mM MES buffer (pH 6.0), which established a specific retention time for identification of the substrate and an integrated area that was proportional to its concentration. The second control sample contained 10 *μ*g of purified enzyme in 50 mM MES buffer (pH 6.0). The purpose of this control sample was to determine the retention times of the peak(s) formed by the enzyme alone. The active samples contained 100 mg/L of organophosphate substrate and 10 *μ*g of purified enzyme in 50 mM MES buffer (pH 6.0). Hydrolysis of active samples was determined by the percent decrease in initial substrate peak area at its defined retention time. Hydrolysis product identification was conducted by matching CE retention times with products formed by basic hydrolysis (3 M NaOH) of authentic organophosphate standards.

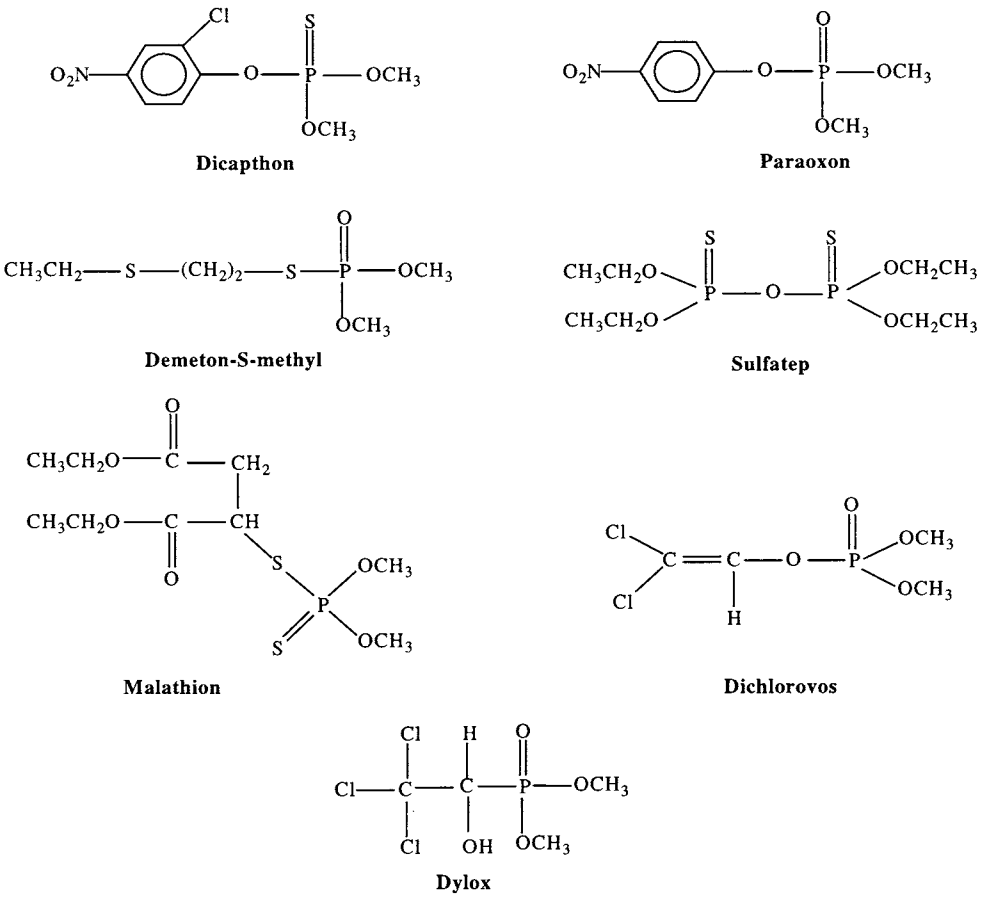

Figure 1. Chemical structure of commercially available insecticides tested for hydrolysis by *S. oligorrhiza* acid phosphatase.

Table 1. Purification Summary for an Acid Phosphatase from 1 kg of *S. oligorrhiza*

purification step	protein (mg)	units activity (U)	specific activity (U/mg)	purification (fold)	yield (units of activity recovered)
crude extract	2457	179	0.07	1	100
salt precipitation	1298	171	0.13	1.85	95
ultrafiltration	112	165	1.40	20	90
phenyl sepharose	38.1	147	3.85	55.0	88
resource-S	0.70	86.0	123	1757	58
seph. S-400	0.57	75.0	131	1871	40

RESULTS

Enzyme extracts from *S. oligorrhiza* grown in phosphate deficient media showed no appreciable increase in acid phosphatase activity as compared to those plants cultivated under phosphate sufficient conditions. As further evidence of the acid phosphatase being constitutively present, acid phosphatase activity was nondetectable in the growth medium under either condition ensuring that induction did not occur.

A summary of the different protein isolation steps carried out to purify the *S. oligorrhiza* acid phosphatase is shown in **Table 1**. This protocol enabled a 1871-fold purification of the enzyme with an overall yield of 40%. The phenyl sepharose affinity column separated two very distinct acid phosphatases. One was eluted at 30% B, while the other was released from the column at 90−92% B (see Materials and Methods). The first-eluted acid phosphatase hydrolyzed *p*-nitrophenylphosphate but not methylparaoxon, whereas the latter acid phosphatase hydrolyzed both *p*-nitrophenylphosphate and methylparaoxon. Because of the enzymatic capability to hydrolyze the organophosphate pesticide methylparaoxon, the more hydrophobic phosphatase was targeted for purification.

The resource-S cation exchange column was a highly efficient step in purification as most contaminants passed through the column in the unbound volume. The acid phosphatase bound to this matrix at a buffer pH of 5.0 ± 0.2 and eluded from the column at a 250 mM KCl concentration. While some enzyme activity was lost when loading the column, the purification fold dramatically increased from 55 to 1757, and the specific activity of the enzyme climbed from 3.85 to 123 U/mg **(Table 1)**. Fractions with acid phosphatase activity were collected and conditioned for gel filtration chromatography. Size exclusion further resolved the acid phosphatase preparation, and it was determined that the purified native enzyme functioned at approximately 120 kDa.

Gel Electrophoresis and Zymagram Assay of a Purified Acid Phosphatase. SDS−PAGE electrophoresis of the 120 kDa size exclusion fraction yielded a single protein band that migrated to approximately 60 kDa (**Figure 2**, lane 2) when compared to the SDS−PAGE protein markers conducted under denaturing conditions (**Figure 2**, lane 1). Electrophoresis of the pure fraction under nondenaturing conditions resulted in a migratory molecular mass > 120 kDa (**Figure 2**, lane 3), which corresponded to the native activity band using the fluorescent zymagram assay (**Figure 2**, lane 4). The discrepency in molecular mass between the size exclusion measurement and the band in lane 3 can likely be attributed to the nondenaturing conditions of the acid phosphatase during gel electrophoresis.

Organophosphate Substrate Studies. The purified *S. oligorrhiza* acid phosphatase hydrolyzed a variety of organophosphate substrates as shown in **Table 2**. This enzyme demonstrated a high affinity for the physiological substrates pyrophosphate,

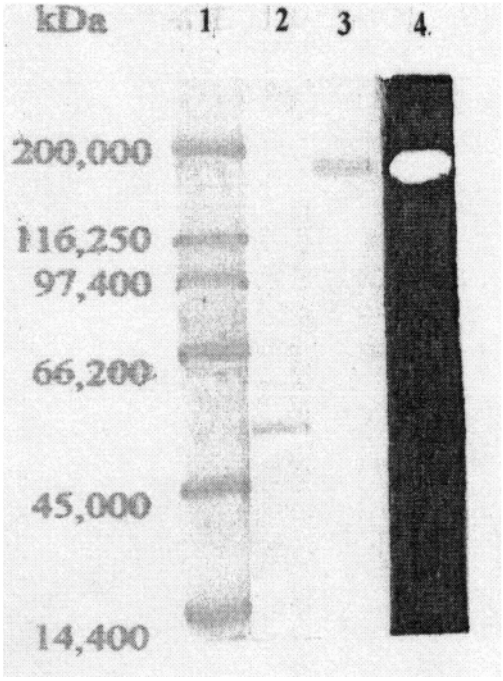

Figure 2. Gel electrophoresis of purified *S. oligorrhiza* acid phosphatase. Proteins were stained with Coomassie Brilliant blue (R-250). Lane 1: Broad molecular weight standard containing rabbit skeletal muscle myosin (200 kDa), *E. coli* β-galactosidase (116.2 kDa), rabbit muscle phosphorylase-b (97.4 kDa), bovine serum albumin (66.25 kDa), hen egg white albumin (45.0 kDa), and hen egg white lysozyme (14.4 kDa). Lane 2: SDS–PAGE of purified acid phosphatase carried out using the Laemmli method. Lane 3: Nondenaturing PAGE of purified acid phosphatase. Lane 4: Acid phosphatase activity stain visualized on nondenaturing PAGE gels after the hydrolysis of the flourogenic substrate, 4-methylumbelliferone phosphate.

Table 2. Summary of *S. oligorrhiza* Acid Phosphatase Activity on Various Organophosphate Substrates

organophosphate substrate[a]	U/mg
pyrophosphate	131 ± 1
ATP	125 ± 2
p-nitrophenyl phosphate[b]	118 ± 2
ADP	97 ± 3
β-glycerophosphate	89 ± 4
phosphoserine	81 ± 2
methylparaoxon[b]	74 ± 3
AMP	156 ± 3
organophosphate insecticide[c]	**degradation (%)**
dicapthon	100 ± 3
dichlorvos	100 ± 4
malathion	95 ± 2
sulfatep	50 ± 4
demeton-*S*-methyl	36 ± 3
dylox	0

[a] Hydrolysis of substrates was detected spectrophotometrically at 660 nm by measuring the release of inorganic phosphate after 6 min at 25 °C. [b] Hydrolysis was monitored spectrophotometrically at 410 nm by measuring the release of 4-nitrophenol after 30 min at 25 °C. [c] Disappearance of parent substrate measured by capillary electrophoresis after 30 min of incubation at 25 °C.

ATP, *p*-nitrophenylphosphate, and ADP. In contrast, the substrates β-glycerophosphate, phosphoserine, and AMP underwent hydrolysis to a lesser extent.

Analysis of organophosphate pesticides for hydrolytic transformation was determined by comparing the ratio of control peak areas vs active samples containing purified acid phosphatase (see Materials and Methods). Parent substrate disappearance and end product identification were observed in all active samples, while positive controls remained unchanged.

Our results demonstrated that the purified acid phosphatase was able to hydrolyze both dicapthon and dichlorvos to undetectable levels and degraded approximately 95% of malathion in 30 min at 25 °C (**Table 2**). The enzyme hydrolyzed 50% of sulfatep and 36% of demeton-*S*-methyl, whereas the insecticide dylox did not undergo transformation.

The enzyme activity of the acid phosphatase with *p*-nitrophenylphosphate was investigated in the presence of various metals, chelators, and orthophosphate as a potential feedback inhibitor (**Table 3**). Substrate hydrolysis was unaltered when either $MgCl_2$, $MnSO_4$, or ZnCl was added to the reaction. The chelators EDTA and EGTA appeared to also have no effect on enzyme activity. Thus, it is likely that no divalent metals are involved with the reaction. The addition of 5 mM sodium phosphate inhibited 54% of the enzyme activity, which was likely due to end product inhibition.

pH Optima and Temperature Stability. The enzyme activity of the acid phosphatase was measured over a range of pH values from 2 to 9 (**Figure 3**). The optimum pH was determined to be 6.0 using *p*-nitrophenylphosphate as a substrate at 25 °C. At pH 5.0, approximately 90% of the optimal enzyme activity was detectable. The acid phosphatase lost 80% of its enzyme activity at pH 8.0. The *S. oligorrhiza* acid phosphatase was stable over a temperature range of 10−40 °C (**Figure 4**).

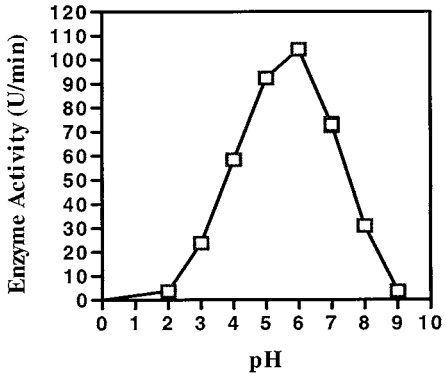

Figure 3. pH optimum of the *S. oligorrhiza* acid phosphatase.

Table 3. Effects of Various Metals and Chelators on Acid Phosphatase Activity

compound (5 mM)	relative activity (%)	compound (5 mM)	relative activity (%)
$MgCl_2$	92 ± 2	$(Na)_3PO_4$	46 ± 3
$MgSO_4$	94 ± 3	EDTA	96 ± 4
$MnSO_4$	92 ± 3	EGTA	90 ± 3
$ZnCl_2$	94 ± 2		

DISCUSSION

In this report, the purification and partial characterization of an *S. oligorrhiza* acid phosphatase have been described. Starting with 1 kg of plants, 0.5 mg of the enzyme was purified 1871-fold with a total *p*-nitrophenylphosphate hydrolyzing capability of 131 U/mg and an overall yield of 40%. A comparable purification protocol for the potato tuber acid phosphatase demonstrated that 0.5 mg of purified enzyme was produced from 0.6 kg of plant tissue, with a total *p*-nitrophenylphosphate hydrolyzing activity of 1250 U/mg (*10*). Although the general

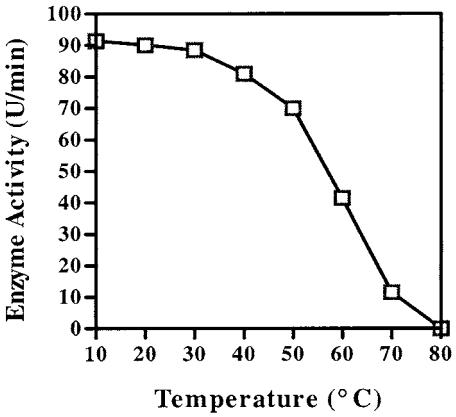

Figure 4. Temperature stability curve of the *S. oligorrhiza* acid phosphatase.

amount of purified enzyme is approximately the same, the specific *p*-nitrophenylphosphate activity of the potato tuber acid phosphatase is approximately 10-fold higher than that observed by the *S. oligorrhiza* acid phosphatase. Such a difference in *p*-nitrophenylphosphate hydrolyzing activity between these two enzymes may be due to different physiological functions, substrate preferences, or reaction conditions.

Induction of acid phosphatase activity was investigated in *S. oligorrhiza* via phosphate depletion in the plant culture medium. When studying protein extracts prepared from plants grown in both phosphate-deprived and phosphate sufficient culture media across a broad pH gradient, no significant change in enzyme activity with the organophosphate pesticide methylparaoxon was observed. The continual presence of this acid phosphatase in enzyme extracts of *S. oligorrhiza* when grown under both media conditions indicates that this enzyme likely functions constitutively. However, the alkaline phosphatase activity, when detected in protein extracts with *p*-nitrophenylphosphate, was significantly enhanced when phosphate was absent from the growth medium. Induction of alkaline phosphatases by phosphate deprivation has previously been reported by Bieleski (*34*). That study revealed that phosphatase activity could be enhanced 50-fold due to the controlled expression of multiple, extracellular alkaline phosphatases.

SDS−PAGE estimated a molecular mass of the *S. oligorrhiza* acid phosphatase to be 60 kDa, and gel filtration chromatography approximated a molecular mass of 120 kDa. These results indicate that the native, functional enzyme is a homodimer, with a molecular mass of 60 kDa for each subunit. In comparison to other purified plant acid phosphatases, the *S. oligorrhiza* acid phosphatase is larger than the acid phosphatases purified from potato tuber (*10*), tomato cell culture (*11*), and barley roots (*12*) but smaller than the acid phosphatase from cotton (*13*) and peanut (*14*). Similarly, the tomato acid phosphatase consists of a homodimer structure with two 51 kDa subunits, whereas the potato tuber acid phosphatase functions as a heterodimer, with 55 and 57 kDa subunits.

The effect of pH on the enzyme activity was investigated in the range of pH 2−9. The enzyme was stable in a range of pH 4−7, with a pH optimum of 6.0. The pH optimum determined for the *S. oligorrhiza* acid phosphatase is largely consistent with other pH optima (5.0−6.0) determined for other known acid phosphatases. The *S. oligorrhiza* acid phosphatase was shown to be functional over a broad range of temperatures and maintained stability up to 40 °C. To determine whether the *S. oligorrhiza* acid phosphatase requires a metal for enzyme activity, various metallic compounds and chelating agents were added to reaction assays. The results revealed no changes in enzyme activity in the presence of either metals or chelators; hence, this enzyme does not likely require a metal for hydrolysis. Unlike the *S. oligorrhiza* acid phosphatase, the enzyme activity of the potato tuber acid phosphatase can be enhanced by 40% with superfluous Mg^{2+} (*10*).

Degradation studies using a variety of physiological and commercial organophosphates were investigated with the *S. oligorrhiza* acid phosphatase. Some of the organophosphates used in this study were physiological substrates, such as ATP, ADP, AMP, β-glycerophosphate, and pyrophosphate, and all were hydrolyzed. Thus, this acid phosphatase is not only capable of recognizing and hydrolyzing monoester organophosphates but also both di- and triester phosphocompounds. It has been reported that other acid phosphatases including 3-phosphoglycerate phosphatase, phosphenolpyruvate phosphatase, and phytase can display unselective enzyme activity on a variety of substrates (*8*). The ability of the *S. oligorrhiza* acid phosphatase to have such broad and unspecific substrate requirements led to the degradation studies of commercial insecticides.

Organophosphate insecticides are similar in molecular structure to other phosphatase substrates. Among the organophosphate compounds studied were the nerve agents, malathion and demeton-*S*-methyl. Both malathion and demeton-*S*-methyl were hydrolyzed by the enzyme, but malathion was degraded to a much greater extent (95%) than observed for demeton-*S*-methyl (36%). Because the bond cleaved (P−S) for both malathion and demeton-*S*-methyl is identical, the difference in hydrolysis may be due to the less stable (P═S) bond present in malathion. Dicapthon and dichlorvos were both hydrolyzed to undetectable levels. The similar extent of hydrolysis for sulfatep and methylparaoxon might be due to the better recognized (P−O) bond that is cleaved, allowing more efficient hydrolysis. However, incubation of acid phosphatase with dylox resulted in no detectable hydrolysis. The inability for the acid phosphatase to hydrolyze dylox may likely be due to the stability of the (P−C) bond that is attached by the enzyme. The different types of substrates that the *S. oligorrhiza* acid phosphatase hydrolyzed include both aromatic and aliphatic organophosphates containing P−O and P−S bonds, phosphorylated amino acids and sugars, and the ability to cleave mono-, di-, and triesterphosphate bonds. It is evident that the *S. oligorrhiza* acid phosphatase has a very broad, unselective capability to degrade a variety of structurally diverse organophosphate compounds.

The similarities in molecular structure of both malathion and demeton-*S*-methyl to the nerve agents 1,2,2-trimethylpropyl methylphosphonofluoridate (soman), *O*-ethyl-*S*-(2-diisopropylaminoethyl (sarin), and diisopropylflourophosphate (DFP) classify them as "nerve gas surrogates." The molecular structures of the organophosphate nerve agents are illustrated in **Figure 5**.

Degradation of soman, sarin, and DFP has been demonstrated by several aquatic plants, including *S. oligorrhiza*. On the basis of the work of Hoskin et al. (*35*), the hydrolysis of DFP and soman was investigated using *S. oligorrhiza*, *Lemna minor* (duckweed), *Vigna radiata* (germinated mung bean), and the slime mold, *Dictyostelium discoidium*. Soman was hydrolyzed by all three macrophytes, and *D. discoidium* hydrolyzed both DFP and soman. That study indicated that the hydrolysis of the nerve agents was enzyme-mediated. It is tempting to suggest that the enzyme responsible for the in vivo transformation of the nerve agents could be an unspecific phosphatase with functional capabilities similar to the *S. oligorrhiza* acid phosphatase.

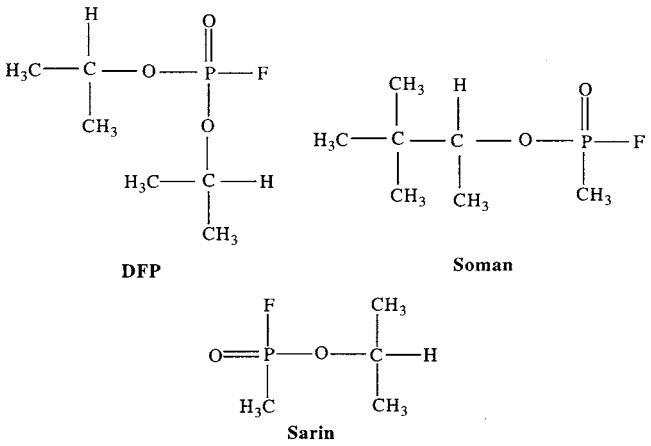

Figure 5. Chemical structure of organophosphate nerve agents.

On the basis of this present work and the studies by Gao et al. (*27*) and Hoskin et al. (*35*), it is evident that certain aquatic plants possess nonspecific hydrolytic enzymes with insecticide remediation potential. Our results indicate that an acid phosphatase from *S. oligorrhiza* hydrolyzes several different organophophate substrates, some of which are structurally similar to nerve agents. In the future, this enzyme may warrant a subsequent genetic investigation with efforts to achieve sequence data so gene cloning, overexpression of the recombinant enzyme, and further characterization will be possible. Such research could reveal characteristics that could provide insight into the enzymes physiological function as well as practical enzyme applications that could be investigated and developed.

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