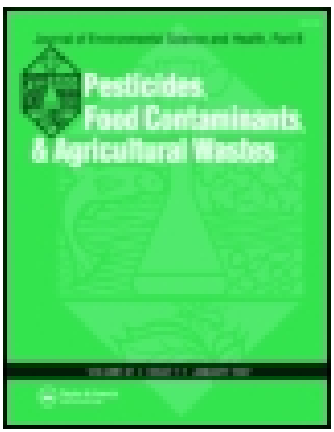

Journal of Environmental Science and Health, Part B: Pesticides, Food Contaminants, and Agricultural Wastes
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lesb20

Characterization of a phosphotriesterase from genetically-engineered Escherichia coli

Yen-Der Cheng [a] , Jeffrey S. Karns [b] & Alba Torrents [a]

[a] Environmental Engineering Program, Department of Civil Engineering , University of Maryland , College Park, MD, 20742

[b] Soil Microbial Systems Laboratory , Room 140 Building 001 BARC-West 10300 Baltimore Ave., Beltsville, MD, 20705-2350

Published online: 21 Nov 2008.

To cite this article: Yen-Der Cheng , Jeffrey S. Karns & Alba Torrents (1998) Characterization of a phosphotriesterase from genetically-engineered Escherichia coli , Journal of Environmental Science and Health, Part B: Pesticides, Food Contaminants, and Agricultural Wastes, 33:4, 347-367

To link to this article: http://dx.doi.org/10.1080/03601239809373150

J. ENVIRON. SCI. HEALTH, B33(4), 347-367 (1998)

Characterization of a Phosphotriesterase from Genetically-Engineered *Escherichia coli*

Key Words: Phosphotriesterase, activity, stability, pesticide-waste

Yen-Der Cheng[1], Jeffrey S. Karns[2*] and Alba Torrents[1]

[1]Environmental Engineering Program
Department of Civil Engineering
University of Maryland
College Park, MD 20742

[2]Soil Microbial Systems Laboratory
Room 140 Building 001 BARC-West
10300 Baltimore Ave. Beltsville,
MD 20705-2350

ABSTRACT

A phosphotriesterase (PTE) capable of hydrolyzing organophosphate esters was purified from *Escherichia coli* strain DH-5α carrying a cloned *opd* gene from *Flavobacterium*. The effects of pH, temperature and metal ion concentrations on enzyme stability and activity were investigated. Optimum conditions for PTE's catalytic activity were determined to be 35°C and pH 8.5. Protein-metal

*To whom correspondence should be addressed.

equilibrium binding experiments showed that PTE could accommodate two equivalents of Co^{2+} or Zn^{2+} ions. PTE protein was found to have higher affinity for Co^{2+}. In addition, Co^{2+} was found to possess the most positive effects in maintaining and restoring PTE's stability and catalytic activity when compared to other divalent metal ions. Assessment of the feasibility of PTE operation in a practical environment was performed in a system designed to mimic a continuously stirred tank reactor (CSTR) with different solution compositions in the flow reservoir. PTE was deactivated in 24 hours when the inflow solution contained 5% ethanol or 1 mM EDTA, while it retained one third of its initial activity in a deionized water stream. When the inflow solution contained 1 mM Co^{2+}, PTE was found to retain activity throughout the 24-hour experiment.

INTRODUCTION

Since the banning of chlorinated hydrocarbon insecticides in the early 70`s, organophosphate pesticides are used extensively worldwide for a wide range of agricultural and household applications. These compounds possess the advantage of quick and effective elimination of pests by the inhibition of acetylcholinesterase. However, this is also the primary cause of toxicity in non-target organisms including humans. Studies have shown that exposure to excess levels of an organophosphorous compound not only has immediate consequences but also can cause delayed cholinergic toxicity and neurotoxicity (Tuovinen *et al.*, 1994; Sawyer *et al.*, 1991). Thus, a comprehensive and effective technique is desired for the detoxification and

disposal of these organophosphate compounds in cases of excess and accidental discharge as well as in manufacturing waste streams.

Munnecke (1976) first reported phosphotriesterase (PTE) as parathion hydrolase. Crude PTE extracts from *Pseudomonas diminuta* were found to be able to hydrolyze ethyl parathion (O,O-diethyl O-4-nitrophenyl phosphorothioate) and other organophosphate compounds (paraoxon, diazinon, methyl parathion, chlorpyrifos, fenitrothion, and cyanophos) with rates 40 to 1000 times faster than that of chemical hydrolysis (with 0.1 N sodium hydroxide) (Munnecke, 1980). Dumas and his coworkers purified PTE to homogeneity (3200 U/mg) and observed 10^{12} times catalytic rate enhancement for paraoxon hydrolysis compared to chemical hydrolysis at pH 7 (Dumas *et al.*, 1990). Due to its broad substrate specificity and high efficiency, PTE is a potent candidate for use in the detoxification of organophosphate wastes.

There have been considerable efforts made to develop various techniques for remediation of organophosphate related agents (Shelton and Karns, 1988; Caldwell and Raushel, 1991; Karns *et al.*, 1992; Havens and Rase, 1993; Grice *et al.*, 1996; Lejeune and Russell, 1996). However, to improve and optimize these techniques, extensive studies of purified PTE's responses to ambient factors such as pH, temperature and possible existing metal ions are of great importance. The present study uses PTE isolated from a recombinant *Escherichia coli* strain DH-5α carrying the *opd* gene from *Flavobacterium* (Mulbry and Karns, 1989). In this paper, we examine the stability and catalytic activity of PTE under various pH conditions and temperatures as well as in the presence of different metal ions. A continuous mixed flow system is used to evaluate the operative stability of PTE in such conditions.

MATERIALS AND METHODS

Phosphotriesterase (PTE) was purified from *Escherichia coli* strain DH-5 containing the cloned *opd* gene isolated from *Flavobacterium* ATCC27551 in plasmid pJK33 (Mulbry and Karns, 1989). Ethyl parathion was obtained from Chem Service® (West Chester, PA) with purity of 99.9% and was used without further purification. All buffers were purchased from Sigma® (St. Louis, MO) and were analytical grade. TSK-DEAE column (2.15 x 15 cm) was purchased from Waters® (Milford, MA.) and TSK-phenyl column (2.15 x 15 cm) was purchased from HP-Genenchem® (South San Francisco, CA.). Metal ion solutions were prepared from pure sulfate or chloride salts obtained from Aldrich® (Milwaukee, WI.). In all experiments, ultra purified water (18 MΩ/cm, Modulab®, Type I HPLC, Continental Water System® Co., Houston, TX) was used.

Preparation of Cell-Free Enzyme

Escherichia coli DH-5α containing plasmid pJK33 was grown in 3 liters of LB broth containing 100 mg ampicilin and 0.01 mM $CoSO_4$. The culture was incubated overnight in a shaker at 200 rpm and 37°C. The culture was harvested by centrifuging at 6,000 xg at 4°C for 20 minutes. Cells were washed with 250 ml 20 mM potassium phosphate (Kpi) buffer (pH 7.0) containing 0.1 mM $CoSO_4$ (buffer A) and recentrifuged at 8,000 xg at 4°C for 15 minutes. Two grams of cells in 15 ml buffer A were lysed in a French press at 1.03×10^8 Pa, 4°C. The lysed cell extract was centrifuged at 8,000 xg to remove the cell debris and then subjected to

ultracentrifugation at 105,000 xg and 4°C for 90 minutes. The supernatant was collected and dialyzed against buffer A overnight.

Purification of Cell-Free Enzyme

Cell-free extract was pumped at 2 ml/min directly onto a TSK-DEAE column equilibrated with buffer A. The column was washed with buffer A at 5 ml/min until all unbound protein was eluted. The flow was then switched to a linear gradient of buffer A containing 1 M NaCl over 60 minutes to elute any bound protein from the column. Effluent fractions were visually tested in a microtiter plate for turning the "assay mix" (25 mM Tris, pH 8.5, 0.1% Triton X-100, 0.01 mM $CoSO_4$, 340 μM parathion) to yellow. The active fractions were then assayed spectrophotometrically as described below. The fractions with higher activities were pooled for further purification.

The DEAE fraction pool was adjusted to 0.5 M $(NH_4)_2SO_4$ by adding solid $(NH_4)_2SO_4$. The pool was pumped at 2 ml/min onto a TSK-Phenyl column equilibrated with buffer A containing 0.5 M $(NH_4)_2SO_4$. The buffer in the system was then switched to buffer A containing 10% methyl sulfoxide through a linear gradient over 120 minutes at 5 ml/min. The eluent fractions were pooled and assayed for their activities as described above. The most active portions were pooled and concentrated by ultrafiltration (Centricell® 30, Amicon® Inc., Beverly, MA).

Protein Assay

Protein concentration was measured by the method of Bradford (1976) using the BioRad® protein-dye binding assay kits with IgG as standard.

Enzyme Activity Assay

The activity of purified PTE was determined spectrophometrically by following the production of *p*-nitrophenol as the result of ethyl parathion hydrolysis at 405 nm. Reaction mixture without enzyme served as a blank. A Beckman® DU-7 HS spectrophotometer in the kinetic mode was used in all experiments. The rate of *p*-nitrophenol formation was calculated by using an extinction coefficient (ε) of 18,800 M^{-1}. cm^{-1} (Mulbry and Karns, 1989). The temperature of the reactions was 25°C unless otherwise noted.

Effect of pH and Temperature

To investigate the effect of pH on the stability of purified PTE, 0.023 mg/ml of PTE was incubated in buffer solutions (50mM Kpi for pH 6, 50mM Tris-HCl for pH 7 and 8, 50mM borate for pH 9 and 10) containing 0.01mM $CoSO_4$ at 25°C. To study the effect of temperature, PTE in 25mM Tris-HCl (pH 8.5) was placed in thermostatically controlled water baths (from 25 to 55°C). At pre-determined incubation intervals, 2 µl aliquots from the enzyme/buffer were withdrawn and assayed as described above. For the determination of K_m and V_{max} at various temperatures, PTE activity was assayed with assay mixtures containing different parathion concentrations.

Effect of metals on enzyme stability

Six metal sulfate solutions ($CaSO_4$, $MgSO_4$, $CoSO_4$, $FeSO_4$, $CuSO_4$ and $ZnSO_4$) containing 25 mM Tris buffer, 0.023 mg PTE protein/ml and 5 mM metal ions were prepared and incubated at 25°C. Samples from each treatment were collected at various times and assayed as described above.

Preparation and Restoration of Apoenzyme

To further examine the effect of metal ions, experiments were conducted to extract and reconstitute PTE bound metals. Five ml of PTE extract (protein concentration 0.023 mg/ml) was dialyzed against 1 liter 25 mM Tris buffer solution (pH 8.5) with 5 mM EDTA for 24 hours (the buffer was replaced every 4 hours during dialysis) to provide the metal-free apoenzyme. PTE was then assayed and dialyzed against 25mM Tris buffer solutions containing 0.1 mM and 1 mM $CoSO_4$ as well as 0.1 mM $MgSO_4$ for 24 hours. The enzyme in each mixture was sampled and assayed at pre-determined intervals.

Determination of Co^{2+} and Zn^{2+} Binding Constants

The PTE apoenzyme and buffers containing different metal ions (Co^{2+} and Zn^{2+}) were placed in opposite halves of equilibrium dialysis cells (Spectrum®, Laguna Hill, CA) with 1 ml space on each side. The chambers of each dialysis cell were separated by a semi-permeable cellulose acetate membrane (Spectrum®, MWCO = 10,000). PTE concentration was adjusted to 1 mM (calculated based on PTE M.W. = 33,000) while the metal concentrations in the other halves were varied (blank, 4 mM, 6 mM, 8 mM and 10 mM). An equal amount of Mg^{2+}, proven not to affect PTE activity, was added to the half with protein as the counter ion to prevent any Donnan effect due to charge inequality. The temperature of the dialysis cell was thermostatically controlled at 4°C and the dialysis cells were rotated at a speed of 20 rpm. Samples were taken after 24 hours from the non-protein half of the cell and metal concentrations were analyzed using atomic absorption spectrophotometer (Perkin-Elmer® 5100ZL). The determination of the equilibrium constant for the metal-

protein binding reaction was similar to that is used in the study of protein-ligand binding. All such reversible binding reactions can be represented by the following general equation (Segel, 1976):

$$P + nM \leftrightarrow PM_n \qquad Ka = \frac{(PM_n)}{(P)(M)^n}$$

(protein) (metal ions) (protein-metal complex)

n is the number of identical but non-cooperative metal binding sites on the protein, K_a is the metal-protein binding (affinity) constant. Applying the mass balance to the equilibrium and then developing the expression for K_a algebraically gives the Scatchard equation:

$$\frac{Y}{M} = K_a (n\text{-}Y)$$

where Y is the ratio between moles of metal ions bound with the protein (PM_n) and moles of protein (P).

Stability of PTE in a Continuous Flow System

A flow-through microdialyzer module (Spectrum®) was applied to simulate a continuous flow system. A 0.5 ml preparation of PTE containing buffer (25 mM Tris with 0.1 mM Co^{2+} and 0.023 mg/ml protein, pH 8.5) was added into the dialysis well. A dialysis membrane (MWCO = 14,000) separated the well from a dialysis chamber (volume 34 ml) flushed with continuous flow. The flow rate was controlled at 2 ml/min by a peristaltic pump creating a hydraulic retention time of 17 minutes in the dialysis chamber. In four independent experiments, non-buffered solutions (5% ethanol, 1 mM EDTA, 1 mM Co^{2+}, deionized water) were introduced into the continuous flow system. Samples were collected at pre-determined intervals and PTE

activity was assayed. PTE activity in a batch system containing 0.1 mM Co^{2+} (25°C) was also monitored and compared with the results observed from continuous flow system.

RESULTS AND DISCUSSION

Effect of pH on PTE Activity and Stability

The pH that showed the best combination of high reaction rate and PTE stability appeared to be between 7 and 9 (Figure 1). After incubation for 24 hours at pH 8, 85% of PTE's initial activity remained whereas 56% remained at pH 6. Decrease of the PTE activity at pHs below pH 8 was possibly due to the ionization of crucial amino acids on the protein structure which disrupted the conformation of its active site. The trend of PTE stability in various pH conditions is consistent with previous findings (Dumas and Raushel, 1990) which suggested that the PTE protein requires a functional group with a pKa value around 6.1 to be deprotonated for its full catalytic activity.

Effect of Temperature on PTE Activity and Stability

A temperature study indicated that the optimal temperature for PTE activity was around 35°C. Enzyme activity decreased by 10% at 25°C and decreased sharply at temperatures above 45°C, possibly due to the break down of the PTE protein (Figure 2). The comparison of PTE activity at different temperatures also showed that K_m (Michaelis constant) and V_{max} (maximum turnover rate) at 35°C was 50% and 17% greater than that of 25°C (Figure 3). This result indicated that although PTE had

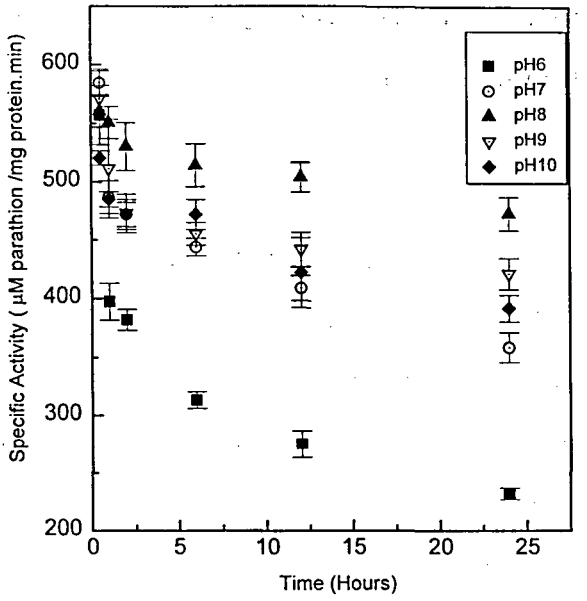

FIGURE 1

Stability of PTE at different pH conditions. Enzyme was sampled and assayed at pre-determined intervals (0.5 hr, 1 hr, 2 hr, 6 hr, 12 hr and 24 hr). The PTE assay mixture contains 340 μM parathion, 25 mM Tris buffer (pH 8.5), 0.1% Triton X-100 and 10 mM $CoSO_4$. The assay temperature was maintained at 25°C. Each data point represents an average of triplicate experiments.

weaker affinity (higher K_m) toward its substrate (ethyl parathion) at 35°C, it still produced *p*-nitrophenol more efficiently than it did at lower temperatures. Determination of the V_{max} and K_m of PTE at temperatures above 35°C was not performed.

Effect of Metal Ions and Chelating Agent on PTE Stability

The effect of metal ions on the activity of PTE is shown in Figure 4. Presence of Co^{2+} in the medium not only prevented PTE from losing activity but also increased

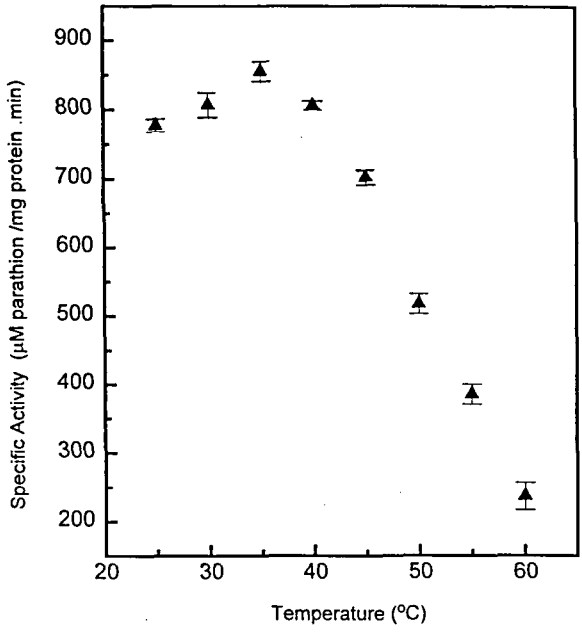

FIGURE 2

Effect of temperature variation on PTE activity. Enzyme was incubated at water bath of different temperatures for 30 minutes before the assay. Assay condition was identical as described above except for temperature adjustment according to the incubation temperature. Each data point represents an average of triplicate experiments.

its initial activity by 24% after 48 hours. Fe^{2+} and Mn^{2+} kept PTE activity slightly higher than the control set (metal free) while no significant difference in activity over the control was observed in the presence of Mg^{2+} and Cu^{2+}. The only significant inhibition on PTE activity was observed in the medium containing Zn^{2+}. In the presence of Zn^{2+}, PTE activity was 33% of that in the no metal control after 36 hours and kept declining to 25% of its initial activity after 48 hours. This observation agrees

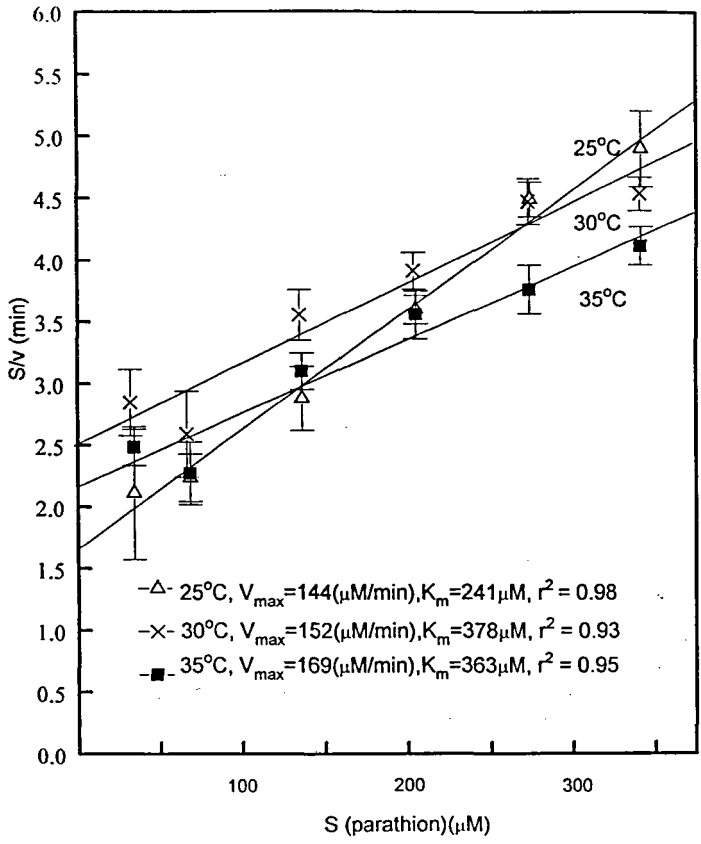

FIGURE 3

Determination of V_{max} and K_m for enzymatic parathion hydrolysis by PTE at three different temperature (25°C, 30°C and 35°C) by Hans-Woolf plot. Measurement of PTE activity was the same as described before. V_{max} and K_m were calculated by means of linear regression of experiment data. Each data point represents an average of triplicate experiments.

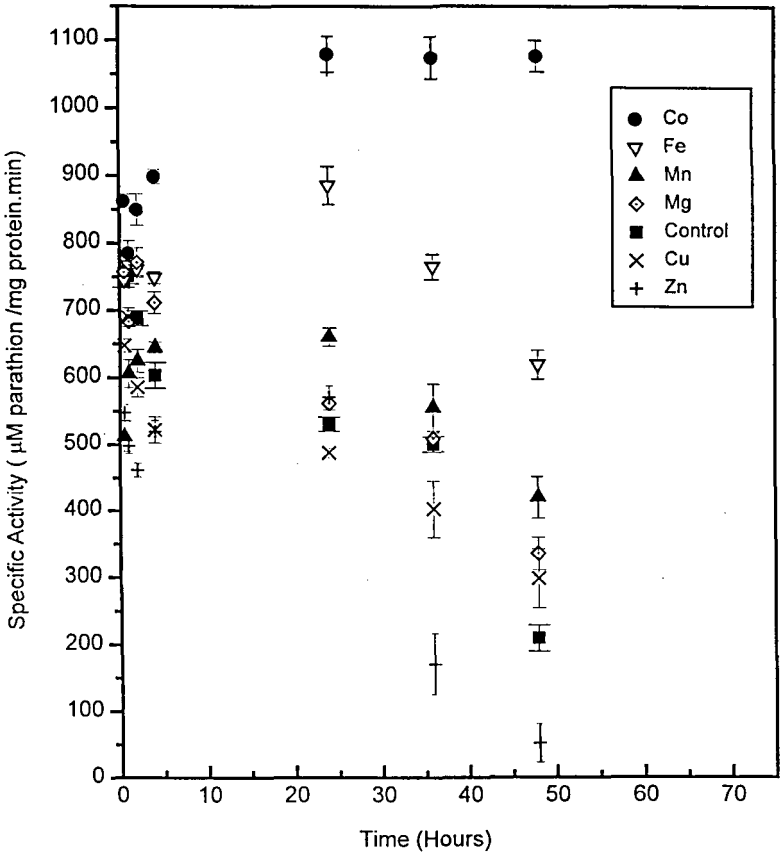

FIGURE 4

Effect of metal ions on PTE activity. PTE was incubated in buffer solutions (25 mM Tris, pH 8.5) containing six different metal sulfate salts (5 mM). During 48 hours incubation, PTE was sampled and assayed in assay mixtures containing identical metal ions as the incubation medium at 25°C. Activity of PTE in buffer without metals was defined as the control. Each data point represents an average of triplicate experiments.

with previous research (Omburo et al., 1992) which showed excess zinc inhibited PTE activity while other divalent ions (Co^{2+}, Ni^{2+}, Cd^{2+}) exhibited no, or even positive effects. Our hypothesis is that excessive amounts of Zn^{2+} can act as a stronger Lewis acid as well as a faster ligand exchanger than most M^{+2} ions (Silva and Willams, 1991). Consequently Zn^{2+} ions might replace metal ions that act as cofactors, such as Co^{2+}, originally bound to PTE during bacterial growth or enzyme purification process. Such replacement and the occupation of some nonspecific sites by Zn^{2+} might distort the conformation of PTE protein structure and cause the inactivation of PTE.

Quick and complete reconstitution of PTE apoenzyme to its initial activity was observed after incubating the metal-stripped enzyme for 12 hours in solutions containing Co^{2+} (Figure 5). No reconstitution was observed when metal-stripped PTE was treated with solution containing either Mg^{2+} or no metal ions. When the PTE apoenzyme was treated with Co^{2+}, competitive adsorption or electronic repulsion among the ions in the more concentrated medium (1 mM compared to 0.1 mM) might be the cause of lower activity recovery of PTE observed at the beginning of the process. The higher activity observed for the reconstituted PTE over its original purified counterpart might be caused the Co^{2+} displacement of non-cofactor ions, which may have been incorporated into the protein structure during the bacterial growth period or during the protein purification process. Figure 6 illustrates the stability of PTE incubated in solutions of high Co^{2+} concentration. In a pH 8 buffer containing 5 mM Co^{2+} for 72 hours, PTE was stable at both 25°C and 35°C and retained 50% of its initial activity at 45°C. Comparing these optimal PTE specific activities with those shown in Figure 2 where Co^{2+} was controlled at 0.01mM

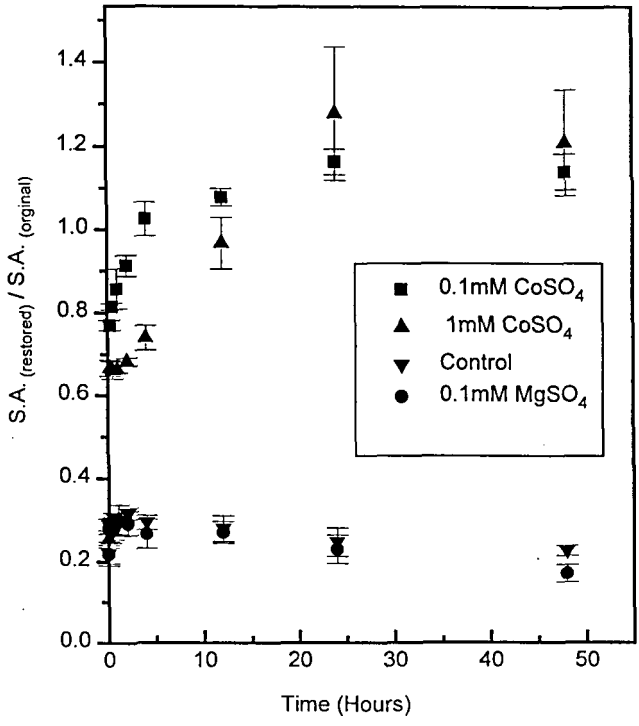

FIGURE 5

Ratio of the specific activity between the reconstituted PTE and its original activity before EDTA stripping was plot over time. Activity of PTE apoenzyme dialyzed in buffer without metals was defined as control set. Each data point represents an average of triplicate experiments.

indicated that Co^{2+} might play a critical role in the PTE protein integrity as well as its catalytic function.

Determination of Binding Constant Between PTE and Co^{2+}, Zn^{2+}

Raushel and his associates suggested that the PTE active site consisted of a binuclear metal center embedded within a cluster of histidine residues which can be

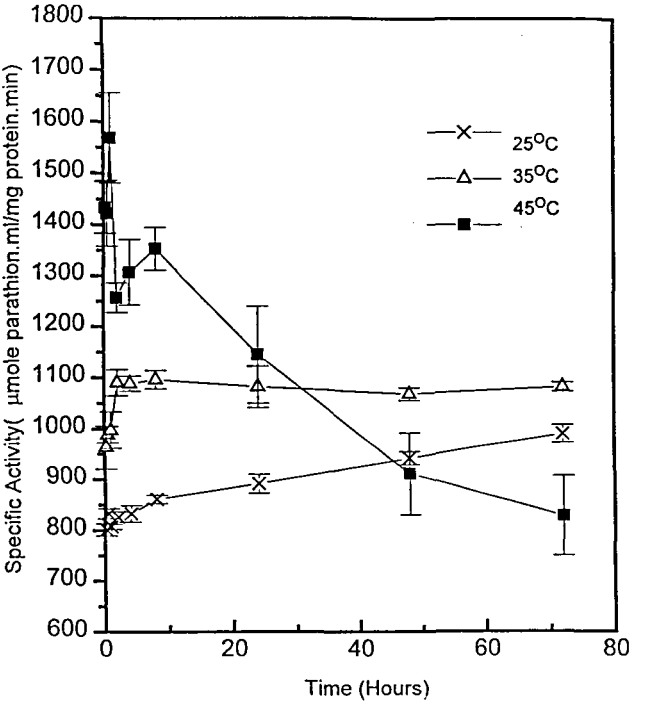

FIGURE 6

PTE stability at different temperatures (25°C, 35°C and 45°C). PTE was incubated in buffer (25mM Tris, pH 8.5) containing 5 mM Co^{2+}. Condition of enzyme activity assay was similar as described before except for the temperature was set identical as the incubation. Each data point represents an average of triplicate experiments.

assembled in an active configuration with either Zn^{2+} or Co^{2+} (Benning et al., 1994, 1995; Hong et al., 1996; Vanhooke et al., 1996). A metalloenzyme like PTE can act as an acidic catalyst due to the Lewis acidity of the metal ions bound to the protein. Benning (1994) and his coworkers suggested a model describing that the activation of a water molecule occurs not via a traditional enzymatic base but rather through

interaction with the binuclear metal center. The phosphoryl oxygen (or sulfur) of the substrate binds to one of the metal ions and displaces the hydroxide ion (from the activated water molecule). The electrophilic center of the phosphotriester is subsequently attacked by the hydroxide ion, causing the expulsion of the aromatic leaving group. Hong and Raushel (1996) postulated that perturbation of the binuclear metal center will not only perturb the catalytic efficiency of the enzyme but also alter the conformations that are required for substrate binding.

The *n* numbers obtained from our binding test (Co^{2+} = 1.8, Zn^{2+} = 2.4) showed that one part of PTE has capacity for binding about two equivalent of divalent metal ions (Figure 7) which agrees with previous findings. Zinc, considering its strong electron affinity, was expected to have a greater affinity toward amino acid residues on protein. However, our study showed instead that PTE protein has a 30% greater affinity constant for Co^{2+} ions than for Zn^{2+} ions (K_a =0.66 compared to 0.49). PTE's poor affinity for Zn^{2+} in this experiment is also contradictory with Raushel and his associates (Omburo et al., 1992; Vanhooke et al., 1996) who suggested that Zn^{2+} was the metal ion related with PTE's active site. To resolve the roles that Co^{2+} and Zn^{2+} play in the PTE catalytic mechanism, further studies are required.

PTE Stability in a Continuous Flow System

To evaluate the applicability of PTE in a practical environment, a continuous stirred flow module was set up and flows containing different surrogate wastes were introduced individually to test the response of PTE. Figure 8 shows that PTE lost more than 50% of its original activity in flow containing either ethanol or EDTA after two hours and was nearly deactivated after 24 hours. In the distilled water flow, PTE

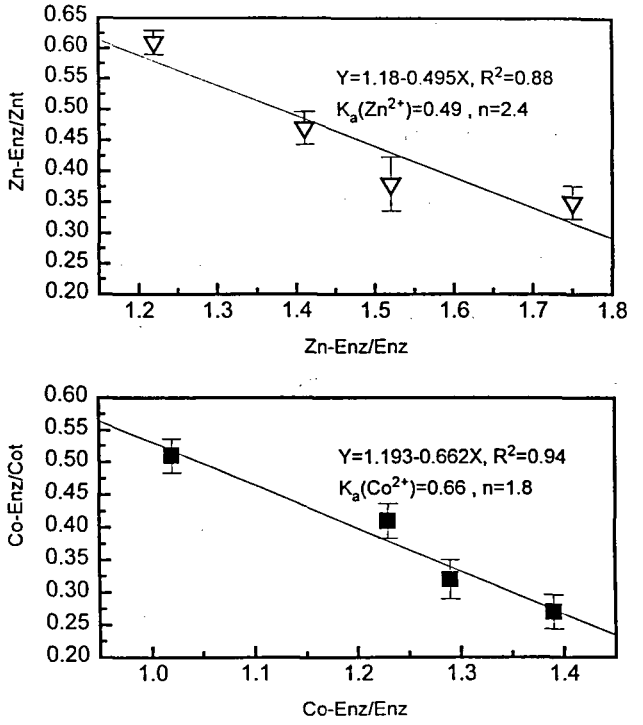

FIGURE 7

(A) Scatchard plot for the determination of Co^{2+}-PTE protein binding constant. (B) Scatchard plot for the determination of Zn^{2+}-PTE protein binding constant.

retained only 30% of its original activity after 24 hours compared to 80% in the closed batch system. Our speculation is that the continuous distilled water flow depleted the Co^{2+} in the enzyme containing portion and created a concentration gradient for PTE to release bound Co^{2+}. Loss of Co^{2+} eventually led to deactivation of the PTE. This hypothesis was consistent with the results of PTE in a Co^{2+} (1 mM) containing flow system where its activity was stable over 24 hours.

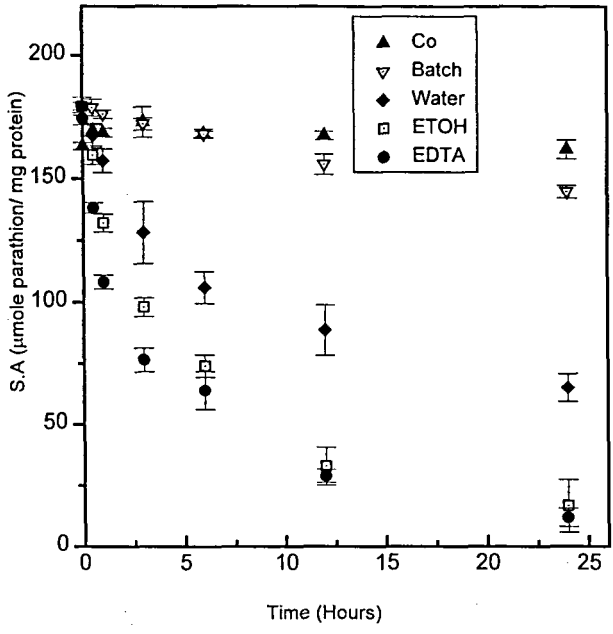

FIGURE 8

Stability of PTE in simulated CSTR systems containing different chemical ingredients. Flow rate and temperature of the system was controlled at 2 ml/min and 25°C. Each data point represents an average of triplicate experiments.

CONCLUSION

A PTE purified from genetically engineered *E.coli* was selected to evaluate the applicability of the enzyme for pesticide waste detoxification. We evaluated its response to variations of solution parameters such as pH, temperature and the presence of metal species. We also examined PTE stability in a simulated continuous stirred flow system in the presence of an organic solvent and a chelating agent. Optimum PTE activities were observed around pH 8 and 35°C. Reconstitution of

PTE apoenzyme and its protein-metal affinity results suggests that Co^{2+} is favored by PTE over other metal ions and might be crucial in maintaining its activity. Contradictions of the role played by Zn^{2+} for PTE between this study and previous findings are to be resolved with further investigations. Although PTE seems to be a very hardy enzyme by most standards, quick loss of PTE activity in the simulated continuous Co^{2+} free flow system suggested that direct application of PTE for waste treatment may require the presence of Co^{2+} to maintain enzyme stability. Previous studies (Havens and Rase, 1993; Lejeune and Russell, 1996) suggested that physical or chemical modifications of PTE could also be an option to enhance its stability in the presence of adverse waste constituents. Apparently, studies examining effects of other waste components on PTE will also be important to evaluate its applicability for practical pesticide waste treatment.

ACKNOWLEDGEMENTS

Support by the Environmental Engineering Program of the National Science Foundation (Grant BES-9702603) directed by Dr. Edward Bryan is gratefully acknowledged.

REFERENCES

Benning, M.M., Kuo, J.M., Raushel, F.M. and Holden, H.M., Biochemistry. 33, 15001-15007 (1994).

Benning, M.M., Kuo, J.M., Raushel, F.M. and Holden, H.M., Biochemistry. 34, 7973-7978 (1995).

Bradford, M.M., Anal. Biochem., 72, 248-252 (1976).

Caldwell, R.S. and Raushel, F.M., Appl. Biochem. & Biotech., 31, 59-72 (1991).

Dumas, D.P. and Raushel, F.M., J. Biol. Chem., 265, 21498-21503 (1990).

Dumas, D.P., Durst, H.D., Landis, W.G., Raushel, F.M. and Wild, J.R., Arch. Biochem. Biophys., 277, 155-159 (1990).

Grice, K.J.; Payne, G.F. and Karns, J.S., J. Agric Food Chem. 44, 351-357 (1996).

Havens, P.L. and Rase, P.F., Ind. Eng. Chem. Res., 32, 2254-2258 (1993).

Hong, S. and Raushel, R.M., Biochemistry., 35, 10904-10912 (1996).

Karns, J.S., Smith, J.M., Payne, G.F. and Lumpkin, J.A., Biotechnol. & Bioengineering., 39, 741-752 (1992).

Lejeune, K. E. and Russell, A.J., Biotechnol. & Bioengineering., 51, 450-457 (1996).

Mulbry, W.W. and Karns J.S., J. Bacteriol., 171, 6740-6746 (1989).

Mulbry, W.W. and Karns, J.S., Appl. Environ. Microbial., 55, 289-293 (1989).

Munnecke, D.M., Appl. Environ. Microbiol., 32, 7-12 (1976).

Munnecke, D.M., J. Agric. Food Chem., 28, 105-111 (1980).

Omburo, G.A., Kuo, J.M., Mullins, L.S and Raushel, F.M., J. Biol. Chem., 267, 13278-13283 (1992).

Sawyer, T.W., Weiss, M.T., Boulet, C.A. and Hansen, A.S., Fundam. Appl. Toxicol., 20, 348-354 (1991).

Segel, I.H., "Biochemical Calculations" John Wiley & Sons Inc. New York, NY (1976).

Shelton, D.R. and Karns, J.S., J. Agric. Food Chem., 36, 831-834 (1988).

Silva, J.R. and William, R.P., "The Biological Chemistry of the Elements" Oxford University Press, London (1991).

Tuovinen, K., Kaliste, K.E., Raushel, F.M. and Hanninen, O., Fundam. Appl. Toxicol., 23, 578-584 (1994).

Vanhooke, J.L., Benning, M.M., Raushel, F.M. and Holden, H.M., Biochemistry., 35, 6020-6025 (1996).

Received: January 26, 1998