COMPATIBILITY OF CW AGENT DEGRADING ENZYMES WITH DISINFECTANTS AND FOAMS

Tu-chen Cheng[1], Vipin K. Rastogi[1], Joseph J. DeFrank[1] and Ilona Fry[2]

1. **The Biotechnology Team, R&T Directorate, U.S. Army-ECBC, APG, MD 21010**
2. **GEO-Centers, Inc., PO Box 68, Gunpowder Branch, APG, MD. 21010**

ABSTRACT

Over the years our group developed a non-corrosive, non-toxic, environmentally safe and user-friendly chemical agent decontaminant (advanced catalytic enzyme system - ACES) in a dry powder form. The ACES is capable of hydrolyzing/detoxifying organophosphorus (OP)-based neurotoxic chemical warfare (CW) agents, i.e. G-type, V-type, and related OP based hazardous industrial materials. Bacterial enzymes, OPH (V- and G-type and pesticides), OPAA (G-type) and squid DFPase (G-type) are the three key biocatalysts capable of detoxifying toxic materials. Retention of catalytic activity of ACES in the presence of several water-soluble and biodegradable commercial fire-fighting agents/foams, degreasers, and laundry detergent, provides effective and efficient decontamination not just for large areas, but also for fire suppressing. In addition, the formulation affords personnel decontamination in skin cream/lotions, and personnel protection through enzyme-impregnated clothing and filters. Here, we report the retention of catalytic activity of ACES in commercial disinfectants (Biocidal ZF® and EcoTru®). Over 50% enzyme activity was retained in the presence of Biocidal ZF®. Whereas a 40% enzyme activity was detected in the presence of EcoTru®, over 65-85% enzyme activity was retained when enzymes were protected using dendritic polymers. Biological decontamination experiments with biological warfare agent (BWA) surrogates showed varying degrees of bactericidal activity (2-10 log kill) when these disinfectants and several commerical foams were tested individually. These commercial compounds were compatible with the ACES enzyme activity. Our findings are consistent with the concept that ACES can be incorporated into commercial disinfectants with a real potential for developing broad range decontamination systems for CBW agents.

INTRODUCTION

Organophosphorus (OP) compounds, including neurotoxic V-type and fluorinated G-type chemical warfare (CW) agents, are extremely toxic acetylcholinesterase (AChE) inhibitors. In the past decade, enzymes detoxifying these inhibitors have been found in both prokaryotes and eukaryotes (1). Among them, three major enzymes, the bacterial *Alteromonas* organophosphorus

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acid anhydrolase (OPAA) *Pseudomonas*/*Flavorbacterium* organophosphorus hydrolase (OPH), and squid *Loligo vulgaris* diisopropylfluorophosphatase (DFPase) are the best characterized enzymes against CW agents. Both OPAA and DFPase have been found to exhibit high levels of activity for detoxifying the G-type nerve agents (2-3), whereas OPH is quite unusual for its ability to hydrolyze V-type, G-type agents and pesticides (4-5). Although the natural function of OPH and DFPase are still unknown, sequence and biochemical analyses of OPAA genes and related enzymes revealed that these enzymes are prolidases, a type of dipeptidase cleaving X-Pro dipeptide bonds with a prolyl residue at the carboxyl terminus (6-7).

The use of enzymes for the decontamination of CW agents has been a subject of study for over 50 years. The enzyme-based decontamination system not only provides rapid removal of CW agents, but is also environmentally safe and could potentially provide effective and efficient decontamination not just for large areas, but also for cleanup operations such as resulting from a possible terrorist incident and chemical agent handling during demilitarization and storage.

The large-scale production of CW agent-degrading enzymes is the first key step in the development of a new generation enzyme-based decontamination system. The genes for the CW agent degrading enzymes OPAA, OPH, and DFPase all have been cloned and sequenced, and can be produced in large quantity with over-expression clones. Similar to commercial laundry detergents containing different enzymes, the incorporation of these enzymes and fire-fighting material in a catalyst-based decontaminant, was referred to as the Advanced Catalytic Enzyme System (ACES). It was developed by the Biotechnology Team at the US Army ECBC. The fire fighting property of ACES is due to the inclusion of ColdFire®, which also enhances/stabilizes the enzymatic activity. Here, we report on the incorporation of various commercial disinfectants or anti-microbial compounds into the CW degrading enzyme/fire-fighting formulation.

EXPERIMENTAL METHODS

Preparation and Purification of Enzymes: Production of recombinant OPAA and OPH was previously described (2,3). The purification of OPAA (1-3) and OPH (6) from recombinant cells was performed similar to those procedures described earlier. DFPase was obtained from BioCatalytics Inc. (Pasadena, CA).

OPAA, OPH and DFPase Assays: OPAA, OPH and DFPase assays were conducted with a fluoride electrode attached to a Corning 355 pH/ion analyzer. Fluoride release rates were followed until a stable release rate was observed over a period of several minutes. The reaction solution contained 0.5% ammonium carbonate, 0.1 mM $MnCl_2$, 3 mM substrate, disinfectant or foam as indicated, and 1-5 µl of enzyme sample or enzyme matrices in a total volume of 2.5 ml. For each assay, the substrate was added last. Assays were run at 25°C in a temperature-controlled vessel with stirring. The rate of fluoride release was corrected for substrate spontaneous hydrolysis under identical conditions. One unit (U) of enzyme catalyzes the release of 1.0 µmole of F^- per minute at 25°C. Specific activity is expressed as U per mg of protein. Protein concentrations were determined using Pierce Coomassie protein assay reagent with bovine serum albumin to generate a standard curve.

Bactericidal testing

(A) *Bacillus anthracis* (vegetative cells): *Bacillus anthracis* NH1-Δ1, a non-pathogenic strain, was grown overnight at 37°C, 220 rpm in 7 ml Oxoid Nutrient Broth #2 (NB#2). Next morning, this culture was used to inoculate 7 ml of NB#2 and was grown to early log phase by monitoring the growth turbidometrically at 600 nm. One ml of this early log culture was transferred to six 1.5 ml microtubes, centrifuged at 5400xg, 24°C, 2', and each cell pellet washed once with phosphate-buffered saline (PBS). The cells were re-suspended in 50 µl PBS and 0.5 ml of the treatment solution added. After 30 minutes at room temperature, 0.8 ml of PBS was added to each tube and the cells washed and spun down at 5400xg, for 1 minute. The pelleted cells were washed 4 more times with 1 ml PBS. The cells were re-suspended in 0.4 ml PBS, and 0.1 ml of the serially diluted cells was spiral plated on NA#2 plates with an Autoplate 4000 (Spiral Biotech, Norwood, MA). The plates were incubated overnight at 37°C. Colonies were counted using the Spiral Biotech Q-count apparatus.

(B) *Francisella philomiragia* and *Yersinia intermedia*: *Francisella philomiragia* (ATCC 25015) and *Y. intermedia* (ATCC 33647) were inoculated into 5 ml of Difco Brain Heart Infusion Broth (BHIB) and incubated overnight at 37°C, 200 rpm. A 0.6 ml aliquot of the overnight culture was harvested at 5400xg, 2' and washed once in 0.8 ml of PBS. The cells were resuspended in 50 µl PBS and 0.5 ml of the treatment solution added/tube. The cells were treated, washed, diluted, plated and counted as described above for *B. anthracis,* except the plating medium for these cells was Brain Heart Infusion Agar (BHIA) and the inoculated plates were incubated for 1-2 d at 37°C before counting colonies.

RESULTS AND DISCUSSION

Production of OPAA, OPH and DFPase

A focus of current decontamination efforts is to develop systems that are rapid and effective in detoxifying CB agents in large areas such as equipment, vehicle, facilities, or spills that may occur at stockpile installations, and for cleanup operations resulting from a possible terrorist incident. The use of non-toxic catalytic enzymes offers obvious advantages for decontamination. The catalyst-based decontaminant containing fire-fighting material, referred to as the Advanced Catalytic Enzyme System (ACES), developed by Biotechnology Team of US Army Edgewood Chemical Biological Center has a dual use role both as a CW agent decontaminant and a fire suppressant (8). It does not impact the effectiveness of the equipment, the environment, or the personnel being decontaminated, and it minimizes the logistical impact on operations.

OPAA, OPH, and DFPase, the key enzymes of ACES, are capable of rapidly detoxifying a wide range of nerve agents. The advent of the ACES depends on the large-scale production of these enzymes and economically feasibility. We have cloned the OPAA and OPH encoding genes into high-expression vectors and developed a recombinant cell line derived from *Escherichia coli*. In addition, we have achieved long-term (over 2 years) stability of the recombinant enzymes, OPH, OPAA in the dry powder form (8-9). Both recombinant OPH and

OPAA enzymes are in the process of commercialization for large-scale production by Genencor International Co. (Rochester, NY). Recombinant DFPase powder is currently produced by Roche Company (Basel, Switzerland) and is available in North America through BioCatalytics Inc. (Pasadena, CA).

Compatibility of Commercial disinfectants and foams with CW degrading enzymes

The ACES has a dual use role both as a decontaminant and a fire extinguisher due to the inclusion of ColdFire® (FireFreeze Worldwide, Piscataway, NJ). ColdFire® is a fire extinguishing agent derived from a variety of plant extracts that combine rapid fire knockdown, an ability to remove heat, and environmental safety. The CW-degrading enzymes, OPH, OPAA and DFPase, were shown to retain high level of activity in ColdFire® and a variety of fire-fighting foams, degreasers, laundry detergent, skin lotion, or other matrices (9-11). The initial ACES formulation is mainly for chemical agent decontamination. ColdFire® was not known to have any bactericidal activity.

In searching for compatible BW agent decontaminants to be used in the ACES formulation, several commercial disinfectants and anti-microbial agents and foams (Table 1) were evaluated for their bactericidal or sporicidal activity against non-pathogenic *B. anthracis* cells and other simulants. Two such products, Biocidal ZF® and EcoTru®, were chosen for their significant broad-range disinfectant/anti-microbial activity. Commercial foams such as Easy Foam and F-500 that may be incorporated into the ACES formulation as additives were also evaluated. The effect of these products on the catalytic potential of OPAA, OPH, and DFPase were tested. In the presence of Biocidal ZF® alone, OPAA, OPH, and DFPase retained 94, 52, and 75% enzymatic activity (Figure 1). Adding 0.5% ammonium carbonate in Biocidal ZF reduced each enzymatic activity almost by half. Previously, we have demonstrated that ammonium carbonate proved to be a superior buffer for these enzymes. It not only enhanced but also stabilized their enzymatic activities. Similarly, OPAA, OPH, and DFPase also required ammonium carbonate to stabilize their enzymatic activities in the presence of Easy Foamor F-500 foam (data not shown). Under the same condition, a reduction of over 85% enzymatic activity of these enzymes was found in the presence of EcoTru® disinfectant (Figure 2). Incorporation of 0.5% ammonium carbonate restored their enzymatic activity by ~40%. Encapsulated OPAA, OPH, and DFPase with a C18-dendritic polymer (provided by Dr. Ray Yin of ANP Technologies, Inc. Aberdeen, MD) greatly improved their enzymatic activity by 70-80% in the presence of EcoTru®. The findings are consistent with the idea that the polymer has stabilizing capability on these enzymes.

The stability of OPAA, OPH, and DFPase in the presence of these disinfectants and foams was determined at room temperature, and the results are shown in Figure 3. Except in the presence of Biocidal ZF® only, EcoTru®, Easy and F-500 foams were all buffered with 0.5% ammonium carbonate. Following 24 hours incubation, full enzymatic activity of C18-dendritic polymer protected enzymes were retained in the presence of EcoTru®. Approximate 50% original enzymatic activity was retained in the presence of Biocidal ZF®. Whereas a 40-60% reduction of original OPAA and OPH activity was found in the presence of Easy Foam and F-500 foam, most their enzymatic activity was retained with F-500 foam (~40%) and a significant reduction of activity was observed with Easy Foam (10-20%) following 24 hours incubation. It

is interesting to note that around 100% and 60% of DFPase activity was stabilized in the presence of either Easy Foam or F-500 foam during 24 hours period. These findings point to the stabilizing capability of these disinfectants and foams.

Anti-microbial activity of commercial disinfectants and foams

The commercial disinfectants and foams listed in Table 1 were tested for their anti-microbial activity against several avirulent bacteria. *Bacillus anthracis* (*B. anthracis* NH 1-Δ1), was used as a surrogate for the anthrax-causing parental strain, *Yersinia intermedia* was used as a surrogate for *Y. pestis*, the causal agent of plaque, and *Francisella philomiragia* was used as surrogate for *F. tularensis*, the causal agent of tularemia. As shown in Figure 4, EcoTru®, Easy Foam, and Biocidal ZF® show a 9-10 log kill of *Y. intermedia*, and *F. philomiragia.* For anti-microbial activity against *B. anthracis* NH 1-Δ1, Biocidal ZF® was more effective with a 7 log kill whereas EcoTru® and Easy Foam had only a 2-3 log kill. F-500 foam has 10 log kill against *F. philomiragia* and 2-3 Log kill against *B. anthracis* NH 1-Δ1, but it has no anti-microbial activity against *Y. intermedia.* Previously, our results demonstrated that one of the quaternary ammonium compounds, dodecyldimethyl (3-sulfopropyl) ammonium hydroxide (DDSAH; Aldrich, WI), greatly enhance (10-20%) and stabilize OPAA, OPH, and DFPase activity(12). Quaternary ammonium compounds are known to frequently possess anti-microbial activity. At a concentration of 1 mg/ml of DDSAH, result showed a greater than 4 log kill of *B. anthracis* NH 1-Δ1. All of these commercial products have no sporicidal activity against *B. anthracis* NH 1-Δ1 spores.

CONCLUSION

A number of commercial disinfectants, foams, and quaternary ammonium compounds known to have significant disinfectant/anti-microbial activity were evaluated for their compatibility with CW agent degrading enzymes. The best candidates currently under development will be those products that can be incorporated in the ACES decontamination system. Such a formulation not only provides a medium to decontaminate BW agent, but also assists in solubilization of the CW agents for enzyme action. Our findings are consistent with the idea that incorporating CW degrading enzymes into disinfectants provides a real impetus for broadening the efficacy of ACES against both CW and BW agents.

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Table 1. Commercial Disinfectants and Foams Used for Evaluating CB Decontamination Compatibility.

Name	Source	Characteristics	Concentration used
EcoTru	EnviroSystems Inc. (San Jose, CA)	Bactericidal-virucidal-fungicidal-tuberculocidal	100%*
Biocidal ZF	WAK-CHEMIE (Steinbach, Germany)	Bactericidal-virucidal-fungicidal-sporicidal	100%
Easy Foam	Enviro Foam Tech. Huntsville, AL	Fire-fighting foam	100%*
F-500	Hazard Control Tech. (Fayetteville, GA)	Fire-fighting foam	0.5%*

***Final concentration of 0.5% ammonium carbonate was added for CB agent testing.**

Figure 1. Effects of Biocidal ZF on the Activity of Different Enzymes

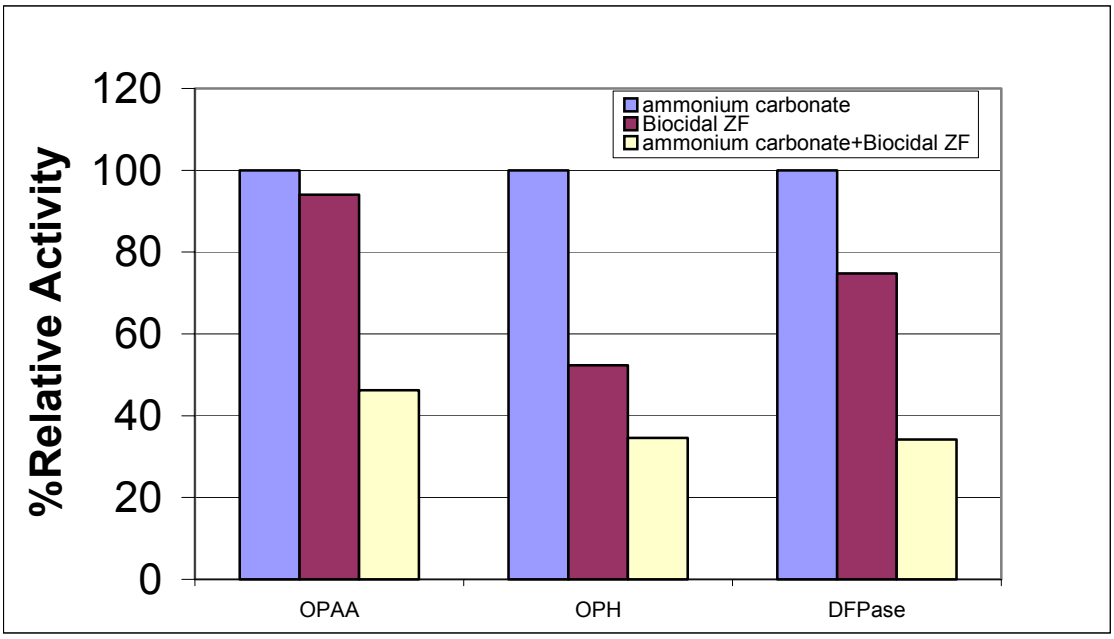

Figure 2. Effects of EcoTru on Different C18-Dendritric Polymer-protected Enzymes

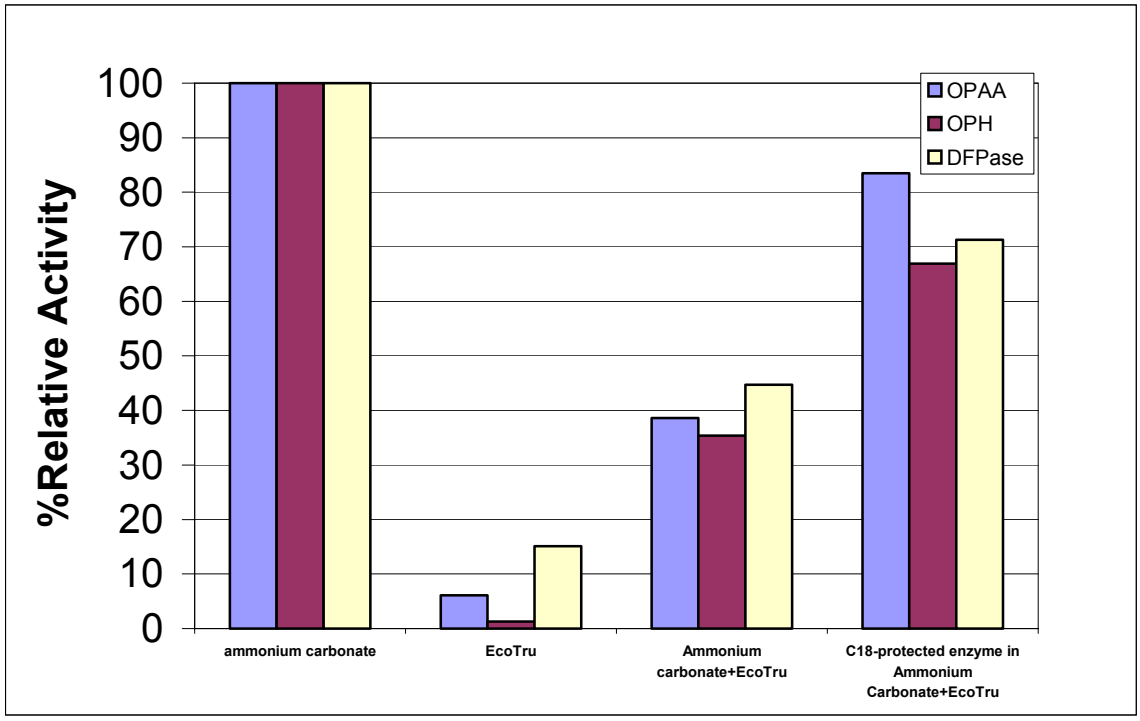

Figure 3. Compare Stability of OPAA, OPH, DFPase activity against DFP in different formulations
1: ammonium carbonate;
2: ammonium carbonate+EcoTru;
3: Biocidal ZF
4: ammonium carbonate+Easy Foam;
5: Ammonium carbonate+F-500
***C18-dendritic polymer-protected enzymes were used.**

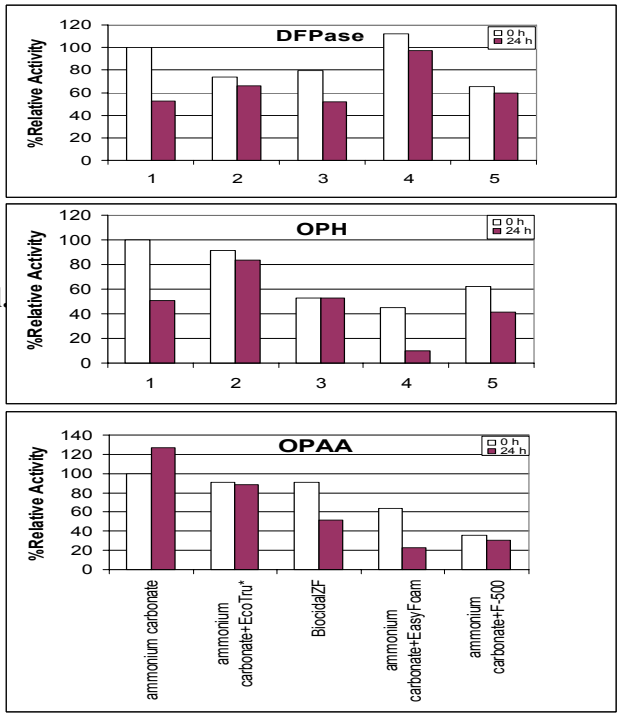

Figure 4: Log Kill (CFU/ml) of *Bacillus anthracis* NH1-Δ1, *Francisella philomiragia,* and *Yersinia intermedia* after 30 minute treatment with different commercial preparations.