Bactericidal Activity of the Myeloperoxidase Antimicrobial Enzyme System Against Vegetative and Spore Forms of *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus subtilis*

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S. Woodhead[1], G. Hill[2], S. Valvani[2], O. Abril-Horpel[2], W. Haag[2], S. Becquerelle[2], **G. A. Denys[3]**
[1]Ricerca Biosciences, Concord, OH, [2]ExOxEmis, Inc., Little Rock, AR, [3]Clarian Health Partners, Inc., Indianapolis, IN

Exoxemis

Contact Information
www.exoxemis.com

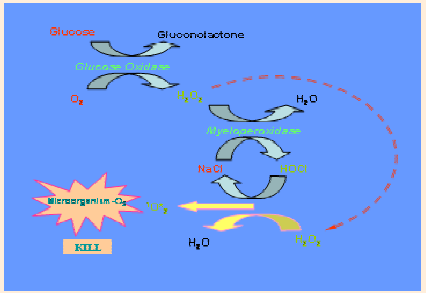
REVISED ABSTRACT

Background: The emergence of new infectious diseases, rise in antibiotic resistance, and threat of bioterriorism and biowarfare has prompted the development of alternative treatment options to antibiotics. An oxidant generating enzyme system containing myeloperoxidase has been developed with a rapid and broad range of antimicrobial activity (ExOxEmis, Inc., Little Rock, AR). The purpose of this study was to investigate the *in vitro* action of the *myeloperoxidase enzyme system* on vegetative and spore forms of Bacillus species closely related to *Bacillus anthracis*. **Methods:** *Bacillus cereus* ATCC 10987, *Bacillus thuringiensis* ATCC 33679, and *Bacillus subtilis* ATCC 19659 were used in this study. A 1.0 ml volume of enzyme formulation plus vegetative growth or spore suspension at a final target concentration of ~10^7 cfu/spores/ml was tested. Treatment vials were incubated at 37ºC in a dry bath. The enzyme reaction was stopped by the addition of 1% catalase after 0, 15, 30, 60, 90 and 120 min. The contents of each vial were inoculated onto isolation media for quantitative culture and incubated for 24 hours at 37ºC. Colonies were then counted and compared to an enzyme diluent control. **Results:** Time kill studies demonstrated that the extent of kill increased with time of exposure to enzyme formulation. Within 60 minutes of treatment, there was a 3-4 log reduction (>99.99% kill) of viable spores. By 120 minutes of treatment there were no surviving germinating spores. **Conclusion:** The *myeloperoxidase enzyme system* demonstrated significant antibacterial activity against Bacillus spores at 60 minutes (>99.99% kill) and bactericidal activity (100% kill) within 120 minutes. The unique mechanism of action and broad spectrum of kill of the *myeloperoxidase enzyme system* shows promise as an agent for the prevention of anthrax infection as well as other infections, which maybe caused by agents used as a biowarfare weapon.

INTRODUCTION

Myeloperoxidase is a major neutrophil protein which is stored in azurophilic granules and released into the phagosome during phagocytosis. The enzyme uses superoxide anions and hydrogen peroxide generated by the neutrophil respiratory burst to form reactive oxidative species that cause cell damage[1]. A cell free oxidant generating enzyme system containing myeloperoxidase has been developed which exploits this natural antimicrobial system (ExOxEmis, Inc., Little Rock, AR). The mechanism of action of the *myeloperoxidase enzyme system* (Figure 1) involves the use of hydrogen peroxide, generated by a balanced formulation *in situ*, to convert chloride into hypochlorous acid. Once generated, hypochlorous acid either participates in the direct halogenation of target cell components, or reacts with a second hydrogen peroxide molecule to yield singlet oxygen[2,3]. Singlet oxygen, a broad spectrum oxygenating agent, effects its microbicidal action via oxidative destruction of the membrane integrity, oxidative inhibition of the enzymes required for metabolic function, and/or oxidative disintegration of nucleic acids required for reproduction. The half-life of singlet oxygen restricts its reactivity to within a 0.2 micron radius at the surface of the targeted bound pathogen. Previous studies have shown that the *myeloperoxidase enzyme system* is rapidly bactericidal to an extensive range of microorganisms including multidrug-resistant strains[4]. Low mutational frequencies of the *myeloperoxidase enzyme system* indicate a minimal potential for resistance development[5]. The objective of this study was to demonstrate the *in vitro* efficacy of the *myeloperoxidase enzyme system* to kill vegetative and spore forms of genetically related strains of *Bacillus anthracis*.

Figure 1. Mechanisms of action of the *myeloperoxidase enzyme system* generating singlet oxygen.



METHODS

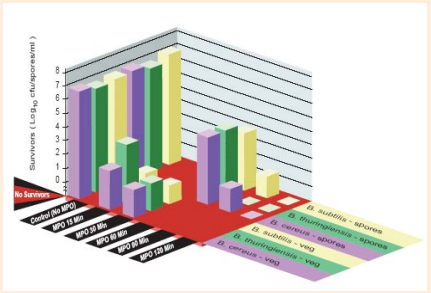
Organisms. *Bacillus cereus* ATCC 10987, *Bacillus thuringiensis* ATCC 33679, and *Bacillus subtilis* ATCC 19659 were used in this study. Strains of *B. cereus* and *B. thuringiensis* were selected because of their genetic relatedness to *Bacillus anthracis*[6].

Spore preparation. All strains were freshly grown on BBL trypticase soy agar (TSA, Becton Dickinson Microbiology Systems, Cockeysville, MD) from freeze-dried stocks and incubated in air at 37°C for 24 h. Isolated colonies were spread onto TSA plates to produce a lawn of growth and incubated at 37°C for 5 days. Cells were then harvested in 3-4 ml of 50% ethanol per plate and stored overnight. One ml samples were washed and resuspended in buffer.

In vitro activity determination. Cell suspensions of vegetative growth were prepared by the shake flask method to achieve late log to early stationary phase growth. Spore and vegetative cell suspensions were pelleted and resuspended in buffer to ~10^9 cfu/spore/ml. A 1.0 ml volume of enzyme formulation containing 6 µg/ml myeloperoxidase, plus organism suspension at a final target concentration of ~10^7 cfu/spore/ml was tested. Treatment vials were incubated at 37°C in a dry bath. The enzyme reaction was stopped by the addition of catalase (100 µl) at 15 and 30 min. for vegetative cells and 60, 90, and 120 min. for spore suspensions. At each appropriate time point, quantitative cultures were performed.

Quantitative culture. The contents of each vial were collected and serially diluted in saline. A 100 µl volume of each dilution was transferred to a TSA plate and spread over its surface. Plates were incubated in air for 24 h at 37°C and colonies on each dilution plate was counted manually.

Figure 2. *In vitro* activity of the *myeloperoxidase enzyme system* against vegetative cells and germinating spores of *B. cereus*, *B. thuringiensis*, and *B. subtilis*



Data analysis. The $\log_{10}$ survivors after treatment with the *myeloperoxidase enzyme system* were compared to a diluent control containing no myeloperoxidase, with 10^0 representing 1 survivor. The bactericidal activity of the *myeloperoxidase enzyme system* was also determined with extended treatment time.

RESULTS

- The *in vitro* activity of the *myeloperoxidase enzyme system* against vegetative cells of *B. cereus*, *B. thuringiensis*, and *B. subtilis* demonstrated rapid and potent kill (~6 log) within 30 min. (>99.999% kill) of exposure (Figure 2).
- The *in vitro* activity of the *myeloperoxidase enzyme system* against germinating spores of *B. cereus*, *B. thuringiensis*, and *B. subtilis* demonstrated a 3-4 log reduction (>99.99% kill) within 60 min. of exposure. There were no survivors after 120 min. of treatment (Figure 2).
- The extent of kill among germinating spores of all three Bacillus species was similar, with greater bacterial killing efficacy achieved with increased exposure time to the *myeloperoxidase enzyme system.*

CONCLUSION

- This study demonstrated that the *myeloperoxidase enzyme system* is highly effective *in vitro* against vegetative cells and germinating spores of closely related strains of *B. anthracis*.
- The broad spectrum of activity and unique mechanism of action of the *myeloperoxidase enzyme system* against Gram-positive and Gram-negative bacteria[4] and Bacillus spores may allow for a single-agent against microorganisms used as a biological weapon.
- Additional studies are needed to assess the *in vivo* efficacy of the *myeloperoxidase enzyme system* for the prevention and treatment of infections as a new adjunct or alternative to traditional antibiotics.

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