UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE

MASTER COPY DTIC FILE COPY FOR REPRODUCTION PURPOSES

(2)

AD-A220 256

DTIC ELECTE APR 10 1990 S D

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S): ARO 23523.4-LS
6a. NAME OF PERFORMING ORGANIZATION: Univ. of Massachusetts	6b. OFFICE SYMBOL (If applicable)
7a. NAME OF MONITORING ORGANIZATION: U. S. Army Research Office	
6c. ADDRESS (City, State, and ZIP Code): Amherst, MA 01002	7b. ADDRESS (City, State, and ZIP Code): P. O. Box 12211, Research Triangle Park, NC 27709-2211
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U. S. Army Research Office	8b. OFFICE SYMBOL (If applicable)
9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAAL03-86-K-0068	
8c. ADDRESS (City, State, and ZIP Code): P. O. Box 12211, Research Triangle Park, NC 27709-2211	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification): Isolation and Characterization of Clostridium Perfringens Spore-Lytic Enzymes

12. PERSONAL AUTHOR(S): Ronald G. Labbe

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 6/2/86 TO 3/1/90	March 1990	3

16. SUPPLEMENTARY NOTATION: The view, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy, or decision, unless so designated by other documentation.

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number): Bacterial Spores, Amino Groups, Amidase, Enzymes; Peptidoglycan Hydrolase, Clostridium Perfringens

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Initiation Protein (IP):

IP hydrolysed spore cortical fragments with the release of free amino groups. End group analysis of hydrolysed fragments indicated the presence of N-terminal alanine but no reducing sugars. Molecular weight analysis of IP- and lysozyme-treated fluorescamine-labelled cortical fragments indicated that IP acts only on peptidoglycan chains containing cross-lined peptide subunits. IP failed to hydrolyse a number of nitrophenyl-conjugated glucopyranosides and galactopyranosides. The results indicate that IP is an N-acetylmuramyl-L-alanine amidase.

(Continued on back)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code) / 22c. OFFICE SYMBOL

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

A sensitive fluorescamine assay was developed for spore-lytic enzyme. The substrate was prepared by treatment of cortical fragments with fluorescamine which reacts with amino terminal groups in the peptidoglycan which are not cross-linked, presumably diaminopimelic acid. Treatment of the labelled substrate with lytic enzymes results in the release of soluble fluorescent products which can be easily measured in a basic fluorometer. The assay is very sensitive, inexpensive and reproducible. As little as 1 μg of lysozyme can be detected by this assay.

Peptidoglycan Hydrolase

An intracellular spore-lytic enzyme (SLE) which causes the germination of spores of Clostridium perfringens has been extracted, partially purified, and characterized. Because intracellular SLE was found in vegetable cells as well as sporulating cells and mutant cells blocked at sporulation state 0 of C. perfringens, this enzyme may not be spore specific. An investigation was made of the effect of this enzyme on vegetative cell walls and determined its mode of action.

(Keywords on previous page)

ARO 23523.4-LS

FINAL REPORT (DAAL03-86-K-0068)

1. Participating Scientific Personnel

Dr. Shirley Tang

Dr. Ronald Labbe

2. List of Publications:

a. Tang, S. and R. Labbe. 1987. Mode of action of Clostridium perfringens initiation protein. Annales of the Pasteur Institute 138:597-608.
b. Tang, S. and R. Labbe 1987. Fluorescamine-labelled cortical fragments as a substrate for lysozyme and initiation protein. Journal of Microbiological Methods 6:185-190.
c. Park, K. and R. Labbe. 1989. Artifacts following gold staining of Western-blotted membranes. Analytical Biochemistry 180:55-58.

3. Brief Statement of the Problem

For many years there has been an active interest in the research community on the mechanism by which bacterial spores germinate. Although the chemical stimuli (e.g. nutrients) which stimulate this process have been known for many years an understanding of the biochemical process has been elusive. While most researchers by far have used aerobic spores (Bacillus) we have chosen an anaerobic model (Clostridium). This is because this (1) genus has been neglected (due to difficulty in obtaining sufficient working amounts) and (2) one species, C. perfringens, has an important role in food-borne illness where large numbers of people are served a common meal as one encounters in military feeding situations. We have been working with this species.

The structure of the spore backbone requires that a lytic enzyme be activated during germination in order to allow the core protoplast to be released and resume vegetative cell growth. The question we sought to answer is what role do spore-lytic enzymes play in the germination process of this organism. What is the nature of such enzymes, their location, properties, modes of action, method of activation, etc. From our previous work we were aware of the existence of two such enzymes from C. perfringens which could cause the germination of its own spores. One (a) was associated with the spore itself (spore-associated spore-lytic enzymes). A second (b) was excreted by the vegetative cell (Initiation Protein) a the third (c) was associated with the cell (peptidoglycan hydrolase).

90 [illegible] 30

4. Summary of Results

a. Spore-Associated Spore-Lytic Enzymes

Our work with this enzyme was completed and reported as part of a previous ARO contract (DAAG 29-83-K-0064).

b. Initiation Protein (IP)

IP hydrolysed spore cortical fragments with the release of free amino groups. End group analysis of hydrolysed fragments indicated the presence of N-terminal alanine but no reducing sugars. Molecular weight analysis of IP- and lysozyme-treated fluorescamine-labelled cortical fragments indicated that IP acts only on peptidoglycan chains containing cross-lined peptide subunits. IP failed to hydrolyse a number of nitrophenyl-conjugated glucopyranosides and galactopyranosides. The results indicate that IP is an N-acetylmuramyl-L-alanine amidase.

As part of this work we developed a sensitive fluorescamine assay for spore-lytic enzyme. The substrate was prepared by treatment of cortical fragments with fluorescamine which reacts with amino terminal groups in the peptidoglycan which are not cross-linked, presumably diaminopimelic acid. Treatment of the labelled substrate with lytic enzymes results in the release of soluble fluorescent products which can be easily measured in a basic fluorometer The assay is very sensitive, inexpensive and reproducible. As little as 1 µg of lysozyme can be detected by this assay.

c. Peptidoglycan hydrolase

We have extracted, partially purified, and characterized an intracellular spore-lytic enzyme (SLE) which causes the germination of spores of Clostridium perfringens. Because intracellular SLE was found in vegetative cells as well as sporulating cells and mutant cells blocked at sporulation stage 0 of C. perfringens, this enzyme may not be spore specific. Therefore, we investigated the effect of this enzyme on vegetative cell walls and determined its mode of action.

The cell walls were prepared from the [illegible] organism. The effect of SLE on vegetative cell walls was determined by observing loss in absorbance when SLE was incubated with cell wall fragments at 45°C. We found that SLE acted on the cell walls to release free amino groups. Release of free amino groups were detected by dinitrophenylation. The amount of amino groups released increased with incubation time and correlated with a decrease in turbidity of the cell wall fragments. However, no reducing sugars were detected. Because this enzyme was effective against vegetative cell walls, the enzyme should be termed a peptidoglycan hydrolase (PGH) instead of a SLE (even though it also acts on spore cortex). Results also showed that PGH is either a peptidase or amidase but not a N-acetyl--glucosaminidase or muramidase. We also found that cell walls treated only with sodium dodecyl sulfate (SDS) were better substrate than walls treated with SDS plus trichloroacetic acid. The latter supposedly removes teichoic acid from bacterial walls.

d. Other Work

As part of our overall research efforts we also made an important observation, i.e., artifacts associated with Western blotting of proteins. We found that when proteins are separated by sodium dodecyl sulfate -polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose membranes, and stained with colloidal gold, artifact bands can appear. These have molecular weights of 58,000, 63,000 and 65,000. Their appearance is due to oxidized dithiothreitol used to denature the proteins prior to electrophoresis. The artifact bands could be avoided by the addition of excess iodoacetamide to the denatured protein solution. Supplies for this work were obtained in part from ARO contract funds.