ISOLATION, PURIFICATION AND CHARACTERIZATION OF *BACILLUS THURINGIENSIS* δ-ENDOTOXIN ACTIVE AGAINST MOSQUITO LARVAE

BY

FRED A. W. WAMUNYOKOLI, BSc (Hons),
UNIVERSITY OF NAIROBI

A thesis submitted in partial fulfilment for the degree of Master of Science in the University of Nairobi.

1992

DECLARATION

I, Fred Alex Wafula Wamunyokoli, hereby declare that this thesis is my original work and the work here has not been submitted to any other university.

FRED A. W. WAMUNYOKOLI
CANDIDATE

This thesis has been submitted for examination with our approval as supervisors

DR. ELLIE O. OSIR
PRINCIPAL SUPERVISOR

DR. JAMES O. OCHANDA
UNIVERSITY SUPERVISOR

DR. NORAH K. OLEMBO
CHAIRMAN,
BIOCHEMISTRY DEPARTMENT

ACKNOWLEDGEMENTS

I am greatly indebted to the German Academic Exchange Service (DAAD) for awarding me the scholarship and to the Director of the Internation Centre of Insect Physiology and Ecology (ICIPE), Prof. Thomas R. Odhiambo for allowing me to use their research facilities at Duduville.

I am profoundly grateful to my principal supervisor, Dr. Ellie O. Osir, for his wise counsel, guidance and constructive criticism throughout the course of this work. I would also like to express my deep appreciation to my other supervisors, Dr. James O. Ochanda and Dr. Norah K. Olembo for their invaluable suggestions given throughout the study.

Special thanks goes to the entire staff of Chemistry and Biochemistry Research Unit (CBRU), especially the Biochemistry staff for their invaluable assistance. My gratitude goes to Mrs. M. Oketch, Dr. M. Imbuga, Dr. M. Vundla, Mr. V. Labongo, Mr. F. Oduol, Mr. D. Ogoyi, Mrs. E. Kenya and my colleagues Gabriel Magoma and Vincent Ochieng' for their able and useful assistance, discussions and encouragement during my research work.

I am deeply grateful to my parents, brother and sisters for their untiring patience, support and encouragement throughout my postgraduate course. This thesis is dedicated to my beloved late mother, Selinah.

CONTENTS

CHAPTER PAGE

ABBREVIATIONS

BCA	-	Bicinchoninic acid
BSA	-	Bovine serum albumin
B.t.	-	*Bacillus thuringiensis*
Con-A	-	Concanavalin-A
DTT	-	Dithiothreitol
FITC	-	Fluorescein isothiocyanate
MFB4/2	-	Solubilized protoxin active against *Chilo partellus*
MPM	-	Modified peptonized milk
NaBr	-	Sodium bromide
Na_2CO_3	-	Sodium carbonate
PAGE	-	Polyacrylamide gel electrophoresis
PAS	-	Periodic acid Schiff reagent
PBS	-	Phosphate buffered saline
SDS	-	Sodium dodecyl sulphate
TCA	-	Trichloroacetic acid
TIKI	-	Solubilized protoxin active against *Glossina* spp.
Tris	-	Tris-(Hydroxy methyl)-amino methane
u.v	-	Ultraviolet
v.v	-	Volume by volume
w.v	-	Weight by volume

UNIT ABBREVIATIONS

α	-	Alpha
β	-	Beta
°C	-	Degree centigrade
cm	-	Centimeter
δ	-	Delta
g	-	Gram
g	-	Centrifugal force
h	-	Hour
KD	-	Kilodalton
M	-	Molar concentration
mA	-	Milliamperes
MD	-	Megadalton
mg	-	Milligram
min	-	Minute
ml	-	Millilitre
mM	-	Millimolar
M_r	-	Molecular weight
ng	-	Nanogram
nm	-	Nanometer
pH	-	-Log. hydrogen ion concentration
sec	-	Second
µg	-	Microgram
µl	-	Microlitre

SUMMARY

During the past decade, growing public awareness of the impact of pesticides on food and enviromental safety has significantly affected the pest control industry. Several organizations (scientific, consumer, enviromental and governmental) have called for more severe restrictions on the use of toxic chemicals and increased funding for the development of alternative pest control methods (Gelernter, 1990). This has been a major stimulus for renewed interest in the use of microbial control agents, which have an excellent safety record and maybe produced using renewable raw-materials. Many of these agents can be integrated with chemical and other pest management techniques. Among the microbial agents that offer great potential in this respect is *Bacillus thuringiensis* (*B.t.*) (Davidson and Sweeney, 1983). *B.t.* based products account for 90-95% of the total bio-pesticide market (Feitelson *et al.*, 1992). Worldwide sales of *B.t.* products have grown from $24 million in 1980 to $107 million in 1989. Annual sales are forecast to expand at a rate of 11% reaching $300 million by the year 1999 (Feitelson *et al.*, 1992).

In this study, a newly isolated *B.t.* strain was cultured by shake flask fermentation involving two inoculum stages and a production stage. The δ-endotoxin crystals so produced were isolated by centrifugation on a continous sucrose gradient (40-70%). Analysis of the amorphous protein

crystals by SDS polyacrylamide gel electrophoresis, revealed three major sub-units of molecular weights ~25 KD, ~66 KD, and ~140 KD. Upon solubilization of the crystal under high pH and reducing conditions, the resulting supernatant solution had one major protein sub-unit band (M_r ~21 KD), while the pellet also had one major sub-unit band (M_r ~66 KD). Upon protease treatment of the solubilized protoxin and insoluble protoxin (pellet) fractions using bovine pancreatic trypsin, α-chymotrypsin, insect larval gut homogenates (*Aedes aegypti*, *Chilo partellus*, *Musca domestica*) and partially purified trypsin like protease from *Glossina* spp midgut, no apparent change in the molecular weight of the major proteins was observed. The crystal was shown to be a glycoprotein with high mannose sugar residues as demonstrated by staining with periodic acid Schiff reagent and fluorescein-isothiocyanate conjugated concanavalin-A, respectively. The carbohydrate content of the crystal estimated by phenol sulphuric acid method was 0.023 ± 0.0016%. Double radial immunodiffusion experiments showed that the antisera raised against the M_r ~21 KD and M_r ~66 KD protein sub-units did not cross-react with the solubilized protoxins active against *Glossina spp* (TIKI) and *Chilo partellus/Spodoptera exempta* (MFB4/2), respectively.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

The order Diptera has insects that are vectors of economically important tropical diseases. For example, some species of the genus *Anopheles* transmit *Plasmodium*, the causative agent of malaria. Similarly, the genus *Simulium* transmit *Onchocerca volvulus*, the causative agent of onchocerciasis, while some species of the genera *Culex*, *Anopheles* and *Mansonia* transmit the causative agents of lymphatic filariasis. The World Health Organization (WHO) estimates that 267 million people in 103 countries are infected with malaria. Of these, the estimated mortality is 1 to 2 million per year (WHO, 1991). The number of people considered to be at risk of contracting malaria is 2,100 million worldwide (WHO, 1991). Similarly the WHO estimates that 17.6 million people in 34 countries suffer from onchocerciasis, while an estimated 90 million people in 76 countries suffer from lymphatic filariasis (WHO, 1991).

Several control measures have been used against either the immature aquatic stages, the adult or both stages of the mosquito vector simultaneously. Chemical control has involved the use of paris green (a copper aceto-arsenite) and organo-phosphates, for example, malathion and fenthion. These have been used to control the larval stages of mosquitoes (Service, 1986). On the other hand, residual house spray using organo-phosphate insecticides such as

fenitrothion and malathion has been used against the adult stages (Service, 1986). The use of chemical insecticides in the control of both the immature and adult stages of the mosquito vector has several disadvantages. For example, *Anopheles albimanus* Weid in several Latin American countries, and *Anopheles stephensi* Giles in India have developed resistance to organo-chlorines, organo-phosphates and carbamates (Margalit and Dean, 1985). Secondly, the persistance of organo-chlorine larvicides in soil, water, animal and plant tissues increases their potential as biological pollutants (Service, 1986). Thirdly, the direct cost of developing and producing these petrochemically-derived chemical insecticides is rapidly increasing (Dulmage, 1987).

Because of the reasons cited above, there is an increasing search for alternative strategies which are cost effective and non-pollutive. One such method involves the use of biological agents. Many entomopathogenic micro-organisms have been suggested for use as microbial insecticides for mosquito control. Examples of biological agents being applied or evaluated for the control of mosquito larvae include viruses, protozoa, fungi and bacteria. Examples of viruses that show potential in the control of mosquitoes are the large iridescent viruses and cytoplasmic polyhedrosis viruses (Kelly, 1981; Payne, 1981). Protozoa that are useful candidates for commercial larvicidal development are *Nosema algerae* Vavra and *Vavraia*

culicis. The former is pathogenic to Anophelines, while the latter to Culicines (Davidson and Sweeney, 1983). Six fungi are presently being evaluated as potential mosquito larvicides. These include *Coelomomyces*, *Lagenidium*, *Leptolegnia*, *Culicinomyces*, *Metarrhizium* and *Tolypocladium*. Of the six, considerable progress has been made towards development of *Culicinomyces* as a microbial larvicide in Australia (Davidson and Sweeney, 1983). Several species of mermithid nematodes are known to parasitize mosquito larvae. Of these nematodes, *Romanomermis culicivorax* has been the subject of the most extensive laboratory and field trials. This nematode parasitizes *Culex*, *Aedes* and *Anopheles* among others. Commercial production of this nematode is being undertaken in the United States of America (Finney, 1981; Davidson and Sweeney, 1983). Fish species of the genera *Gambusia*, *Porcilia*, *Sarotherodon* and *Panchax* have been used to control mosquito larvae in South East Asia and Pacific Islands (Service, 1986). The most promising bacteria species that are currently being used or evaluated for mosquito control are *Bacillus sphaericus* and *Bacillus thuringiensis* (*B.t.*). *B.t.* variety *israelensis* is being used as an exclusive pesticide in natural preserves as well as in all large water treatment plants in Israel (Margalit and Dean, 1985). However, for more effective control of insect pest and disease vectors, there is an increasing need to use integrated control programs which combine both biological and chemical control methods.

1.2 *Bacillus thuringiensis*

Bacillus thuringiensis is a ubiquitous soil bacterium that has a worldwide distrubution (Martin and Travers, 1989). The impetus of studying *B.t.* comes from the ability of the bacterium to produce an insecticidal parasporal crystalline inclusion during the sporulation phase of its life cycle. The crystalline inclusions exhibits specific toxicity towards certain susceptible insects. For example, the crystalline inclusions of *B.t.* subspecies *kurstaki* HD-73 are toxic to only lepidopteran larvae while those of *B.t.* subspecies *kurstaki* HD-1 are toxic to both lepidopteran and dipteran larvae. The crystalline inclusions of *B.t* subspecies *tenebrionis* are toxic to only coleopteran larvae (Ellar, 1990). *B.t.* subspecies *israelensis* produces crystals that are highly toxic to larvae of mosquitoes and blackflies (Goldberg and Margalit, 1977), and have proven to be effective against 72 species of mosquito larvae and 22 species of simulium blackfly larvae (Margalit and Dean, 1985). Five unclassified isolates of *B.t.* pathogenic to nematodes have been reported (Gelernter, 1990)

Bacillus thuringiensis was first isolated in 1901 by Ishiwata from diseased silkworm larvae. This isolate was subsequently named *Bacillus thuringiensis* variety *sotto* in current nomenclature (Dulmage, 1981). The name *Bacillus thuringiensis* dates from 1911, when Berliner isolated a crystal-containing organism from diseased floor moth

(Whiteley and Schnepf, 1986). Despite the fact that B.t. was initially isolated from insect cadavars, its preferred habitat is the soil. The methodology used to isolate B.t. from soil samples involves the use of liquid medium containing sodium acetate which selects B.t. from other *Bacillus* spp. (Travers *et al.*, 1987). The classification schemes of B.t. have been based on their morphological, biochemical, insect host range and crystal protein antigen characteristics. However, the universally acceptable scheme is based on comparing antibodies to their flagellar proteins - the "H" antigens (de Barjac, 1981; Dulmage, 1981). Currently, 25 "H" serotypes are known (de Barjac, 1989).

Bacillus thuringiensis undergoes two phases of development during its life cycle. A rapid vegetative phase is followed by a sporulation phase during which the crystalline inclusion and endospore are formed (Fast, 1981). The vegetative cells are gram positive rods (2-5 x 1.0-1.5 µm) that have peritrichous flagella (Luthy *et al.*, 1982; Dulmage, 1987). They divide by binary fission and frequently occur in chains (Dulmage, 1987). At the end of the sporulation phase, the cells lyse releasing the crystal and the endospore. The latter is resistant against extreme enviromental conditions and can preserve its viability over many years (Huber and Luthy, 1981).

Bacillus thuringiensis is a chemoheterotroph that can be cultured using various artificial media. The media should have carbon and nitrogen sources supplemented with mineral

sources of Mg^{2+}, Mn^{2+}, Fe^{2+}, Zn^{2+}, Ca^{2+} and K^{+} if these minerals are absent in the carbon and nitrogen sources (Dulmage, 1989; UNDP/WORLD BANK/WHO, 1990). The optimal δ-endotoxin production occurs at 28° C to 32° C. However, the normal fermentation temperature is 30° C (Dulmage, 1987). Adequate aeration is very important for the successfull fermentation of *B.t.* (Dulmage, 1989).

Several different toxins produced by *B.t.* strains have been described. Alpha-exotoxin (Phospholipase "c") also called lecithinase is secreted by *B.t.* varieties *finitimus* and *alesti*. This water-soluble and heat-labile toxin accumulates during the exponential growth phase and has been found to be toxic to *Plutella xylostella* (Dulmage, 1981 and 1987). Beta-exotoxin (thuringiensin) is a water soluble, heat stable adenosine derivative secreted by *B.t.* varieties *aizawai* and *morrisoni* during their exponential growth phase. It has been found to be toxic be to larvae of several fly species (Dulmage, 1981 and 1987). Gamma-exotoxin is produced by *B.t.* variety *entomocidus* (Dulmage, 1987). Other toxins are "labile toxin" produced by *B.t.* variety *thuringiensis*; water soluble toxin produced by *B.t.* variety *alesti*; "louse factor" produced by *B.t.* variety *kurstaki* and delta-endotoxin produced by *B.t.* variety *israelensis* (Dulmage, 1981 and 1987).

1.3 δ-Endotoxin

The δ-endotoxin of *B.t.*, which is a protoxin, is the major component of the proteinaceous, parasporal crystal. The biosynthesis of the crystal takes place concomitantly with sporulation, starting simultaneously with septum formation in stage II and terminating after sporulation stage IV (Huber and Luthy, 1981). The crystal accounts for between 20-40% of the cellular dry weight (Luthy *et al.*, 1982; Ellar, 1990). The crystalline inclusion(s) deposits either within or outside the exosporium. There is usually one inclusion per cell but there may be two or more (Aronson *et al.*, 1986).

Several techniques for purifying the crystals based on the differences in buoyant density, surface properties and solubility of the crystals and spores have been developed. These include a two phase system (Delafield *et al.*, 1968); density gradient centrifugation in caesium chloride (Fast, 1972); isopynic density gradient centrifugation in Renografin (methylglucamine 3, 5 diacetylamino - 2, 4, 6 triiodobenzoate) (Sharpe *et al.*, 1975); zonal gradient centrifugation with sodium bromide (Ang and Nickerson, 1978) and differential ultracentrifugation through a discontinous sucrose density gradient (Thomas and Ellar, 1983). The crystal is characterically bipyramidal in shape. However, irregular, roundish or spherical (Mikkola *et al.*, 1982) ovoid, cubical, rhomboid, elliptical and parallelogramic shapes have been observed (Abdel-Hameed *et al.*, 1990). The

crystal is held together by hydrophobic interactions and interchain disulphide bonds (Fast, 1981). The crystal is insoluble in water and organic solvents. Dissolution of crystals with retention of toxicity has relied on highly alkaline solutions with or without reducing agents. However, there has been success in dissolving crystals of variety *kurstaki* K-1 at pH 7.8 with full retention of toxicity (Fast and Milne, 1979). The crystal is moderately thermostable, withstanding exposure to short periods at 100° C.

The molecular weight of the native and denatured crystal proteins differs significantly (Huber and Luthy, 1981). The molecular weight of the native crystal varies between M_r ~177 Kilodaltons (KD) and 500 KD (Huber and Luthy, 1981). The precise number and size distribution of the crystal proteins also depends upon the particular strain and the conditions used to dissolve the crystal (which may allow further proteolysis) (Whiteley and Schnepf, 1986). Upon solubilization using various conditions, B.t. variety *israelensis* produces polypeptides of molecular weight between M_r ~26 KD and 135 KD (Tyrell *et al.*, 1981; Thomas and Ellar, 1983; Aronson *et al.*, 1986). *B.t.* subspecies *kurstaki* produces polypeptides of molecular weight between M_r ~55 KD and 135 KD upon solubilization (Thomas and Ellar, 1983; Aronson *et al.*, 1986). *B.t.* subspecies *tenebrionis* produces crystal polypeptides of molecular weights between M_r ~46 KD and 73 KD (Carroll *et al.*, 1989). Digestion of the solubilized protoxin using trypsin or α-chymotrypsin yields

polypeptides of less than M_r ~20 KD to 58 KD (Haider *et al.*, 1986; Ibarra and Federici, 1986). Upon treatment of solubilized protoxin using various gut extracts of susceptible insect larvae, stable protease-resistant toxic core polypeptides of varing molecular weight have been observed (Aronson *et al.*, 1986; Haider *et al.*, 1986 and Knowles *et al.*, 1986)

The carbohydrate content of the crystals detected using various methods ranges from 0-12% (Fast, 1981). The crystals of *B.t.* subspecies *israelensis* have been reported to contain 2.7% sugars consisting of 1.0% neutral sugars and 1.7% amino sugars (Muthukumar and Nickerson, 1987) while the crystals of *B.t.* subspecies *kurstaki* have been reported to contain 5.6% carbohydrate, 3.8% glucose and 1.8% mannose (Bulla *et al.*, 1977). The crystals of *B.t.* subspecies *thuringiensis* have been found to contain 12% carbohydrate, mainly glucose with lesser quantities of mannose, xylose, and arabinose but with no amino sugar (Fast, 1981). Neutral sugars such as glucose, mannose, fucose, rhamnose, xylose (Tyrell *et al.*, 1981) and amino sugars such as glucosamine and galactosamine (Pfannenstiel *et al.*, 1987) have been detected. Amino-acid composition of the crystals of various *B.t.* strains has been determined. High proportions of glutamic and aspartic acid in the crystals have been reported (Tyrell *et al.*, 1981).

Genes which encode insecticidal toxins in the *Bacillus thuringiensis* have been found on plasmids present in these bacteria, although copies of such genes have been found in

the chromosomal DNA of some strains of the bacteria. The number of plasmids ranges from 2 (*B.t.* subspecies *entomocidus*) to 77 (*B.t.* subspecies *finitimus*) per strain (Aronson *et al.*, 1986). The size of the plasmids ranges from ~1.4 Megadaltons (MD) (*B.t.* subspecies *kurstaki* HD-1) to ~180 MD (*B.t.* subspecies *berliner*) (Gonzalez *et al.*, 1981; Aronson *et al.*, 1986). Crystal protein genes have been located in plasmids of various sizes, but in all cases the plasmids are more than 30 MD (Aronson *et al.*, 1986). For example, crystal toxin production in *B.t.* variety *israelensis* requires a single large transmissible plasmid of 75 MD, the absence of which is associated with loss of crystal production and toxicity to mosquito larvae (Gonzalez and Calton, 1984). Plasmid transfer experiments using acrystalliferous *B.t.* variety *israelensis* strain as the recipient, showed that acquisition of the 75 MD plasmid from a crystal-producing donor converted the acrystalliferous strain to a crystal producing strain (Gonzalez and Calton, 1984). Acrystalliferous *Bacillus cereus* have been converted into crystal producers by means of plasmid transfer experiments (Gonzalez *et al.*, 1982). Protoxin genes have been detected exclusively in the chromosomal genes of *B.t.* subspecies *dendrolimus* and *wuhanensis* (Aronson *et al.*, 1986).

The toxicity of δ-endotoxin is highly dependent on the *B.t.* variety and crystal type. It is thought at least two mechanisms could effect such specificity, namely, that

different varieties produce significantly different protoxins and/or that the same protoxin is hydrolysed to unique toxic fragments by different insect species (Dulmage, 1987). Upon ingestion by a susceptible insect, the crystalline inclusions undergo solubilization and activation into toxic fragments by proteolytic processing in the larval midgut which is highly alkaline in the case of Diptera and Lepidoptera and less so in the case of Coleoptera larvae (Ellar, 1990). For several B.t. toxins, specific high affinity binding sites or receptors have been demonstrated on the midgut epithelium of susceptible insects (Hofmann *et al.*, 1988 and Van Rie *et al.*, 1990). This could, at least in part explain the extreme specificity of these toxins. It has been suggested that the binding of the toxin to the receptor is via a lectin-like binding mechanism (Knowles *et al.*, 1984; Muthukumar and Nickerson, 1987). It is thought that the binding of the toxin to the receptor/binding site results in a change in the conformation of the toxin. This change leads to the toxic domain of the toxin to insert into the cell membrane. Oligomerization of the toxin molecules in the cell membrane induces the formation of small non-specific pores (0.5 - 1.0 nm) in the membrane of susceptible cells of the midgut epithelium. The result is a net influx of water and cations or other small molecules. Consequently, the micro-villi present on the luminal surface of the midgut and the midgut epithelial cells swell and lyse. This leads to ion and pH imbalance in the hemolymph resulting in

paralysis of the insect gut followed by body paralysis and death of the larva (Aronson *et al.*, 1986; Hofte and Whiteley, 1989; Gill *et al.*, 1992).

The use of commercial preparations of *B.t.* in the control of insect pests and medical vectors has increased steadily since 1970 (Luthy *et al.*, 1982). Despite this, several disadvantages exist in the use of *B.t.* formulations. Several *B.t.* crystal producing subspecies (*finitimus*, *pakistani*, *dakota* and *indiana*) are not lethal to commonly tested lepidopteran and dipteran larvae (Whiteley and Schnepf, 1986). Secondly, *B.t.* formulations exhibit a narrow host spectrum, hence no single isolate is active against all pest species. Thirdly, *B.t.* exhibit limited field stability. Formulations of *B.t.* spores and crystals applied on foliage are washed off by rain and may be inactivated by sunlight so that only relatively short term protection from pest populations is obtained from single applications. Thus, the formulation must be applied repeatedly for long-term protection (Aronson *et al.*, 1986). Fourthly, the effectiveness of *B.t.* formulations is dependant on their ingestion by target insect. For example, a major problem with use of *B.t.* variety *israelensis* is the requirement for formulations which remain in the feeding zone of the mosquito larvae. In some cases, this feeding zone is just below the water surface while in others suspension at a greater depth is necessary, hence the settling of spores or inclusions (or both) to the bottom of streams and ponds

(Aronson *et al.*, 1986). Fifthly, some insects, for example, the indian meal moth (*Plodia interpunctella*) larvae have developed resistance against commercial spore-crystal formulations of B.t. subspecies *kurstaki* HD-1 (McGaughey, 1985). The diamond-back moth (*Plutella xylostella*) larvae have developed resistance to commercial formula-complex of B.t. subspecies *kurstaki* NRD-12 (Tabashnik *et al.*, 1990). However, the use of B.t. has proven to be a valuable alternative to conventional insecticides due to the following reasons. Due to its high specificity, B.t. is remarkably safe to non-target organisms. Secondly, B.t. is adaptable to many types of formulations, hence its potential to be incorporated into feeding stimulants or baits to increase its effectiveness. Thirdly, there is the probability of producing more potent formulations at reduced cost through the use of locally available raw materials and improved fermentation technology. Fourthly, δ-endotoxin genes from B.t. varieties *kurstaki* and *tenebrionis* have been successfully transferred into crop plants like tomatoes, potatoes, tobbaco and cotton (Gelernter, 1990). Fifthly, there is the high probability of producing strains that have increased activity and a wider toxicity spectrum through molecular biology and genetic engineering techniques (Dulmage, 1987).

1.4 Importance of study

This study is the first we know of that uses a combination of techniques to characterize a δ-endotoxin from a new local isolate of *B.t.* shown to be toxic to mosquito larvae, and provides the necessary background for further investigations of the δ-endotoxins in the bacteria.

Aim and objectives of the study

1.5.1 Overall aim

To establish the culture, isolation, purification, solubilization conditions and characterization of the newly isolated *B.t.* δ-endotoxin active against mosquito larvae.

1.5.2 Specific objectives

(i) Establish the culture conditions of the *Bacillus thuringiensis* strain.

(ii) Establish the conditions for the isolation, purification and solubilization of the crystalline δ-endotoxin.

(iii) Carry out physical-chemical characterization of the protoxin and protease-treated protoxin preparations.

(iv) Carry out immunological studies on the toxin preparations.

et al., 1978). The agar was incubated at 30° C for 24 h in an incubator (LabLine Instruments, Melrose park, USA). The purity of the M.37.2 colonies was monitored by use of Hucker method for Gram stain (Finegold *et al.*, 1978). A thin film of a colony was made on a microscope slide. The film was air dried and then heat fixed for 1 min. This was followed by flooding the film with crystal violet staining reagent (20 g (85%) crystal violet, 100 ml (95% (v.v) ethanol, 1 g ammonium oxalate, 100 ml distilled water) for 1 min. The film was then washed in a gentle indirect stream of running water before being immersed in Gram iodine solution (1 g iodine crystals, 2 g potassium iodide) for 10 sec. The film was rinsed in running water and decolourized using 95% (v.v) ethanol/acetone (1:1) for 20 sec, before being counterstained (2.5% (w.v) safranine O, 95% (v.v) ethanol) for 10 sec. After washing in running water and blotting between Whatman filter paper, the film was examined under oil immersion objective (1000 x). Crystal staining method (Smirnoff, 1962) was also used to check for the presence or absence of crystals. A thin film of the M.37.2 colony was made on a microscope slide. The film was air dried and heat fixed as stated above before staining it with reagent A (1.5 g Amido Black 10B, 50 ml of 98% (v.v) methanol, 40 ml of 96% acetic acid, 40 ml distilled water) for 70 sec. After rinsing in running water, the film was covered with reagent B (30% (v.v) (1 g basic fuschin, 95% (v.v) ethanol, 5 g phenol, 90 ml distilled water), 70% (v.v) distilled water) for 20 sec. The film was washed in running water before blotting using

Whatman filter paper. The film was then examined under oil immersion objective (1000 x). After this purity determination, the bacterial growth was preserved at 4° C.

In the second inoculum preparation, a 500 ml Erlenmeyer flask containing 100 ml of tryptone soya broth was steam sterilized using a model 50/70/1 vertical laboratory autoclave (KSG, Olching, Germany) at 121° C for 15 min. After cooling to room temperature, a loopful of M.37.2. growth from the first inoculum preparation was used to seed the broth under sterile conditions. The broth was incubated on an orbital shaker (LabLine Instruments, Melrose park, USA) set at 200 rpm, 30° C for 16 h. Purity of the broth culture was monitored by use of Gram stain and crystal staining methods. A total cell count of the broth culture was carried out using the pour plate method (Finegold *et al.*, 1978). The broth culture (0.5 ml) was serially diluted in steam sterilized saline (0.85% (w.v) NaCl) (4.5 ml) upto the dilution of 10^{-9}. The mixtures (1 ml) from the 10^{-7} and 10^{-8} dilutions was thoroughly vortexed for 1 min then mixed with nutrient agar (at 50° C) by gentle swirling for 1 min. After the solidification of the agar, the plates were incubated at 30° C in an incubator. The number of colonies in each plate were recorded after 24 h.

The production medium used was modified peptonized milk broth (Ibarra and Federici, 1986). The broth consisted of (Glucose; 10.00 g, Peptone; 10.00g, Yeast extract; 2.0 g, $MgSO_4.7H_2O$; 0.30 g, $MnSO_4.4H_2O$; 0.02 g, $FeSO_4.7H_2O$; 0.02 g, $ZnSO_4.7H_2O$; 0.02 g) in 1000 ml distilled water. The pH of

medium was adjusted to 7.3 using 0.1 M sodium hydroxide before aliquoting 100 ml into, 500 ml Erlenmeyer flasks. The flasks were sterilized by autoclaving using a vertical laboratory autoclave at 121° C for 15 min. After cooling to room temperature, 1.0 ml of the starter culture (second inoculum preparation) was used to seed each flask under sterile conditions. The flasks were incubated on a orbital shaker set at 200 rpm, 30° C for 72 h. The purity, sporulation and lysis of the bacterial cells was monitored by use of Gram stain and crystal staining methods. The biomass of the bacterial growth was determined by obtaining the total yield of the spore/crystal mixture. The total yield was determined by pelleting and washing (3) the spore/crystal mixture (as described in 2.3.1) from the lysed cultures. The mixture was freeze-dried and its weight recorded

2.2.2 Growth Media

Modified peptonized milk broth was evaluated against semi-defined media (Glucose; 3.0 g, Ammonium sulphate; 2.0 g, Yeast extract; 2.0 g, $K_2HPO_4.3H_2O$; 0.50g $MgSO_4.7H_2O$; 0.2 g, $CaCl_2$; 0.08 g, $MnSO_4.4H_2O$; 0.05 g, 1000 ml of distilled water) (Kreig, 1981) and nutrient broth (Glucose; 1.0 g, tryptone; 5.0 g, Yeast extract; 5.0 g, K_2HPO_4; 0.8 g, 1000 ml of distilled water) (Singer and Rogoff, 1968). The purity, sporulation and lysis of the bacterial cells in each medium was monitored by use of Gram stain and crystal staining methods. The biomass of the bacterial growth in

each media were determined as described above. Bioassays were carried out using freeze-dried spore/crystal suspension (~20, 2 and 0.2 ng/ml) obtained from each medium on late third instar *Aedes aegypti* larvae, respectively.

2.3 Isolation, purification and solubilization of the crystal δ-endotoxin

2.3.1 Isolation and purification of the crystals

Spores and crystals were harvested by transferring the culture medium into 250 ml Nalgene tubes and pelleting them by centrifugation (10,000 x g, 10 min, 4° C) in a Sorvall GSA rotor using a model RC-5C Sorvall centrifuge (Du Pont, Delaware, USA). The sporangial debris were removed by washing the pellet three times with distilled water by centrifugation (9,000 x g, 10 min, 4° C) in a Sorvall GSA rotor using a Sorvall centrifuge. The final pellet was resuspended in 6.0 ml of phosphate buffered saline (PBS) (0.005% (w.v) $NaH_2PO_4.2H_2O$, 0.004% (w.v) $Na_2HPO_4.2H_2O$, 0.015% (w.v) NaCl) and kept at 4° C. The crystals were separated from the spores using a modified method of Thomas and Ellar, (1983). Six, 10 ml, 40-70% (w.w) linear sucrose gradient was cast using a gradient maker (Buchler Instruments, USA). The crystal-spore suspension (1 ml) was carefully layered on top of the sucrose gradients using a Pasteur pipette. The gradients were centrifuged (8,000 x g , 30 min, 4° C) in a Beckman SW 41 Ti rotor using a model L8-M Beckman ultracentrifuge (Beckman, Palo Alto, USA). The

crystal interphase was carefully pipetted off using a Pasteur pipette, and washed three times in PBS by centrifugation (10,000 x g, 5 min, 4° C) in a Sorvall SS-34 rotor using a Sorvall centrifuge to remove the sucrose. The pellet obtained from this step was resuspended in PBS (1 ml) and layered onto a second 40-70% (w.w) linear sucrose gradient. The gradient was centrifuged (8,000 x g, 90 min, 4° C) in a Beckman SW 41 Ti rotor using a Beckman ultracentrifuge. The pellet obtained from this step was washed three times in PBS by centrifugation (as for the interphase) to remove sucrose. The purity of the crystals at the end of each step of the isolation procedure was monitored using crystal staining method. The purified crystals were suspended in PBS (1 ml) and stored at -20° C. This purification technique was evaluated against the methods of differential ultracentrifugation using discontinous sucrose gradient (Thomas and Ellar, 1983) and centrifugation using discontinous sodium bromide gradient (Ibarra and Federici, 1986).

2.3.2 Protein estimation

Total protein was determined using Bicinchoninic acid (BCA) assay (Smith *et al.*, 1985). The sample (10 µl) was added to a mixture of BCA working reagent (Pearce, Rockford, USA) (2 ml) (50 parts of reagent A and 1 part of reagent B) and distilled water (90 µl). The mixture was incubated in a waterbath (LabLine Instruments, Melrose park, USA) at 37° C for 30 min. Absorbances were measured at 562 nm wavelength using a model

DU spectrophotometer (Beckman, Palo Alto, USA). Bovine serum albumin (BSA) fraction V (Serva, Heidelberg, Germany) was used as the standard protein.

2.3.3 Solubilization of the crystal

The purified crystals (0.5 mg) were treated with 50 mM Na_2CO_3.HCl pH 9.5 and 10 mM DTT. The mixture was incubated in waterbath at 37° C for 14 h using a modified method of Haider *et al.*, (1986). Insoluble material was removed by centrifugation (4,000 x *g*, 10 min, 4° C) in a model 12 Beckman Microfuge (Beckman, Palo Alto, USA). The insoluble protoxin fraction was washed (4) in PBS by centrifugation (4,000 x *g*, 10 min, 4° C) to remove the Na_2CO_3.HCl and DTT. The solubilized protoxin was dialysed for 6 h in PBS at 4° C to remove the Na_2CO_3.HCl and DTT. Protein estimation was carried out on both the solubilized protoxin and insoluble protoxin fractions as described above. Both fractions were stored at -20 °C.

2.3.4 Protease treatment

The solubilized protoxin was dialysed in 0.1 M Tris-HCl, pH 8.0 at 4° C for 24 h, while the insoluble protoxin fraction was washed (3) in the above buffer by centrifugation (4000 x *g*, 10 min, 4° C). Each of the above samples was treated using bovine pancreatic trypsin and α-chymotrypsin (Serva, Heidelberg, Germany) and partially purified trypsin-like protease from *Glossina* spp midgut (Onyango, Personal

communication). Crude gut homogenates (fifth instar *Chilo partellus*, late third instar *Aedes aegypti* and third instar *Musca domestica*) were prepared by dissecting out the guts, homogenizing (1 min) them in an ice cooled hand homogenizer containing ice cold 0.1 M Tris-HCL, pH 8. This was followed by centrifugation (12,000 x g, 10 min, 4° C) in a Microfuge. The trypsin activity of the supernatant solutions was determined using the chromogenic substrate, α-N-benzoyl-DL-arginine-p-nitroanilide (BApNA) (Serva, Heidelberg, Germany) (Osir *et al.*, unpublished). The assay consisted of 0.1 M Tris-HCl, pH 8.0 (860 µl) and the sample (supernatant solution) (40 µl). The mixture was incubated in a waterbath set at 30° C for 3 min. The reaction was initiated by addition of 2 mM BApNA dissolved in 66% (w.v) dimethylformamide (100 µl). Changes in absorbance were monitered at 410 nm using the spectrophotometer after every min. The amount of substrate hydrolysed was determined from the change in molar extinction at 410 nm (A_{410} = 8800). The protoxins (35 µg) were treated with trypsin or α-chymotrypsin (165 µg). Protoxin (20 µg) was treated with gut homogenates (*Chilo partellus*, 40 µl; *Musca domestica*, 35 µl; *Aedes aegypti*, 20 µl; partially purified trypsin form *Glossina* spp midgut, 10 µl) (volume equivalent to an activity of 5 µM/ml/min). The enzyme/protoxin mixtures were incubated in a water bath at 37° C for 60 min. The enzyme treated samples were kept at 4° C.

2.4 Physical-chemical characterization of the crystal

2.4.1 Electrophoresis

2.4.1.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970). Gradient gels (4-15%) were cast using a gradient maker (BRL, Gaithersburg, USA). The gels were layered with butanol/water (1:1). After polymerization, the butanol was washed off with distilled water and a stacking gel (3% acrylamide) was cast on top of the resolving gel. Samples for electrophoresis were mixed with an equal volume of SDS-PAGE sample buffer (0.13 M Tris-HCl, 20% (w.v) glycerol, 0.002% (w.v) bromophenol blue, 4% (w.v) SDS, 1% (v.v) β-mercaptoethanol, pH 6.8) and boiled for five minutes in a boiling water bath, prior to loading onto the gel. Electrophoresis was carried out using a running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% (w.v) SDS, pH 8.3) at a constant current of 25 mA at room temperature until the tracker dye reached the bottom of the gel. After electrophoresis, the gels were stained for proteins with 0.6% (w.v) Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany) in a solution of acetic acid, methanol and distilled water (in the ratio 9.2 : 40 : 50.8, respectively) overnight. The gels were then treated with several changes of destaining

solution I (acetic acid, methanol, distilled water; 9.2 : 50 : 40.8) for several hours at room temperature. After complete destaining, the gels were immersed in destaining soution II (acetic acid, methanol, distilled water; 7.5 : 5.0 : 87.5) for 6 h at room temperature. Destained gels were stored in 7% (v.v) acetic acid until they were photographed.

2.4.1.2 Molecular weights determination

SDS-PAGE was used to determine the molecular weights of various protein sub-units using protein standards (Bio-Rad laboratories, Richmond, USA). The standards used were; myosin (M_r ~200 KD), lysozyme (M_r ~116.25 KD), phosphorylase b (M_r ~97.4 KD), bovine serum albumin (M_r ~66.2 KD), hen egg white ovalbumin (M_r ~45 KD) bovine carbonic anhydrase (M_r ~31 KD), soyabean trypsin inhibitor (M_r ~21.5 KD) and hen egg white lysozyme (M_r ~14.4 KD). The protein standards were loaded in wells adjacent to the respective samples. After staining the gels with Coomassie Brilliant Blue R-250 and destaining as outlined above, estimates of molecular weights of the protein sub-units was determined from a plot of log. molecular weight versus relative migration of the protein standards.

2.4.1.3 Preparative gel electrophoresis

The solubilized protoxin and insoluble protoxin fractions were separated by 4-15% gradient SDS polyacrylamide gel with stacking gels (3% acrylamide). After staining and destaining the vertical slices of the gels for protein using

Coomassie Brilliant Blue R-250, the gels were cut at parts corresponding to the protein sub-units of M_r ~21 KD and M_r ~66 KD, respectively. The gel slices corresponding to each protein sub-unit were transferred into separate electroelution tubes. The proteins were electroeluted using buffer (25 mM Tris, 192 mM glycine and 0.1% (w.v) SDS, pH 8.3) in a model 422 electroeluter (Bio-Rad laboratories, Richmond, USA) at a constant current of 20 mA for 6 h with stirring at room temperature. The success of the electroelution was ascertained by SDS-PAGE. The samples were dialysed in PBS using a microdialyser (Pierce, Rockford, USA) at 4° C for 24 h to remove some of the SDS. The dialysed samples were concentrated by freeze-drying and stored at -20° C.

2.4.2 Staining for carbohydrates

The presence of covalently-bound carbohydrates was tested by staining 4-15% gradient SDS polyacrylamide gel containing the crystal (50 µg), electroeluted M_r ~21 KD (20 µg) and M_r ~66 KD (50 µg) protein sub-units and lipophorin (*Schistocerca gregaria*) (50 µg) with periodic acid-Schiff reagent (PAS) (Kapitany and Zebrowski, 1973). After electrophoresis the gel was fixed in 12.5% (w.v) trichloroacetic acid (TCA) for 2 h followed by several washes in distilled water for 2 h. The gel was then immersed in 1% (w.v) periodic acid for 2 h. This was followed by several washings of the gel in 15% (v.v) acetic acid for 2 h. The gel was stained overnight with PAS reagent.

This was followed by destaining of gel using several changes of 7% (v.v) acetic acid.

The presence of carbohydrates with high mannose sugar residues was determined by staining with Fluorescein-isothiocyanate (FITC) conjugated Concanavalin-A (Con-A) (Sigma, St. Louis, USA) (Furlan *et al.*, 1979). The crystal was separated on a 4-15% gradient SDS polyacrylamide gel. After electrophoresis, the proteins were electrophoretically transferred onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) as described by Towbin *et al.*, (1979) and Burnette, (1981). The transfer was carried out using a model 2117-250 NovaBlot transfer unit (Pharmacia LKB, Bromma, Sweden), using buffer (39 mM glycine, 48 mM Tris, 0.0375% (w.v) SDS, 20% (v.v) methanol in 1000 ml of distilled water), at a constant current of 125 mA at room temperature for 90 min. After the electrophoretic transfer, the success of the transfer was ascertained by staining with Rouge Ponceau solution (50 mg in 3% (v.v) TCA). The stain was removed by washing in distilled water and then stained with with FITC-Con-A buffer (4 mg of FITC in 4 ml of Con-A buffer (0.05 M NaCl, 0.05 M Tris-HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.0) at room temperature for 15 h. This was followed by destaining in Con-A buffer for over 15 h at room temperature. The membrane was observed in the dark under ultraviolet light (u.v.).

2.4.3 Determination of carbohydrate content

The percentage carbohydrate content of the crystal was determined according to the method described by Dubois *et al.*, (1956). D-mannose sugar (BDH Chemicals, Poole, England) was used as the standard carbohydrate. To freeze-dried crystal powder(~1.0), distilled water (0.4 ml) and phenol (0.4 ml) (2.0 g in 0.5 ml of distilled water) were added. This was followed by addition of concentrated (96%) sulphuric acid (1 ml). After shaking, the tubes were allowed to stand for 30 min at room temperature. Absorbances were then measured at 480 nm. The carbohydrate content of the crystal was estimated from the plot of absorbance versus concentration of the standard carbohydrate.

2.5 Bioassay

Bioassays were carried out using late third instar *Aedes aegypti* larvae provided by the Insect and Animal Breeding Unit (IABU), ICIPE. The samples used in the bioassay were purified crystal, denatured (boiled for 5 min) crystal, solubilized protoxin, insoluble protoxin, and the enzyme treated protoxin preparations, respectively. Ten larvae were transferred into a 250 ml plastic beaker containing distilled water (50 ml) using a Pasteur pipette, taking care to add as little water as possible. The beakers were prepared in duplicate, with an eleventh beaker serving as a control. The samples (20 µg/ml) were added to their respective beakers. Mortality was recorded

every 30 min by counting the dead larvae. All the tests were replicated three times on different days.

2.6 Immunological studies

2.6.1 Production of antibodies

Antibodies were raised against the protein sub-units of M_r ~21 KD and M_r ~66 KD in two different male New Zealand white rabbits. A primer dose (~1.0 mg) was emulsified in an equal volume of Freund's complete adjuvant (Gibco Laboratories, Grand Island, New York, USA). The dose was administered subcutaneously and at 4 different sites on the thighs. After 4 weeks, a booster dose (0.5 mg) in incomplete Freunds adjuvant, was administered. The rabbits were bled 2 weeks later through the main ear artery. To obtain the antisera, the fresh blood was left to stand at room temperature for 1 h for clot formation, then kept overnight at 4° C. The serum was decanted from the clot and then centrifuged (1000 x g, 30 min, 4° C) in a Microfuge. The supernatant solution was pipetted off and 0.1% (w.v) sodium azide added before being stored at -70° C.

2.6.2 Double radial immunodiffusion

Double radial immunodiffusion was carried out as described by Ouchterlony (1968). Molten agarose (1% (w.v) in PBS, with 0.08% sodium azide) was carefully poured onto a glass plate (10 cm x 10 cm) on a level stand and left to solidify. Six wells were punched peripherally around a central well using a

gel punch. This technique was used to check for the presence of antibodies against the antigen and cross-reactivity between the antisera and crystal, soluble and insoluble protoxins, electroeluted sub-units and the solubilized TIKI and MFB4/2 protoxins, respectively. Antisera (10 µl) was placed in the central well and the antigen (20 µg) in the peripheral wells. Diffusion was allowed to take place in a humid chamber at room temperature for 24 h after which the gel was washed in PBS for 4 h to remove the unprecipitated proteins. The gel was dried by blotting with Whatman filter paper before staining with Coomassie Brilliant Blue R-250 and destaining as described above.

CHAPTER 3

RESULTS

3.1 Fermentation studies

The first inoculation gave rise to cream coloured, flat, spreading, rough edged bacterial colonies. The three media were each seeded with 1.0 ml ($2.9 \pm 0.8 \times 10^8$ bacterial cells) of starter culture (**Fig. 1**). The M.37.2 cells in each medium started sporulating 24 h after seeding (**Fig. 2**). Lysis of the sporulating bacterial cells in each medium began 48 h after seeding and the process was virtually complete within 72 h of seeding (**Fig. 3**). Crystal staining of the lysed cells in each culture medium showed that the crystals were spherical to amorphous in shape, while the spores were ovoid in shape. The total yield of the spore/crystal mixture obtained from 1000 ml of lysed cultures from each medium is as shown (**Table 1**). The results of bioassays carried out using spore/crystal mixture (concentrations of ~20, 2, and 0.2 ng/ml) obtained from each of the three different media types, on ten late third instar *Aedes aegypti* larvae are as shown (**Table 2**).

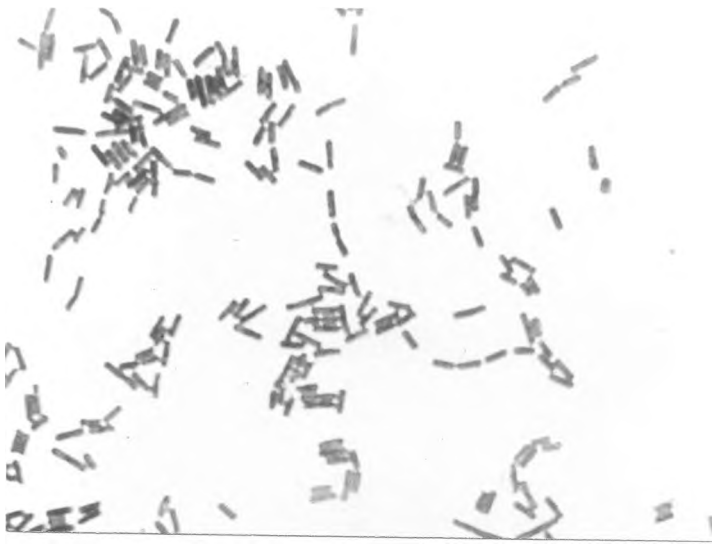


Fig. 2 **Sporulating M.37.2. cells 36 h after seeding.**
The production medium was seeded with starter culture containing M.37.2 cells. After incubating the cultures for 36 h, the sporulating cells were stained using the crystal stain. (4000 x)

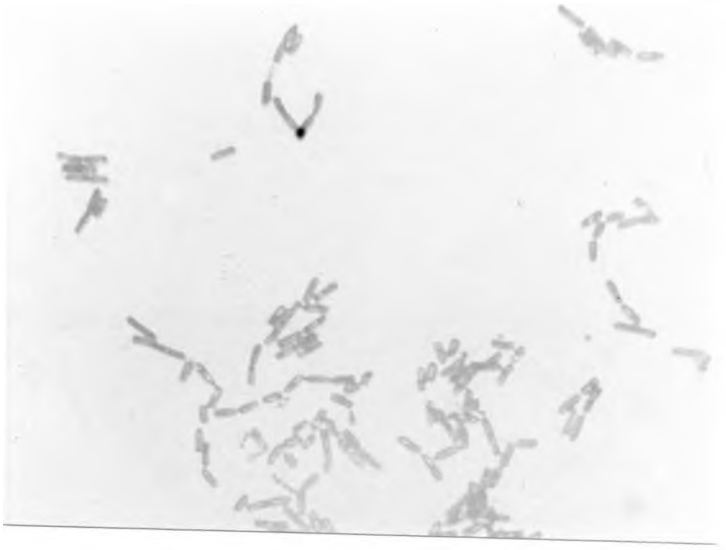


Fig. 3 **Completely lysed M.37.2. cells 72 h after seeding.** The cultured M.37.2 cells virtually autolysed within 72 h of seeding the production medium. The autolysed cells were stained using the crystal stain. (4000 x)

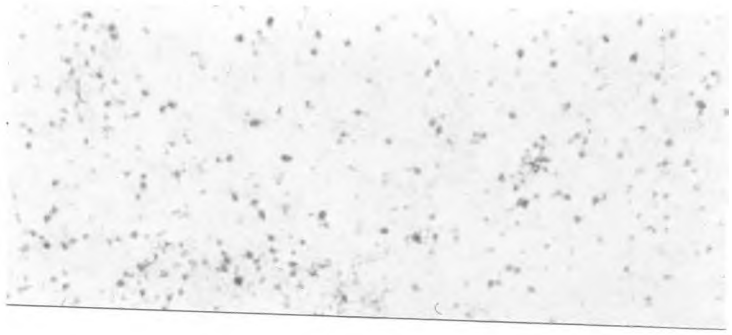


Table 1: Total yield of the spore/crystal mixtures. The spore/crystal mixture obtained from each medium was washed in PBS (3), freeze-dried and weighed.

Medium	Total yield of spore/crystal(g/l)
Modified peptonized milk	0.97 ± 0.008
Semi-defined	0.55 ± 0.004
Nutrient broth	0.32 ± 0.003

Table 2: Bioassay of the spore/crystal mixture.

The toxicity of a suspension of the spore/crystal mixture from each medium was determined using late third instar *Aedes aegypti* (60). Larval mortality was recorded after 60 min.

Medium	Concentration of spo/cry (ng/ml)	Percentage larval mortality (after 60 min)
Modified peptonized milk broth	20	100
	2	67
	0.2	0
Semi-defined	20	100
	2	37
	0.2	0
Nutrient broth	20	100
	2	30
	0.2	0
Control	-	0

KEY

Spo/cry - suspension of the freeze dried spore/crystal mixture

3.2 Isolation, purification and solubilization of the crystal

Several techniques were attempted in the isolation of crystals from the crystal/spore mixture. Using sodium bromide gradients centrifuged in a Beckman SW 41 Ti rotor (52,000 x g, 60 min, 20° C) (Ibarra and Federici, 1986), no separation of the crystals from the crystal/spore mixture was achieved (**Table 3**). Similarly, no separation was achieved using sucrose gradients centrifuged in a Beckman SW 41 Ti rotor (80,000 x g, 14 h, 4° C) (Thomas and Ellar, 1983) (**Table 4**). However, when a linear sucrose gradient (40-70% (w.w)) was used, and the centrifugation time reduced to 30 min, separation of the crystals from crystal/spore mixture was achieved. Despite this achievement, the crystals were still contaminated with some cell debris (**Table 5**). The cell debris were removed from the crystal/cell debris fraction by centrifugation (8,000 x g, 90 min, 4° C). The pellet obtained from this step was highly enriched with crystals while the supernatant solution contained mostly cell debris. The crystals obtained from this latter step were stained using crystal stain (**Fig. 4**).

Partial solubilization of the crystal was achieved using alkaline-reducing conditions. The solubilized protoxin retained its toxicity to late third instar *Aedes aegypti* larvae.

Table 3: Isolation of the crystals from the crystal/spore mixture using NaBr gradients (Ibarra and Federici, 1986). The gradients were centrifuged (52,000 x g , 60 min, 20° C) in a Beckman Ultracentrifuge. The contents of the supernatant solution and pellet were stained using the crystal stain as described under *Materials and Methods*.

Sodium Bromide concentration (w.v)	Gradient type	Supernatant contents	Pellet contents
24-34%	step	cell debris	spores, crystals
24-34%	linear	cell debris	spores, crystals
24-40%	step	cell debris	spores, crystals
24-40%	linear	cell debris	spores, crystals
24-40%*	linear	cell debris	spores, crystals

*This gradient was centrifuged at 8,000 x g for 30 min at 20° C

Table 4: Isolation of the crystals from the crystal/spore mixture using sucrose gradients (Thomas and Ellar, 1983). The gradients were centrifuged (80,000 x g, 14 h, 4° C). The contents of the supernatant solution and pellet were stained using crystal stain.

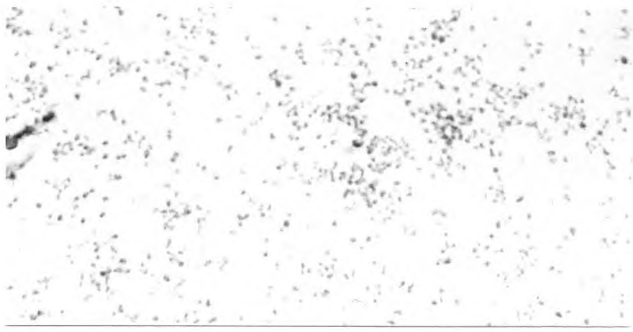
Table 4: Isolation of the crystals from the crystal/spore mixture using sucrose gradients (Thomas and Ellar, 1983). The gradients were centrifuged (80,000 x g, 14 h, 4° C). The contents of the supernatant solution and pellet were stained using crystal stain.

Table 4: Isolation of the crystals from the crystal/spore mixture using sucrose gradients (Thomas and Ellar, 1983). The gradients were centrifuged (80,000 x g, 14 h, 4° C). The contents of the supernatant solution and pellet were stained using crystal stain.

Table 5: Isolation of the crystals from the crystal/spore mixture using sucrose gradients (modified method of Thomas and Ellar, 1983). The gradients were centrifuged (8,000 x g, 4° C) for various times. The contents of the supernatant solution and pellet were stained using the crystal stain.

Sucrose concentration (w.w)	Gradient type	Centrifugation time (min)	Supernatant contents	Pellet contents
40-70%	linear	90	cell debris	spores, crystals
40-70%	linear	60	cell debris crystals	crystals, spores
40-70%	linear	30	cell debris crystals	crystals, spores

Fig. 4 **Isolated crystals.** The crystals were isolated from the spore/crystal mixture using a linear sucrose gradient (40-70 %) as described in ***Materials and Methods***. The contents of supernatant solution and pellet were stained using the crystal stain.



3.3 Physical-chemical characterization of the crystal

3.3.1 SDS-PAGE electrophoresis

The electrophoretic pattern of the crystal on SDS-PAGE revealed the presence of several protein sub-units, with three major sub-units of M_r ~25, 66 and 140 KD (**Fig. 6**). The pattern of the solubilized protoxin was similar to that of the crystal. However, there was a notable absence of the M_r ~25 and 140 KD sub-units. The pattern revealed the presence of a prominent M_r ~21 KD band and a relatively smaller M_r ~66 KD band (**Fig. 7**). The electrophoretic pattern of the insoluble protoxin revealed the presence of a major sub-unit of molecular weight of M_r ~66 KD (**Fig. 7**).

The SDS-PAGE patterns of the insoluble protoxin treated using bovine pancreatic trypsin and α-chymotrypsin revealed no apparent changes in the molecular weight of the major M_r ~66 KD protein sub-unit (**Fig. 8**). Similarly, the SDS-PAGE patterns of the solubilized protoxin treated using the samples above revealed no apparent changes in the molecular weight of the major M_r ~21 KD sub-unit, respectively (**Fig. 8**)

The electrophoretic patterns of the insoluble protoxin treated using partially purified trypsin from *Glossina* spp midgut and other insect gut homogenates (*Aedes aegypti*, *Chilo partellus*, and *Musca domestica*) revealed no apparent change in the molecular weight of the major M_r ~66 KD sub-unit (**Fig. 9a**). Similarly, the SDS-PAGE pattern of the

solubilized protoxin treated using above samples revealed no apparent change in the molecular weight of the major M_r ~21 KD sub-unit (**Fig. 9b**).

3.3.2 Carbohydrate analysis

The presence of covalently-bound carbohydrate on the crystal and electroeluted protein sub-units was demonstrated by staining with PAS reagent. The results showed that all the protein sub-units contained carbohydrates (**Fig. 10**). The crystal is a glycoprotein with high mannose sugar residues as demonstrated by staining with FITC Con-A (result not shown). The carbohydrate content of the crystal was estimated at 0.023 ± 0.0016% using the phenol sulphuric acid method (**Fig. 11**).

3.4 Bioassay results

All the samples used in the bioassay with the exception of the boiled (5 min) crystal caused larval mortality. The purified crystal resulted in the highest mortality in the shortest time duration (30 min), while the solubilized, insoluble protoxins and their enzyme digested fractions exhibited reduced toxicity in comparison with the native crystal (**Table 6**).

Table 6: Bioassay results of the crystal, protoxin and toxin preparations. Protein sample (20 µg) was added to a beaker containing 50 ml of distilled water and 10 late third instar *Aedes aegypti* larvae. Larval mortality in each beaker was recorded at 30 min intervals.

KEY

Conc. = Concentration

Insol. = Insoluble protoxin

Sol = Solubilized protoxin

Bioassay results

Sample	Number fed	Conc (µg/ml)	Larval mortality {cumulative percentage at time (min.)}												
			0	30	60	90	120	150	180	210	240	270	4h	6h	24h
Native crystal	60	0.4	0	100	-	-	-	-	-	-	-	-	-	-	-
Crystal (boiled)	60	0.4	0	0	0	0	0	0	0	0	0	0	0	0	0
Sol. protoxin	60	0.4	0	0	23	25	52	79	100	-	-	-	-	-	-
Digested sol. (trypsin)	60	0.4	0	0	21	33	64	91	97	100	-	-	-	-	-
Digested sol. (α-chymotrypsin)	60	0.4	0	0	26	48	65	78	100	-	-	-	-	-	-
Insol. protoxin	60	0.4	0	0	32	41	66	93	100	-	-	-	-	-	-
Digested insol. (trypsin)	60	0.4	0	0	20	43	79	88	100	-	-	-	-	-	-
Digested insol. (α-chymotrypsin)	60	0.4	0	0	15	34	50	86	89	100	-	-	-	-	-
Sol + insol.	60	0.4	0	11	44	62	100	-	-	-	-	-	-	-	-
Control	60	-	0	0	0	0	0	0	0	0	0	0	0	0	0

3.5 Immunological studies

3.5.1 Double radial immunodiffusion

The antiserum raised to the M_r ~21 KD protein sub-unit reacted with its homologous antigen and the M_r ~21 KD sub-unit obtained from the solubilized protoxin (previously untreated using trypsin enzyme). The antisera did not cross react with the crystal, insoluble and solubilized protoxins (Fig. 12a). Similarly, the antiserum raised to the M_r ~66 KD protein sub-unit reacted with its homologous antigen and the M_r ~66 KD sub-unit obtained from the insoluble protoxin (previously untreated using trypsin enzyme). The antisera did not cross react with the crystal, insoluble and soluble protoxins (Fig. 12b). The antisera raised to the M_r ~21 KD did not cross react with the M_r ~66 KD sub-unit, and vise versa (Figs. 12a and b). The antisera raised to the M_r ~21 KD and ~66 KD protein sub-units did not cross-react with the solubilized TIKI and MFB4/2 protoxins, respectively (Fig. 13a and b).

Fig. 5 Standard curve of log. molecular weight against relative mobility on a gradient SDS-PAGE (4-15%). Both high and low range molecular weight standards (Bio-Rad) were used in the construction of the curve.

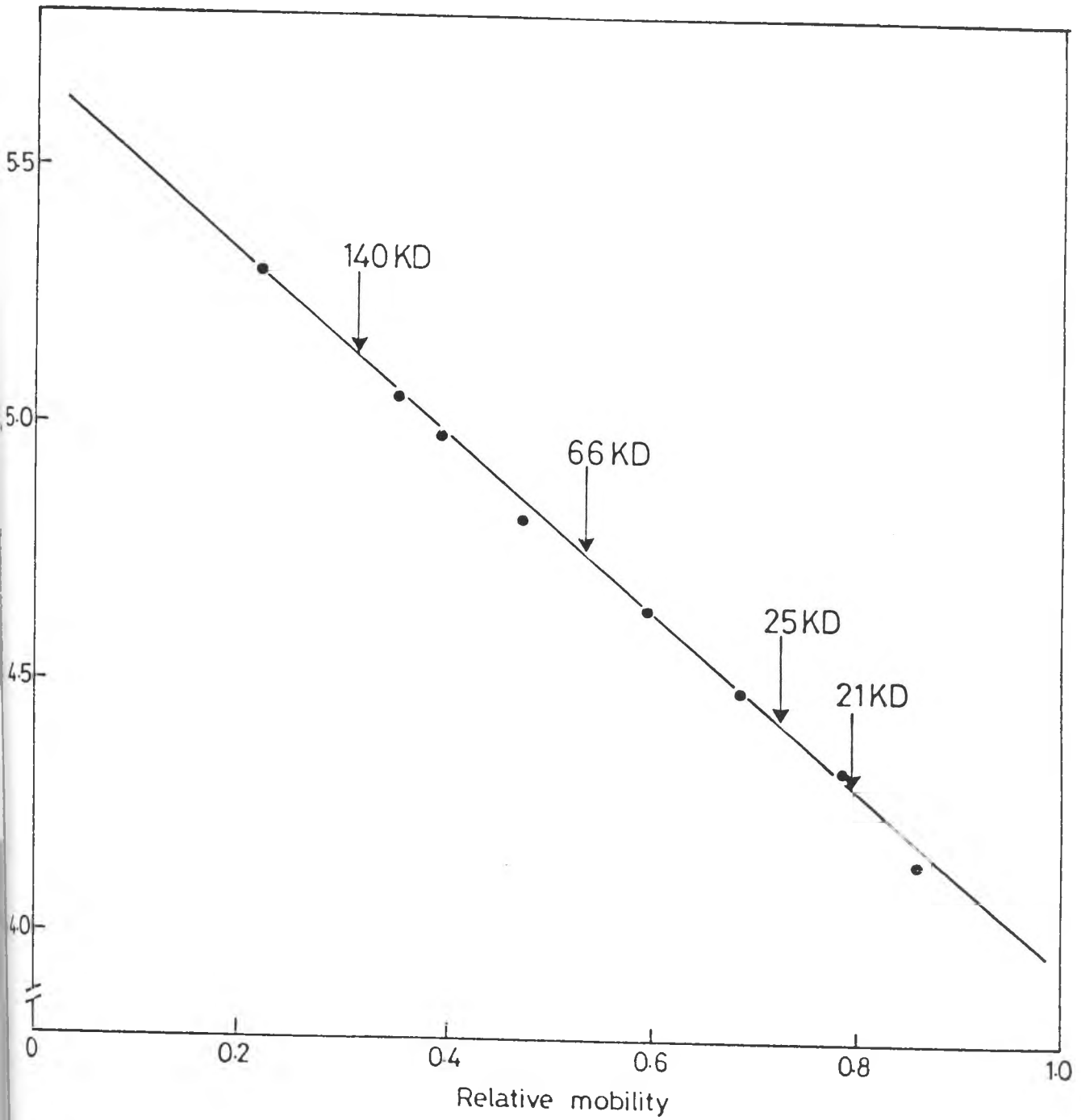

5·5
5·0
4·5
4·0
140 KD
66 KD
25 KD
21 KD
0
0·2
0·4
0·6
0·8
1·0
Relative mobility

Fig. 6 Polyacrylamide gel electrophoresis of the isolated crystals. Protein samples were separated on a gradient SDS-PAGE (4-15%) for 4 h at 25 mA. The gel was stained for proteins using Coomassie Brilliant Blue as described under *Materials and Methods*

1 and 3 - High and low range molecular weight standards (Bio-Rad, Richmond, USA), respectively

2 - Isolated crystals (35 µg)

The arrows:

I - The M_r ~140 KD protein sub-unit

II - The M_r ~66 KD protein sub-unit

III - The M_r ~25 KD protein sub-unit

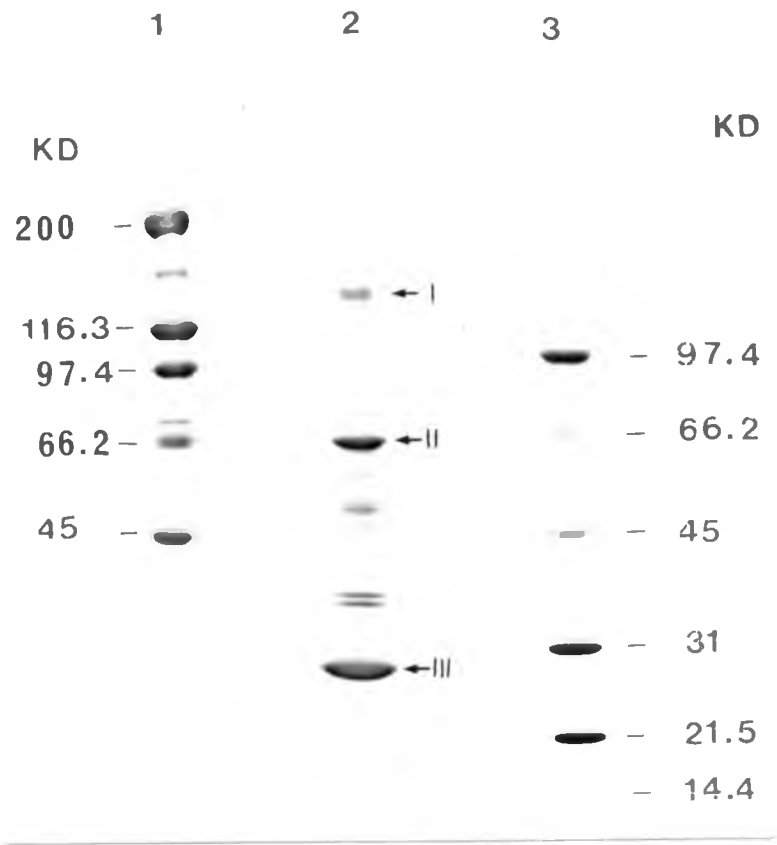

1
2
3
KD
KD
200
116.3
97.4
66.2
45
I
II
III
97.4
66.2
45
31
21.5
14.4

Fig. 7 Polyacrylamide gel electrophoresis of the solubilized and insoluble protoxins. The crystal was solubilized using 50 mM Na_2CO_3.HCl and 10 mM DTT pH 9.5 buffer, as described under ***Materials and Methods***. The protein samples were separated on gradient SDS-PAGE (4-15%) for 3.5 h at 25 mA. The gel was stained for protein.

1 and 5 - High and low range molecular weight markers, respectively.

2 - Crystal (40 µg).

3 - Solubilized protoxin (35 µg)

4 - Insoluble protoxin (35 µg)

The arrows:

I and III - The M_r ~66 KD protein sub-unit

II - The M_r ~21 KD protein sub-unit

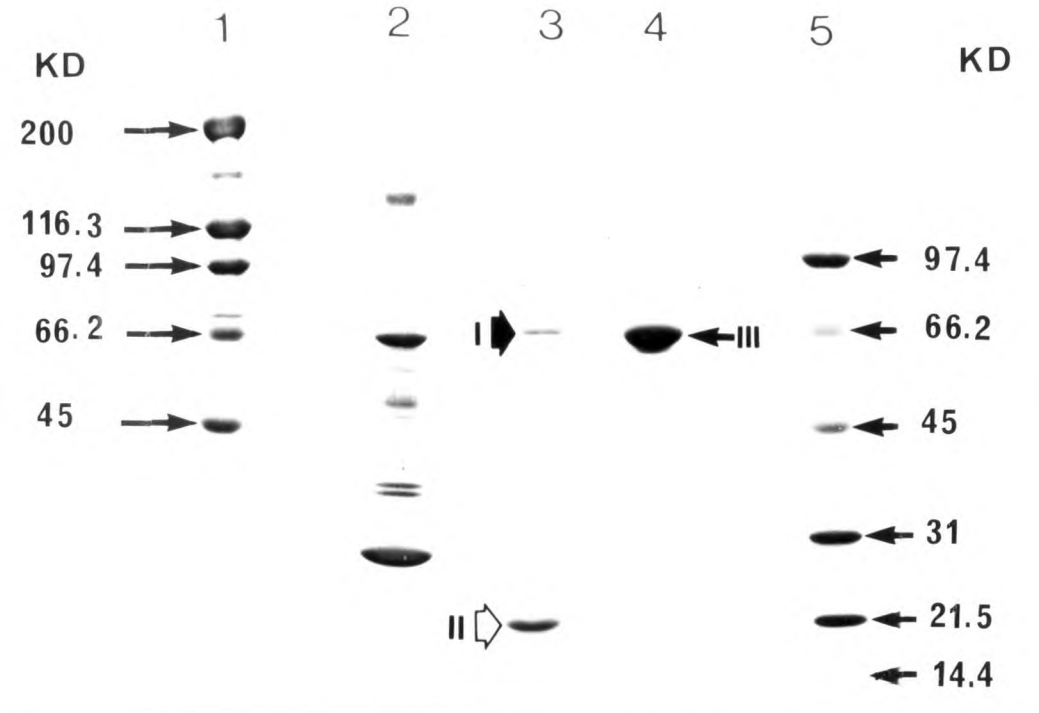

1
2
3
4
5
KD
KD
200
116.3
97.4
66.2
45
I
II
III
97.4
66.2
45
31
21.5
14.4

Fig. 8 Polyacrylamide gel electrophoresis of the solubilized and insoluble protoxins treated with bovine pancreatic trypsin and α-chymotrypsin enzymes. The protoxins (35 µg) were digested using enzyme (165 µg) for 60 min at 37° C, prior to being separated on gradient SDS-PAGE (4-15%) as described under ***Materials and Methods***.

1 - High range molecular weight standard.

2 - α-chymotrypsin (40 µg).

3 - Trypsin (40 µg).

4 - Insoluble protoxin digested using α-chymotrypsin.

5 - Insoluble protoxin digested using trypsin.

6 - Insoluble protoxin (35 µg)

7 - Solubilized protoxin digested using α-chymotrypsin.

8 - Solubilized protoxin digested using trypsin.

9 - Solubilized protoxin (35 µg).

10 - Crystal (40 µg).

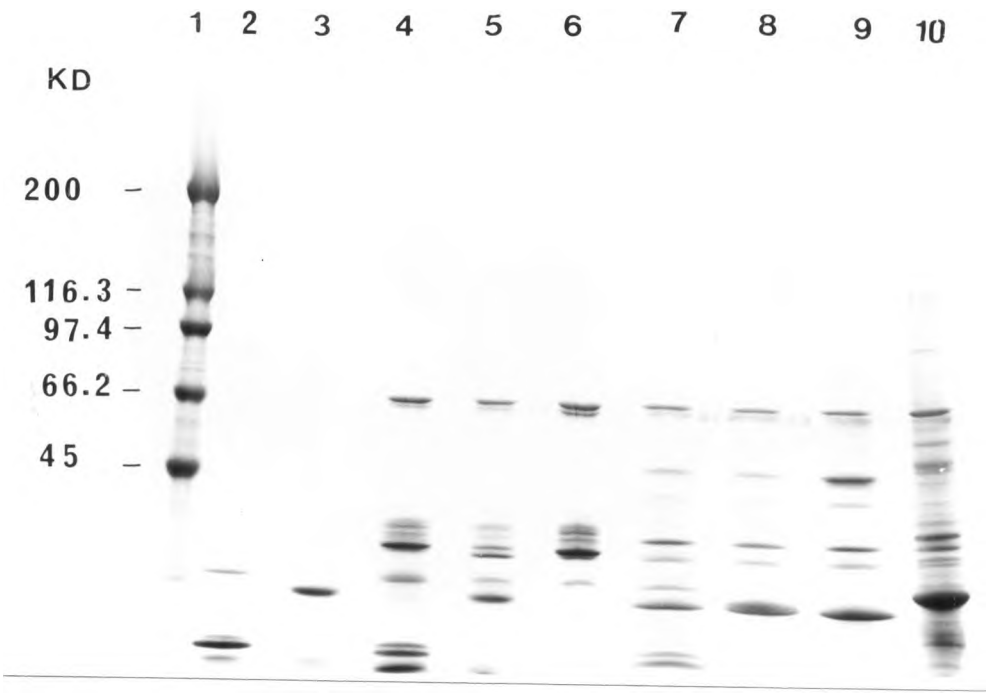

1 2 3 4 5 6 7 8 9 10
KD
200
116.3
97.4
66.2
45

Fig. 9a Polyacrylamide gel electrophoresis of the insoluble protoxin treated with insect gut homogenates. Protoxin (20 µg) was digested using insect larval gut homogenates as described under ***Materials and Methods.*** The digests were separated by gradient SDS-PAGE (4-15%) for 4 h at 25 mA.

1 - High range molecular weight standard.

2 - Gut homogenate from fifth instar larvae of *Chilo partellus* (40 µl).

3 - Protoxin digested using *Chilo partellus* gut homogenate.

4 - Gut homogenate from third instar larvae of *Musca domestica* (35 µl).

5 - Protoxin digested using *Musca domestica* gut homogenate.

6 - Partially purified trypsin from *Glossina* spp. midgut (10 µg).

7 - Protoxin digested using partially purified trypsin from *Glossina* spp.

8 - Gut homogenate from late third instar larvae of *Aedes aegypti* (20 µl).

9 - Protoxin digested using *Aedes aegypti* gut homogenate.

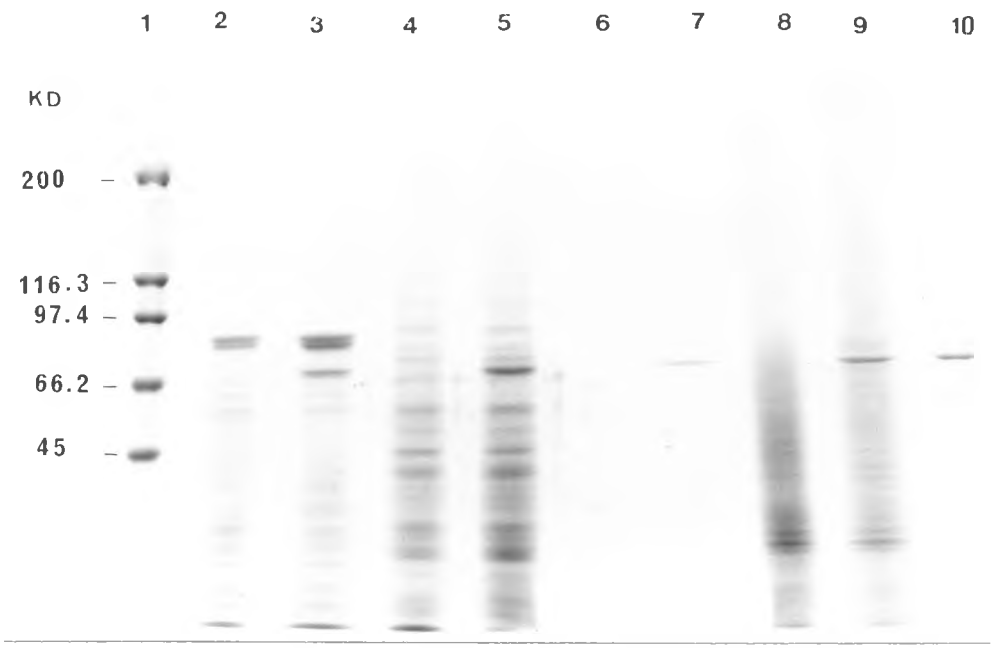

1
2
3
4
5
6
7
8
9
10
KD
200
116.3
97.4
66.2
45

Fig. 9b Polyacrylamide gel electrophoresis of the solubilized protoxin treated with insect gut homogenates. Protoxin (20 µg) was digested using insect gut homogenates as described under ***Materials and Methods***, prior to separation on gradient SDS-PAGE (4-15%).

1 - High range molecular weight standard.

2 - Gut homogenate from *Musca domestica* (35 µl).

3 - Protoxin digested using *Musca domestica* gut homogenate.

4 - Gut homogenate from *Chilo partellus* (40 µl).

5 - Protoxin digested using *Chilo partellus* gut homogenate.

6 - Partially purified trypsin from *Glossina* spp midgut (10 µl).

7 - Protoxin digested using partially purified trypsin.

8 - Gut homogenate from *Aedes aegypti* (20 µl)

9 - Protoxin digested using *Aedes aegypti* gut homogenate.

10 - Solubilized protoxin (20 µg).

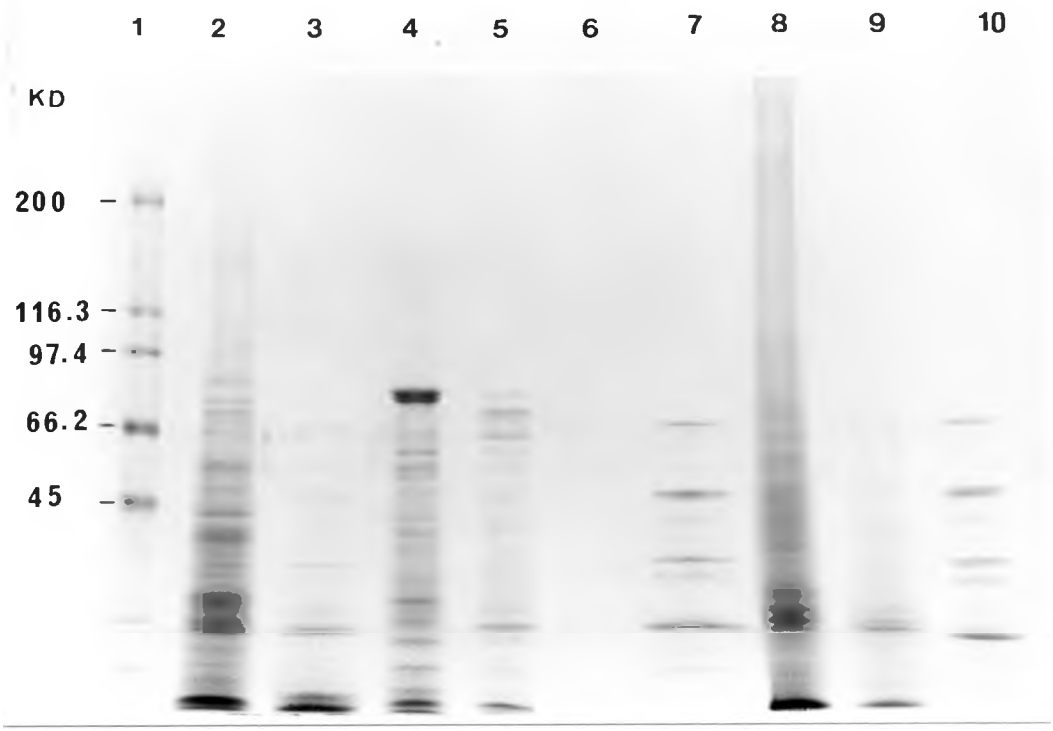

1
2
3
4
5
6
7
8
9
10
KD
200
116.3
97.4
66.2
45

Fig. 10 Polyacrylamide gel electrophoresis of the crystal and protein sub-units stained with PAS for carbohydrates. The protein samples were separated on gradient SDS-PAGE (4-15%). This was followed by staining of the gel using PAS reagent as described under ***Materials and Methods***.

1 - Crystal (50 µg).

2 - M_r ~66 KD sub-unit (50 µg).

3 - M_r ~21 KD sub-unit (20 µg).

4 - Lipophorin (*Schistocerca gregaria*) (50 µg).

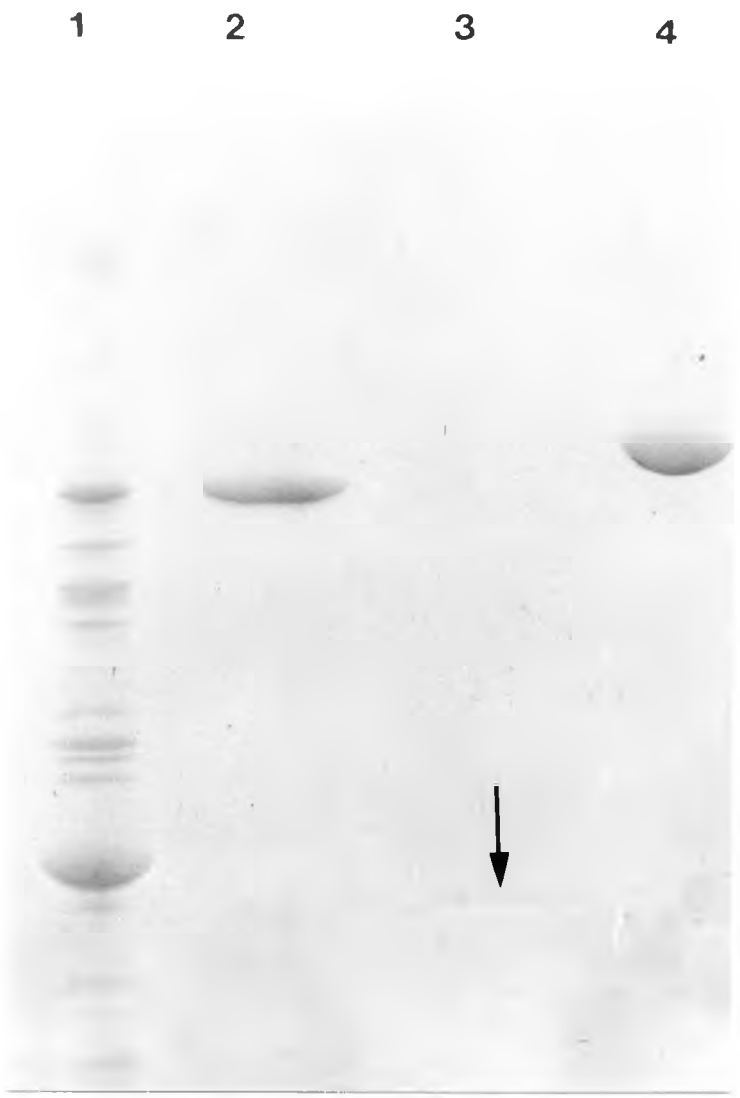

1
2
3
4

Fig 11 **Standard curve for the estimation of percentage carbohydrate using the phenol sulphuric acid method.** D-mannose sugar (BDH, Loope, England) was used as the standard.

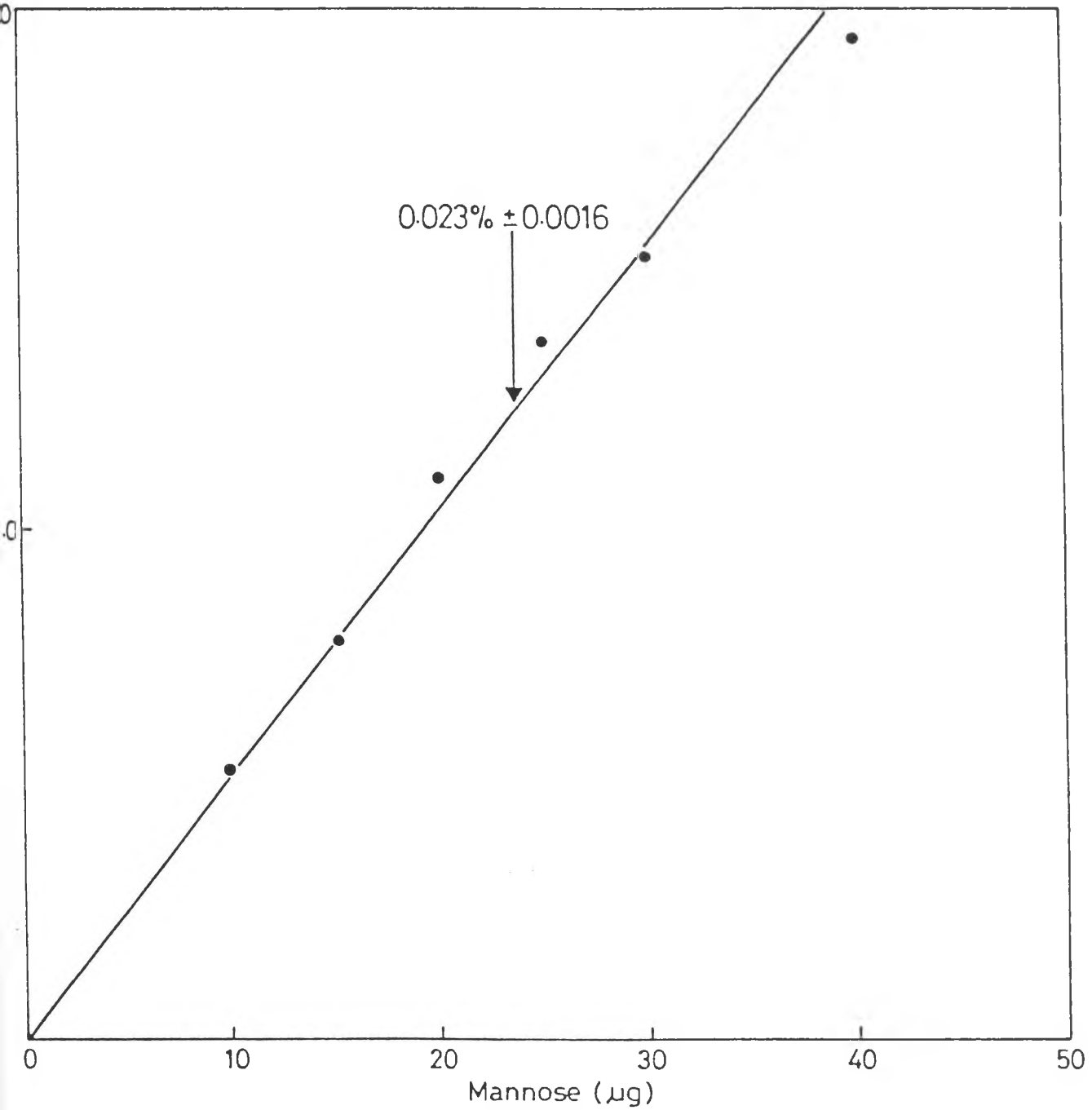

0.023% ± 0.0016
0
10
20
30
40
50
Mannose (µg)

Fig 12a **Double radial immunodiffusion of the crystal and protoxins against antisera raised to the M_r ~21 KD protein sub-unit.** The immunodiffusion was carried out as described under ***Materials and Methods***.

A - Antisera raised to the M_r ~21 KD protein sub-unit (10 µl).

1 - Crystal (20 µg).

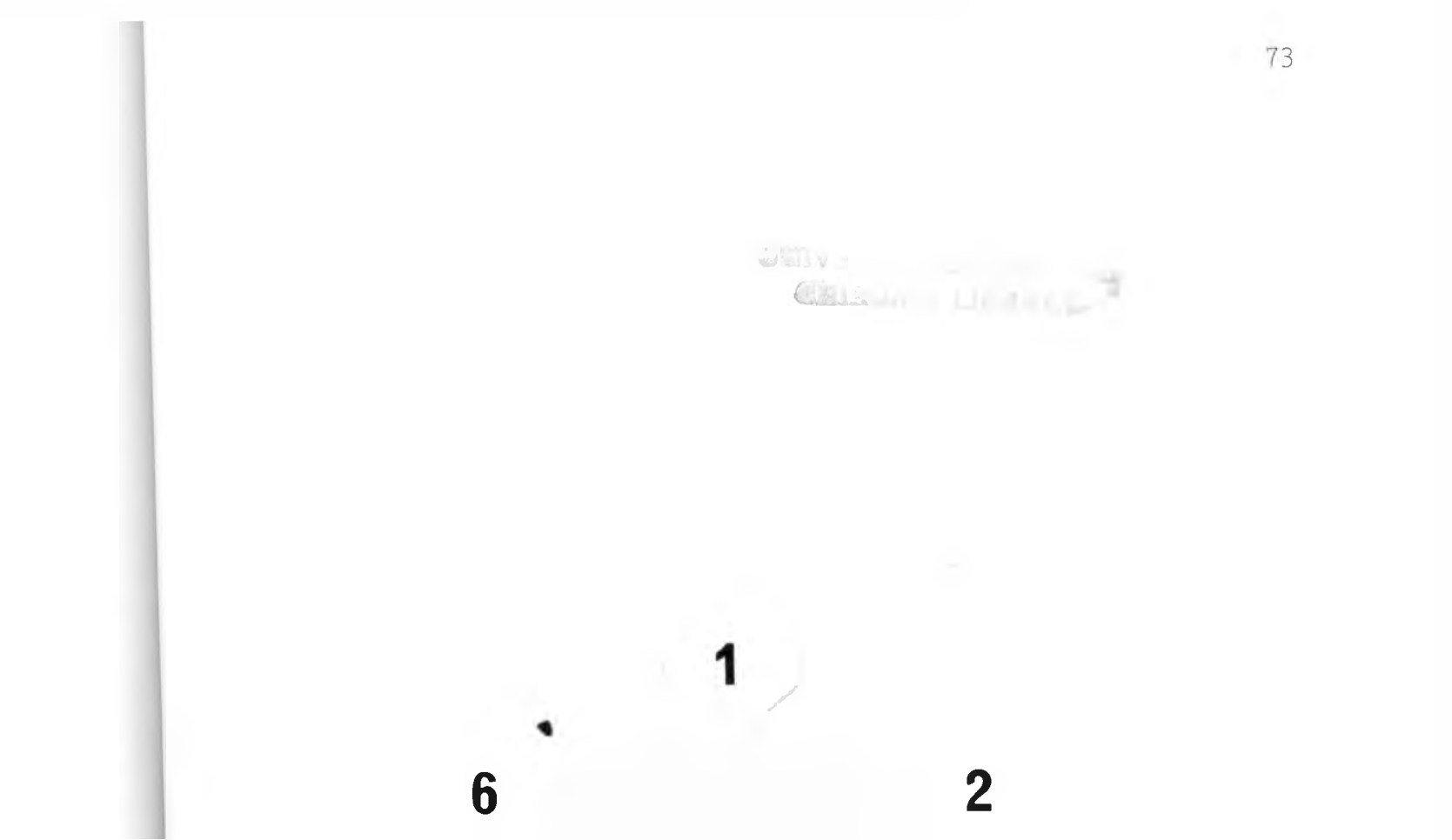
2 - Solubilized protoxin (20 µg).

3 - M_r ~21 KD protein sub-unit (20 µg).

4 - M_r ~66 KD protein sub-unit (20 µg).

5 - Mr ~21 KD protein sub-unit (20 µg). (from trypsin untreated solubilized protoxin).

6 - Insoluble protoxin (20 µg).


1
6
2

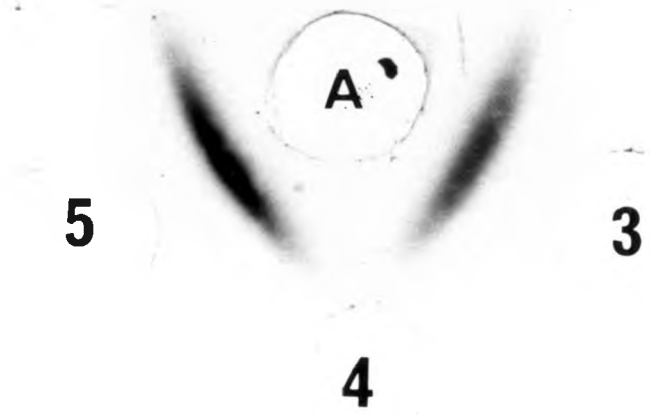

A
5
3
4

Fig 12b Double radial imunodiffusion of the crystal and protoxins against antisera raised to the M_r ~66 KD protein sub-unit.

A - Antisera raised to the M_r ~66 KD protein sub-unit (10 µl).

1 - Crystal (20 µg).

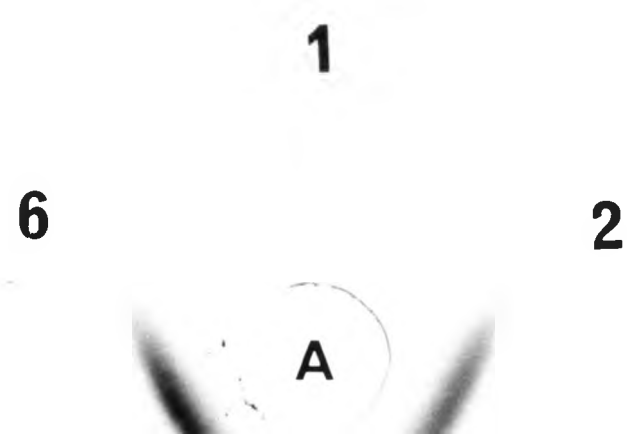
2 - Insoluble protoxin (20 µg).

3 - The M_r ~66 KD protein sub-unit (20 µg).

4 - The M_r ~21 KD protein sub-unit (20 µg).

5 - The M_r ~66 KD protein sub-unit (20 µg). (from trypsin untreated insoluble protoxin)

6 - Solubilized protoxin solution (20 µg).


1
6
2
A

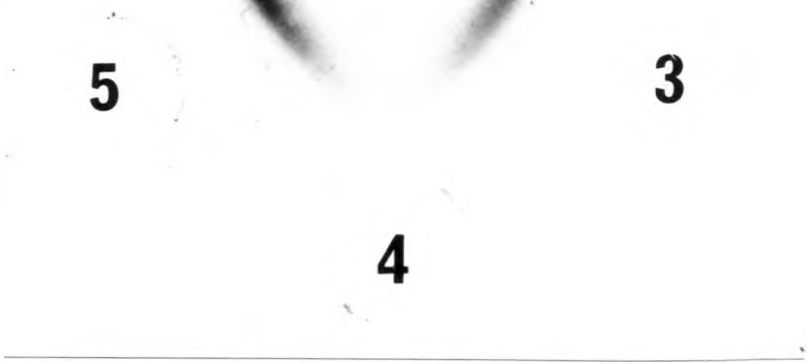

5
3
4

Fig 13a Double radial immunodiffusion of the solubilized TIKI and MFB4/2 protoxins against antisera raised to the M_r ~21 KD protein sub-unit.

A - Antisera to the M_r ~21 KD protein sub-unit (10 µl).

1 - solubilized TIKI protoxin (20 µg).

2 - The M_r ~21 KD protein sub-unit (20 µg).

3 - solubilized MFB4/2 protoxin (20 µg)

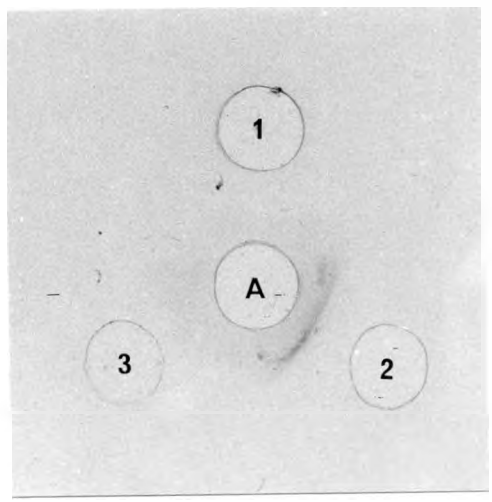

1
A
3
2

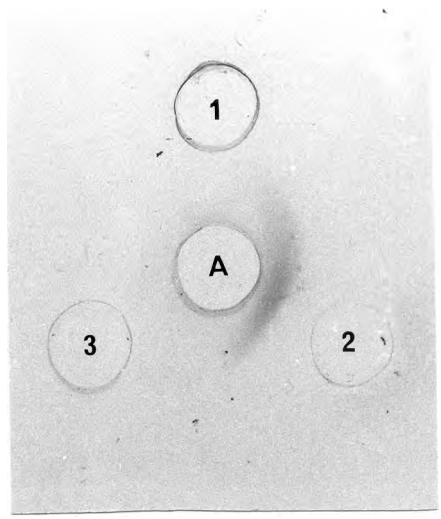
Fig 13b Double radial immunodiffusion of the solubilized TIKI and MFB4/2 protoxins against antisera raised to the M_r -66 KD protein sub-unit.

A - Antisera to the M_r -66 KD protein sub-unit (10 µl).

1 - solubilized TIKI protoxin (20 µg).

2 - The M_r -66 KD protein sub-unit (20 µl).

3 - solubilized MFB4/2 Protoxin (20 µg)


1
A
3
2

CHAPTER 4

DISCUSSION

The shake flask method is among the several fermentation techniques used to culture *B.t.*. This method is the most ideal technique for screening large numbers of *B.t.* isolates and when using smaller levels of nutrient medium (UNDP/WORLD BANK/WHO, 1990). The technique has been used successfully in the fermentation of various *B.t.* strains. For example, *B.t.* variety *israelensis* (*B.t.i.*) (Ibarra and Federici, 1986; Pearson and Ward, 1988), *B.t.* subspecies *galleriae* (Yousten and Rogoff, 1969), *B.t.* subspecies *kurstaki*, *tolworthi*, *alesti* and *berliner* (Tyrell *et al.*, 1981).

The criteria of selection of the three media used in this study was the minimal interference of medium inputs in the recovery process of the δ-endotoxin crystals, cost effectiveness, availability, and previous use of each medium. Generally, the three media supported the growth of M.37.2 resulting in complete lysis of the sporulating cells 72 h after seeding. Similarly, a complete autolysis period of 72 h after seeding of *B.t.* has been previously reported (Yousten and Rogoff 1969; Huber *et al.*, 1981; Ibarra and Federici, 1986). The modified peptonized milk (MPM) broth produced the highest spore/crystal yield, followed by semi-defined and nutrient broths, respectively.

A plausable explanation for these differences in the total yields (**Table 1**) is, the glucose content of each medium. It has been previously reported that glucose not only stimulates cell growth but also increases cell yields (Dulmage, 1989). An increase in glucose concentration to an optimum of 6-8 g/l, has been reported to result in increase of toxicity, size and yield of the δ-endotoxin crystals (Scherer *et al.*, 1973). Secondly, inclusion of yeast extract in the medium has been reported to increase cell growth and spore yield (Dulmage, 1987). Five ions, namely, Mg^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+} and Ca^{2+}, have been reported to be of importance in the growth and sporulation of B.t. (UNDP/WORLD BANK/WHO, 1990). The reasons cited above may explain why MPM broth provided the highest yield while the simple nutrient broth resulted in a poor yield (media composition shown in **Materials and Methods**).

It is also important to be able to measure the quality of the fermentation product. This is because the product should exhibit high purity and be biologically active against its target organism. This was carried out by determining the toxicity of the spore/crystal mixtures to late third *Aedes aegypti* larvae. The mixture from the MPM broth resulted in the highest larval mortality (**Table 2**). The reason for this could be the high glucose content of the medium which resulted in increased crystal yield and toxicity (Scherer *et al.*, 1973). As a result of the highest yield and toxicity of

the spore/crystal mixture obtained from the MPM broth, this medium was selected for routine fermentation of this strain.

The M.37.2 isolate produced ovoid shaped spores and irregularly or amorphously shaped δ-endotoxin crystals. These shapes are not unexpected as they have been previously reported for *B.t.i.* (Mikkola *et al.*, 1982) and *B.t.* isolates from Egypt (Abdel Hameed *et al.*, 1990). These shapes are seemingly unique to *B.t.* active against mosquitoes. (Goldberg and Margalit, 1977; Abdel Hameed *et al.*, 1990).

Gradient ultracentrifugation technique using two different media, namely sucrose in one case and sodium bromide in the other were tried in the isolation of the crystals from the crystal/spore mixture. This technique is simple and has been used successfully to isolate *B.t.i.* crystals (Thomas and Ellar, 1983; Ibarra and Federici, 1986). However, the use of sodium bromide medium and discontinous sucrose gradients proved unsuitable for the isolation of the crystals. A possible explanation is that the δ-endotoxin produced by this strain has unique properties such as shape and size. These properties influence the density of the crystal. Since the density of the crystal determines its separation in the gradient, the gradient range may not have been large enough to allow separation. Secondly, clumping of spores to crystals has been reported to impair the separation of the former from the latter (Sharpe *et al.*, 1975). Hence, for this particular isolate there may have been increased clumping of the spores

to the crystals resulting in poor separation. However, a change from the use of discontinous to continous sucrose gradients (40-70%) resulted in some separation. Despite this success, the crystal yield was low. This can be attributed to the tendency of the spores and crystals to clump together, making the separation poor.

Analysis of the isolated crystals on gradient (4-15%) SDS-PAGE revealed the presence of several protein sub-units. The three major sub-units were of molecular weights ~25 KD, ~66 KD and ~140 KD. The electrophoretic patterns of several *B.t.* crystals have been previously described. For example, native *B.t.i.* crystals reveal a broad band at 28 KD, a triplet at 38, 39 and 40 KD, doublets at 68 and 70 KD and 135 and 140 KD (Pfannenstiel *et al.*, 1984). A similar pattern that contains three major proteins of 27, 65 and a doublet of 130 KD has been reported (Chilcott and Ellar, 1988). *B.t.* variety *dermstadiensis* 73-E10 2 produces crystal proteins of molecular weight 27, 50, 69, 77, 79, 83, and 125 KD (Drobniewski and Ellar, 1989) while *B.t.* variety *fukuokaensis* has crystal proteins of molecular weight of 27, 37, 48, 50, 72, 83, 86, amd 90 KD (Yu *et al.*, 1991). The electrophoretic pattern of *B.t.* variety *kyushuensis* crystals has revealed the presence of polypeptides of molecular weight of 25, 50, 66, 70, 80, 85 and 140 KD (Knowles *et al.*, 1992). A common feature of these mosquitocidal strains is the complex protein pattern profiles of their crystals and the presence of a 25-27 KD and 65-69 KD sub-unit. The

presence of the ~25 and ~66 KD sub-units produced by this newly isolated strain enables it to be classified in this unique category of *B.t.* strains. The electrophoretic pattern of the crystals from mosquitocidal strains is markedly different from the patterns obtained for coleopteran and lepidopteran-specific strains. For example, *B.t.* variety *tenebriones* (coleopteran-specific) crystal pattern shows a major 67 KD polypeptide and minor polypeptides of 73, 72, 55 and 46 KD (Carroll *et al.*, 1989). The crystal patterns of *B.t.* varieties *kurstaki*-P1 and aizawai (lepidopteran-specific) are almost identical with a major polypeptide of 130 KD and a minor component of 63 KD (Knowles *et al.*, 1986). These observations show that the δ-endotoxin of M.37.2 has proteins that are more similar in SDS-PAGE pattern and molecular weight range to those of mosquitocidal rather than lepidopteran and coleopteran-specific strains.

Partial solubilization of the crystal was only achieved using alkaline-reducing conditions. There was no appreciable solubilization achieved using alkaline conditions only. The interesting observations from this experiment were the complete solubilization and degradation of the M_r ~140 KD sub-unit. Secondly, the M_r ~66 KD was sparingly soluble under the above condition, while the SDS-PAGE pattern of the solubilized protoxin revealed the presence of a prominent M_r ~21 KD sub-unit hitherto absent in the native crystal pattern. Complete solubilization of *B.t.i.* crystals has also been achieved using alkaline conditions of pH 11.7

(Pfannenstiel *et al.*, 1986). However, partial solubilization of the same crystals has been achieved using alkaline conditions in the absence of a reducing agent (Thomas and Ellar, 1983). The insoluble protoxin obtained using the latter conditions contained 28, 52, 65, 93.5 and 98 KD sub-units, while the solubilized protoxin contained the 28 and 65 KD sub-units (Thomas and Ellar, 1983). Partial solubilization of *B.t.* variety *kyushuensis* using alkaline conditions in the absence of a reducing agent, results in a solubilized protoxin containing sub-units of predominantly the 14, 15, 25 and 85 KD (Knowles *et al.*, 1992). Solubilization of *B.t.* varieties *kurstaki* and *aizawai* under alkaline-reducing conditions results in the complete solubilization of the M_r 130 KD sub-unit but the 63 KD sub unit remains insoluble (Knowles *et al.*, 1986). The entire crystal of *B.t.* variety *thuringiensis* was solubilized using a similar alkaline-reducing regime (Knowles *et al.*, 1986). From the solubilization results, and in comparison with other *B.t.* crystals, it is difficult to postulate the fate of the M_r ~140 KD sub-unit, whether it degrades either into the ~21 or the ~66 KD sub-units. However, it can only be speculated that the source of the ~21 KD sub-unit is the ~25 KD sub-unit. This is because the latter is conspicously absent from both the insoluble and soluble protoxins, suggesting that it is degraded. The solubilization pattern, unlike the SDS-PAGE pattern of the M.37.2 crystal is neither similar to the solubilization pattern produced by the

lepidopteran nor dipteran (mosquitocidal) specific B.t. crystals.

Treatment of the solubilized and insoluble protoxins with bovine pancreatic trypsin or α-chymotrypsin results in no apparent change in the molecular weight of the major M_r ~21 and ~66 KD sub-units, respectively. Similarly, no apparent change in the molecular weights of the minor sub-units between M_r ~21 and ~66 KD was observed. These results show some similarities and differences to those obtained from the treatment of the *B.t.i.* protoxin with trypsin and α-chymotrypsin. For example, treatment of the *B.t.i.* protoxin with trypsin resulted in four major protease-resistant polypeptides (M_r 23, 30, 50, and 65 KD), while treatment with α-chymotrypsin resulted in three sub-units (M_r 22, 31, and 65 KD) (Pfannenstiel *et al.*, 1986). It has been suggested that treatment of *B.t.i.* protoxin with the above enzymes leads to successive degradation of the 28 KD sub-unit into polypeptides of M_r 22, 23, 25 and 26 KD (Pfannenstiel, *et al.*, 1987). A similar conclusion was drawn, when it was found that the 28 KD sub-unit of *B.t.i.* degrades into multiple bands in the region of M_r ~25 KD (Armstrong *et al.*, 1985). It has also been proposed that the M_r ~135 KD sub-unit of the *B.t.i.* protoxin is degraded successively to protein bands of 72, 94 and probably, 65 KD (Pfannenstiel *et al.*, 1987). Treatment of the solubilized crystal of *B.t.* variety *colmeri* with trypsin resulted in the degradation of the major 130 KD sub-unit into polypeptides

of M_r 55 and 58 KD (Haider *et al.*, 1986). The conclusions that can be drawn from the results are that the solubilization process produces protease-resistant polypeptides that are not further affected by proteolytic treatment. Secondly, protease treatment using the above enzymes may result in the cleavage of very small peptides whose effect shows no apparent change in the molecular weights of the sub-units as judged by SDS PAGE. Thirdly, the above enzymes may have no proteolytic effect on the major sub-units of the protoxins.

Similarly, enzymatic treatment of the solubilized and insoluble protoxins using insect gut homogenates revealed no apparent change in the molecular weights of the major ~21 and ~66 KD sub-units. Treatment of *B.t.i.* protoxin with third instar *Aedes aegypti* gut homogenates has been reported to duplicate the results obtained from the treatment using α-chymotrypsin (Pfannenstiel *et al.*, 1987). Treatment of the *B.t.* variety *colmeri* soluble protoxin with third instar *Aedes aegypti* gut extracts causes the degradation of the 130 KD sub-unit into 53, 58 and slightly less than 52 KD sub-units (Haider *et al.*, 1986). The conclusions that can be drawn from my results are similar to the those stated above for the enzymatic treatment of the protoxins (insoluble and soluble) with trypsin and α-chymotrypsin enzymes.

The crystal was shown to be a glycoprotein with high mannose sugar residues with a carbohydrate content of 0.023 ± 0.0016%. The carbohydrate content of various *B.t.* crystals

has also been determined. For example, *B.t.i.* has been reported to contain 2.7% sugars (Muthukumar and Nickerson, 1987), *B.t.* variety *kurstaki* contains 5.6% (Bulla *et al.*, 1977) while *B.t.* variety *thuringiensis* contains less than 0.05% (Huber *et al.*, 1981). Neutral sugars such as glucose, mannose, fucose and rhamnose have been reported (Tyrell *et al.*, 1981). The exact role of the carbohydrate moeity of the toxin is not known. However, it has been suggested that the moieties are involved in the insecticidal activity of the toxins. For example, the distinct lectin binding patterns of the crystals suggests that host specificity is determined in part by the carbohydrate portion of the glycoprotein (Muthukumar and Nickerson, 1987). The carbohydrate content of the crystal under study was low compared to the contents of the above *B.t.* crystals. This may partly be explained by the repeated washing of the isolated crystal to remove the sucrose. It has been previously reported that prolonged washing of the crystal steadily decreases the amounts of unspecific sugars bound non-covalenty to the crystal (Huber *et al.*, 1981).

The rationale for carrying out bioassays was to ascertain whether the crystal and the various protoxin preparations were biologically active, rather than to determine the concentrations that kill 50% of the larvae. The results showed that the crystal produced the highest mortality in the shortest time. However, the protoxins and their enzyme treated preparations showed reduced toxicity. This reduction

can be attributed to the filter feeding behaviour of the larvae, hence taking longer to concentrate the samples (Chilcott and Ellar, 1988; de Barjac, 1989). The boiled crystal showed no toxicity to the larvae. The conclusion that can be drawn from this observation is that the crystal a thermo-labile and the biological activity is lost by boiling at temparatures of ~100° C for five minutes.

The antisera raised to the M_r ~21 KD protein sub-unit immunologically cross-reacted with the protein (M_r ~21 KD) obtained from solubilized protoxin not previously treated with trypsin. A similar result was obtained with the M_r ~66 KD sub-unit. This result suggested that treatment of the protoxin with trypsin did not modify the antigenic epitopes of the M_r ~21 and ~66 KD sub-units. The antisera raised to the M_r ~21 KD sub-unit did not cross react with the M_r 66 KD sub-unit and vise versa. The antisera against the M_r ~21 KD sub-unit did not cross-react with the TIKI and MFB4/2 protoxins, respectively. A similar result was obtained for the antisera against the M_r ~66 KD sub-unit. It has been previously reported that the 28, 70 and 135 KD sub-units of *B.t.i.* are immmunologiclly distinct (Pfannenstiel *et al.*, 1986). The 130 KD (lepidopteran-specific) and 63 KD (dipteran-specific) sub-units of *B.t.* variety *kurstaki* have also been reported to be immunologically distinct (Thomas and Ellar, 1983). Antisera against *B.t.* variety *kurstaki* has been reported to cross react with *B.t.* variety *colmeri* native crystal (Haider *et al.*, 1986). *B.t.i* crystals have

been reported not to cross-react with antisera against *B.t.* varieties *kurstaki*, *berliner*, *alesti* and *tolworthi*. However, the crystals from the latter varieties cross-reacted with each other (Tyrell *et al.*, 1981). The 23-28 KD proteins from *B.t.* varieties *israelensis*, *darmstadiensis* and *fukuokaensis* are antigenically related to each other (Knowles *et al.*, 1992). Several conclusions can be drawn about the immunological relationships between the M_r ~21 and ~66 KD sub-units and their antigenic relationship to the solubilized TIKI and MFB4/2 protoxins. Basically, the M_r ~21 and ~66 KD sub-units are immunologically distinct. Secondly, the lack of antigenic relatedness between the M_r ~21 and ~66 KD sub-units and the MFB4/2 protoxin (lepidoteran-specific) is not unexpected as the dipteran specific *B.t.* crystals have been shown to be antigenically distinct from the lepidopteran-specific crystals (Tyrell *et al.*, 1981; Thomas and Ellar, 1983). The lack of cross reactivity between the antisera raised to the samples and the crystal, is because of the large size of the crystal. This large size is attributed to the presence of inter-chain disulphide linkage between the polypeptides constituting the crystal (Fast, 1981). Therefore, the crystal needs to be denatured using reagents like β-mercaptoethanol to yield the individual polypeptides that can diffuse in agarose. The same reason can be advanced for the absence of cross-reactivity between the antisera and their respective protoxins. Solubilization of the crystal does not result in the cleavage of the

disulphide linkages hence, both of the protoxins remain too large in size to diffuse through the agarose gel.

Despite this basic biochemical and immunological characterization of this crystal, very many unexplored aspects about this crystal remain. For example, the toxin-receptor studies. This field involves the identification, location, and characterization of the receptor. It will be interesting to elucidate the toxin-receptor interaction, and whether the presence or absence of the carbohydrate moeity is involved in the interaction. Secondly, elucidating the mode of action of the toxin is also important. Other areas include the molecular biology of the *B.t.* For example, determining the number of plasmids and specifiying which plasmid is responsible for the production of which δ-endotoxin polypeptide.

REFERENCES

Abdel-Hameed, A., K. Lounatmaa, G. Carlberg, and O. M. El Tayeb. (1990). Studies on *Bacillus thuringiensis* H-14 strains isolated in Egypt-II. Ultrastructure studies. *World J. Microbiol. Biotechnol.* 6, 305-312.

Amstrong, J. L., G. F. Rorhmann, and G. S. Beaubreau. (1985). Delta endotoxin of *Bacillus thuringiensis* subsp. *israelensis*. *J. Bacteriol.* 161, 39-46.

Ang, B. J., and K. W. Nickerson. (1978). Purification of the protein crystal from *Bacillus thuringiensis* by zonal gradient centrifugation. *Appl. Enviro. Microbiol.* 36, 625-626.

Aronson, A. I., W. Beckman, and P. Dunn. (1986). *Bacillus thuringiensis* and related insect pathogens. *Microbiol. Rev.* 50, 1-24.

Bulla, L. A., Jr., K. J. Kramer, and L. I. Davidson. (1977). Characterization of the entomocidal parasporal crystal of *Bacillus thuringiensis*. *J. Bacteriol.* 130, 375-383.

Burnette, W. N. (1981). "Western blotting" electrophoretic transfer of proteins from sodium dodecyl sulphate

polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodonated protein-A. *Anal. Biochem.* 112, 195-203.

Carroll, J., J. Li, and D. J. Ellar. (1989). Proteolytic processing of a coleopteran-specific δ-endotoxin produced by *Bacillus thuringiensis* var. *tenebrionis*. *Biochem. J.* 261, 99-105.

Chilcott, C. N., and D. J. Ellar. (1988). Comparative toxicity of *Bacillus thuringiensis* var. *israelensis* crystal proteins *in vivo* and *vitro*. *J. Gen. Microbiol.* 134, 2551-2558.

Davidson, E. W., and A. W. Sweeney. (1983). Microbial control of vectors. A decade of progress. *J. med. entomol.* 20, 235-247.

de Barjac, H. (1981). Identification of H-serotypes of *Bacillus thuringiensis*. pg 35-43. *In* H. D. Burges (ed). Microbial control of pests and plant diseases 1970-1980. Academic press inc. New York, USA.

de Barjac, H. (1989). New facts and trends in bacteriological control of mosquitoes. *Mem. Inst. Oswaldo Cruz, Rio de Janiero vol.* 84, 101-105.

Delafield, F. P., H. J. Sommerville and S. C. Rittenburg. (1968). Immunological homology between crystal and spore protein of *Bacillus thuringiensis*. *J. Bacteriol.* 96, 713-720.

Drobniewski, F. A., D. J. Ellar. (1989). Purification and properties of a 28-kilodalton haemolytic and mosquitocidal protein of *Bacillus thuringiensis* subsp. *darmstadiensis* 73-E10-2. *J. Bacteriol.* 171, 3060-3067.

Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. (1956). Colorimetric method for determination of sugars and related substances. *Anal. Biochem.* 28, 350-356.

Dulmage, H. T. (1981). Insecticidal activity of isolates of *Bacillus thuringiensis* and their potential for pest control. pg 193-222. *In* H. D. Burges (ed). Microbial control of pests and plant diseases 1970-1980. Academic press inc. New York, USA.

Dulmage, H. T. (1987). Bioprocess developments in the production of bioinsecticides by *Bacillus thuringiensis*. *CRC. Crit. Rev. Biotech. vol.* 6, 87-121.

Dulmage, H. T. (1989). Production and use of *Bacillus thuringiensis* perspective from 1989. *Mem. Inst. Oswaldo Cruz Rio de Janeiro, vol. 84*, 113-122.

Ellar, D. J. (1990). Pathogenic determinants of entomopathogenic bacteria pg 298-302. *In* D. E. Pinnock (ed). Proceedings of the fifth international colloquim on invertebrate pathology and microbial control.

Fast, P. G., and I. K. Morrison. (1972). The δ-endotoxin of *Bacillus thuringiensis*. the effects of δ-endotoxin on ion regulation by midgut tissue of *Bombyx mori* larvae. *J. Invert. Pathol.* 18, 135-138.

Fast, P. G. (1981). The crystal toxin of *Bacillus thuringiensis* pg 223-248. In H. D. Burges (ed). Microbial control of pests and plant diseases 1970-1980. Academic press inc. New York, USA.

Fast, P. G., and R. Milne. (1979). *Bacillus thuringiensis* toxin dissolution of crystal with retention of toxicity. *J. Invert. Pathol.* 34, 319.

Feitelson, J. S., J. Payne, and L. Kim. (1992). *Bacillus thuringiensis*: Insects and beyond. *Bio/technology*. 10, 271-275.

Finegold, S. M., W. J. Martin, and E. G. Scott. (1978). Bailey and Scott's diagnostic microbiology. pg 17-22, and 477. C. V. Mosby company, Missouri, USA.

Finney, J. R. (1981). Potential of nematodes for pest control pg 603-620. *In* H. D.Burges (ed). Microbial control of pests and plant diseases 1970-1980. Academic press , New York, USA.

Furlan, M, B. A. Perret, and E. A. Beck. (1979). Staining of glycoproteins in polyacrylamide and agarose gels with flourescent lectins. *Anal. Biochem.* 96, 208.

Gelernter, W. D. (1990). *Bacillus thuringiensis*, bioengineering and the future of bioinsecticides. *Brighton crop protection conference - Pests and diseases*. 7A-3, 617-624.

Gill, S. S., A. E. Cowles, and P. V. Pietrantonio. (1992). The mode of action of *Bacillus thuringiensis* endotoxins. *Ann. Rev. Entomol.* 37, 615-636.

Goldberg, L. H., and J. Margalit. (1977).A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii, Uranotaenia unguiculata, Culex univittatus, Aedes aegypti* and *Culex pipiens*. *Mosq. News* 37, 355-358.

Gonzalez, J. M., Jr., H. T. Dulmage, and B. C. Carlton. (1981). Correlation between specific plasmids and δ-endotoxin production in *Bacillus thuringiensis*. *Plasmid* 5, 351-365.

Gonzalez, J. M., Jr., B. S. Brown, and B. C. Carlton. (1982). Plasmid transfer of *Bacillus thuringiensis* plasmids coding for δ-endotoxin among strains of *B. thuringiensis* and *B. cereus*. *Proc. Natl. Acad. Sci. USA* 79, 6951-6955.

Gonzalez, J. M., Jr., and B. C. Carlton. (1984). A large transmissible plasmid is required for crystal toxin production in *Bacillus thuringiensis* variety *israelensis*. *Plasmid* 11, 92-98.

Haider, M. Z., B. H. Knowles, and D. J. Ellar. (1986). Specificity of *Bacillus thuringiensis* var. *colmeri* insecticidal δ-endotoxin is determined by differential proteolytic processing of the protoxin by the larval gut proteases. *Eur. J. Biochem.* 156, 531-540.

Hofmann, C., H. Vanderbruggen, H. Hofte, J. Van Rie, S. Jansen, and H. Van Mellaert. (1988). Specificity of *Bacillus thuringiensis* δ-endotoxin is correlated with the presence of high affinity binding sites in the

brush border membrane of target insect midguts. *Proc. Natl. Acad. Sci. USA* 85, 7844-7848.

Hofte, H., and H. R. Whiteley. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* 53, 242-255.

Huber, H. E., and P. Luthy. (1981). *Bacillus thuringiensis* delta-endotoxin composition and activation pg 209-234. *In* E. W.Davidson (ed). Pathogenesis of invertebrate microbial diseases. Allenheld Osmun Publishers, Totowa New Jersey, USA.

Huber, H. E., P. Luthy, H. R. Ebersold, and J. L. Cordier. (1981). The linkage of the parasporal crystal of *Bacillus thuringiensis*: Size, linkage and toxicity. *Arch. Microbiol.* 129, 14-18.

Ibarra, J. E., and B. A. Federici. (1986). Isolation of a relatively non-toxic 65-kilodalton protein inclusion from the parasporal body of *Bacillus thuringiensis* subsp. *israelensis*. *J. Bacteriol.* 165, 527-533.

Kapitany, R. A., and E. J. Zebrowski. (1973). A high resolution PAS stain for polyacrylamide gel. *Anal. Biochem.* 56, 361-369.

Kelly, D. C. (1981). Non occluded viruses pg 39-60. *In* E. W. Davidson (ed). Pathogenesis of invertebrate microbial diseases. Allenheld osmun publishers, Totowa, New Jersey, USA.

Knowles, B. H., W. E. Thomas, and D. J. Ellar. (1984). Lectin-like binding of *Bacillus thuringiensis* var. *kurstaki* lepidopteran-specific toxin is an initial step in insecticidal action. *FEBS Lett.* 168, 197-202.

Knowles, B. H., P. H. Francis, and D. J. Ellar. (1986). Structurally related *Bacillus thuringiensis* δ-endotoxin display major differences in insecticidal activity *in vivo* and *in vitro*. *J. Cell. Sci.* 84. 221-236.

Knowles, B. H., P. J. White, C. N. Nicholls, and D. J. Ellar. (1992). A broad spectrum cytolytic toxin from *Bacillus thuringiensis* var. *Kyushuensis*. *Proc. R. Soc. Lond. B.* 248, 1-7.

Kreig, A. (1981). The genus *Bacillus*: Insect pathogens pg 1743-1755. *In* M. P. Starr, H. Stolp, H. G. Truper, A. Balows and H. G. Schelgel (ed). The prokaryotes. A handbook on habitats, isolation and identification of bacteria Vol II. Springer-Velag-Berlin-Heidelberg publishers, New York, USA.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the heads of bacteriophage T_4. *Nature* 227, 680-685.

Luthy, P., J. L. Cordier, and H. M. Fischer. (1982). *Bacillus thuringiensis* as a bacterial insecticide: Basic considerations and applications pg 35 74. *In* E. Kurstah (ed). Microbial and viral pesticides. Marce Bekker, New York, USA.

Margalit, J. and Dean, D. (1985). The story of *Bacillus thuringiensis* variety *israelensis* (B.t.i). *J. Am. mosq. control. Ass.* 1, 1-37.

Martin, P. A. W., and R. S. Travers. (1989). Worldwide abundance and distrubution of *Bacillus thuringiensis* isolates. *Appl. Environ. Microbiol.* 55, 2437-2442.

McGaughey, W. H. (1985). Insect resistance to the biological insecticide *Bacillus thuringiensis*. *Science* 299, 193-195.

Mikkola, A. R., G. A. Carlberg, T. Vaara, and H. G. Gyllenberg. (1982). Comparison of inclusions in different *Bacillus thuringiensis* strains. An electron microscopy study. *FEMS. Microbiol. Lett.* 13, 401-408.

Muthukumar, G, and K. W. Nickerson. (1987). The glycoprotein toxin of *Bacillus thuringiensis* subsp. *israelensis* indicates a lectin-like receptor in the larval mosquito gut. *Appl. Enviro. Microbiol.* 53, 2650-2655.

Ouchterlony, O. (1958). Antigen-antibody reaction in gels. Handbook of immunodiffusion and immunoelectrophoresis. Ann. Arbor. Science Publishers, Ann. Arbor, Michigan, USA.

Payne, C. C. (1981). Cytoplasmic polyhedrosis viruses pg 61-100. In E. W.Davidson (ed). Pathogenesis of invertebrate microbial diseases. Allenheld osmun publishers, Totowa, New Jersey, USA.

Pearson, D., and O. P. Ward. (1988). Effect of culture conditions on growth and sporulation of *Bacillus thuringiensis* subsp. *israelensis* and development of media for production of the protein crystal endotoxin. *Biotechnol. Lett.* vol. 10, 451-456.

Pfannenstiel, M. A., E. J. Ross, V. C. Kramer, and K. W. Nickerson. (1984). Toxicity and composition of protease inhibited *Bacillus thuringiensis* var. *israelensis* crystals. *FEMS Microbiol. Lett.* 21, 39-42.

Pfannenstiel, M. A., G. A. Couche, E. J. Ross, and K. W. Nickerson. (1986). Immunological relationships among proteins making up the *Bacillus thuringiensis* Subsp. *israelensis* crystalline toxin. *Appl. Environ. Microbiol.* 52, 644-649.

Pfannenstiel, M. A., G. Muthukumar, G. A. Couche, and K. W. Nickerson. (1987). Amino sugars in the glycoprotein toxin from *Bacillus thuringiensis* subsp. *israelensis*. *J. Bacteriol.* 169, 796-801.

Scherer, P., P. Luthy, and B. Trumpi. (1973). Production of δ-endotoxin by *Bacillus thuringiensis* as a function of glucose concentration. *Appl. Microbiol.* 25, vol. 4 , 644.

Service, M. W. (1986). Lecture notes on medical entomology. Blackwell scientific publications, Great Britian.

Sharpe, E. S., K. W. Nickerson, L. A. Bulla. Jr., and J. N. Aronson. (1975). Separation of spores and parasporal crystals of *Bacillus thuringiensis* in gradients of certain X-ray contrasting agents. *Appl. Microbiol.* 30, 1052-1053.

Singer, S., and M. H. Rogoff. (1968). Inhibition of growth of *Bacillus thuringiensis* by amino acids in defined

media. *J. Invert. Pathol.* 12, 98-104.

Smirnoff, W. A. (1962). A staining method for differentiating spores, crystals and cells of *Bacillus thuringiensis* (*Berliner*). *J. Invert. Pathol.* 4, 384-386.

Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76-85.

Sweeney, A. W. (1981). Fungal pathogens of mosquito larvae pg 401-427. *In* E. W. Davidson (ed). Pathogens of invertebrate microbial diseases. Allenheld osmun publishers, Totowa, New Jersey, USA.

Tabashnik, B. E., N. C. Cushing, N. Finson, and M. W. Johnson. (1990). Field development of resistance to *Bacillus thuringiensis* in Diamondback Moth (Lepidoptera:*Plutellidae*). *J. Econ. Entomol.* 83, 1671-1676.

Thomas, W. E., and D. J. Ellar. (1983). *Bacillus thuringiensis* var. *israelensis* crystal δ-endotoxin:

Effects on insect and mammalian cells *in vitro* and *in vivo*. *J. Cell Sci.* 60, 181-197.

Towbin, H., T. Staehelin, and J. Gordon. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and applications. *Proc. Natl. Acad. Sci. USA.* 76, 4350-4354.

Travers, R. S., P. A. W. Martin, and C. F. Reichelderfer. (1987). Selective process for the efficient isolation of soil *Bacillus* spp. *Appl. Enviro. Microbiol.* 53, 1263-1266.

Tyrell, D. J., L. A. Bulla, Jr., R. E. Andrews, Jr., K. J. Kramer, L. J. Davidson, and P. Nordin. (1981). Comparative biochemistry of entomocidal parasporal crystals of selected *Bacillus thuringiensis* strains. *J. Bacteriol.* 145, 1052-1062.

UNDP/WORLD BANK/WHO. (1990). Fermentation procedures pg 33-45. *In* Dulmage, H. T., A. A. Yousten, S. Singer, L. A. Lacey (ed). Guidelines for production of *Bacillus thuringiensis* H - 14 and *Bacillus sphaericus*. *TDR/BCV/90.1.*

Van Rie, J., S. Jansen, H. Hofte, D. Degheele, and H. Van Mellaert. (1990). Receptors on the brush border membrane of the insect midgut as determinants of the specificity of *Bacillus thuringiensis* δ-endotoxin. *Appl. Enviro. Microbiol.* 56, 1378-1385.

Whiteley, H. R., and H. E. Schnepf. (1986). The molecular biology of parasporal crystal body formation in *Bacillus thuringiensis*. *Ann. Rev. Microbiol.* 40, 549-576.

World Health Organization. (1991). Malaria pg. 20-40. *In* Godal, T (ed). Tropical diseases, progress in research, 1989-1990. World Health Organization, Geneva, Switzerland.

Yousten, A. A., and M. H. Rogoff. (1969). Metabolism of *Bacillus thuringiensis* in relation to spore and crystal formation. *J. Bacteriol.* 100, 1229-1236.

Yu, Y. M., M. Ohba, and S. S. Gill. (1991). Characterisation of mosquitocidal activity of *Bacillus thuringiensis* subsp. *fukuokaensis* crystal proteins. *Appl. Environ. Microbiol.* 57, 1075-1081.