MECHANISMS FOR SELECTIVE TOXICITY OF LOCALLY ISOLATED STRAINS OF *BACILLUS THURINGIENSIS* (BERLINER).

By

EUCHARIA UNOMA KENYA

B.Sc. Zoology, UNN

M.Sc Parasitology, ASUTECH

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FACULTY OF SCIENCE,

RIVERS STATE UNIVERSITY OF SCIENCE AND TECHNOLOGY,

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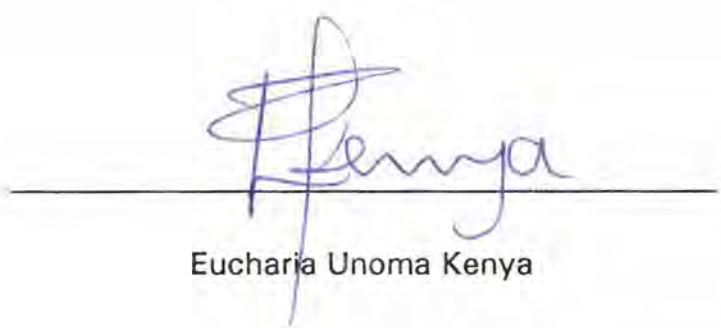
DEDICATION

THIS WORK IS DEDICATED TO MY CHILDREN,

ESSAO KENYA AND AMANDA KENYA.

DECLARATION

THIS IS TO CERTIFY THAT THE WORK REPORTED HERE IS MY ORIGINAL STUDY. IT HAS NOT BEEN SUBMITTED TO THIS OR ANY OTHER UNIVERSITY FOR ANY OTHER DEGREE. ALL HELPS ARE DULY ACKNOWLEDGED.



Eucharia Unoma Kenya

CERTIFICATION

WE CERTIFY THAT THIS WORK WAS CARRIED OUT BY

EUCHARIA UNOMA KENYA

AT THE INTERNATIONAL CENTER OF INSECT PHYSIOLOGY AND ECOLOGY (ICIPE), NAIROBI, KENYA, UNDER OUR GUIDANCE AND SUPERVISION.

DR. E. O. OSIR

ICIPE, P.O. BOX 30772,

Nairobi, Kenya.

DR. B. A. OKWAPKAM

Rivers' State Univ.of

Science and Technology,

Port.Harcourt., Nigeria.

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ABSTRACT

Bacillus thuringiensis is a gram-positive soil-dwelling bacterium. It produces a crystalline insecticidal protein (delta-endotoxin), during the sporulation phase of its growth. Several strains have been isolated and the specificity in its insecticidal action on different hosts is an attractive aspect for its use in biological control. Investigations were carried out into the fermentation of two isolates, M37/2 and TIKKI. In order to optimize the culture conditions in shake-flask cultures, a range of media were studied with respect to bacterial growth and δ-endotoxin production (i.e. biological activity). Of all the media tested, M3 and M4 recorded highest production of M37/2 and TIKKI respectively. Biological activity did not correlate to total bacterial numbers. Physical studies were carried out on three isolates of *B.thuringiensis*: TIKKI, MF4B/2 and M37/2. Each has insecticidal activity against *Glossina* species (Diptera: Glossinidae), *Chilo partellus* Swinhoe (Lepidoptera: Pyralidae) and *Spodoptera exempta* Walker (Lepidoptera: Noctuidae), as well as Culicine and Anopheline mosquitoes. Electrophoretic separation of crystals from the three isolates revealed major bands of $M_r \sim$ 140-25 kd. Further detailed study on the TIKKI isolate showed that the crystal had two main bands of $M_r \sim$ 120 kd and 66 kd. Solubilized crystal (protoxin) revealed a band of $M_r \sim$ 64 kd while the activated toxin was approximately $M_r \sim$ 62 kd. Immunological experiments using antibodies raised against TIKKI toxin gave a strong cross reactivity

between TIKKI and MF4B/2 isolates, but not with M37/2 isolate. Brush border membrane vesicles (BBMV) and soluble proteins prepared from miguts of *G. m. centralis* Machado and *C. partellus* were used to study the interaction with *B. thuringiensis* protoxin/toxin. The Protoxins/toxins were ^{125}I-radioiodinated. Studies with BBMV from *C. partellus* showed three bands of M_r ~ 68 kd, 64 kd and 28 kd for MF4B/2 isolate. A single band of M_r ~ 64 kd was observed for *G. m. centralis* and TIKKI isolate. Homologous competition experiments showed that labelled toxin retained its ability to interact with BBMV even in the presence of a 100-fold higher concentration of unlabelled toxin. Gel filtration techniques using solubilized midgut proteins incubated with ^{125}I-toxin showed interaction with detergent extracted aqueous protein but not with buffer soluble proteins. Histopathological studies showed that all cells (except the giant cells of the mycetome) were susceptible to both TIKKI and MF4B/2 toxins, with the latter being more pathogenic. M37/2 toxin was not toxic to midgut cells. The extent of cell damage can be related to length of exposure to, as well as, the concentration of the toxin. Studies carried out on the inhibitory effects of *B. thuringiensis* crystal from TIKKI isolate on the activity of midgut membrane ATPase showed a non-competitive inhibition. The V_{max} of the enzyme activity was effectively lowered by >50%. The concentration of K^+ ion in the ATPase assay medium was found to affect activity with a peak at 80 Mm. The concentration of Na^+ ion on the other hand had no apparent effect on ATPase activity.

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CHAPTER 1.

INTRODUCTION.

1.1 General introduction.

Bacillus thuringiensis (Berliner) is a soil bacillus, distinguishable from the closely related species *B.cereus*, by a large insecticidal parasporal inclusion (crystal) that appears during sporulation. This is the singular most important characteristic of *B.thuringiensis* because it confers it with the unique insecticidal properties. *B.thuringiensis* is a gut poison and acts within the insect midgut after ingestion. The bacillus was first isolated in Japan by Ishiwata, in 1901, from diseased silkworms (*Bombyx mori*).

Within several decades, numerous field applications with *B.thuringiensis* were carried out with varied success. However, the development of *B.thuringiensis* remained in the shadow of successful chemical insecticides. The subsequent development of resistance by many of the target pests to the chemicals, the disturbance in the balance of insect populations and the well cited environmental impact of many chemical insecticides served to renew interest in *B.thuringiensis* research.

Several commercial preparations of *B.thuringiensis*-based products, which are basically spore/crystal formulations are available following the first; Sporine (*B.t.thuringiensis*) formulated in 1939 (Luthy *et al.*, 1982). Most successes

with field applications have been against lepidopteran and dipteran pest species. *B.thuringiensis* has several benefits as a biocide, the most important of these being its biodegradability (Burges *et al.*, 1975), safety and species specificity. It is also relatively easy to culture large quantities while the final product which is delivered to the user in several forms (wettable-powder, liquid or flowable briquette or granular formulations) is stable for about one year.

B.thuringiensis therefore provides an attractive alternative to harmful chemical insecticides. In addition, the realization that the gene was plasmic encoded provided a straight forward approach to cloning the gene. This in turn has opened up a new area for genetic manipulations of the organism thus providing a more effective plant protection strategy.

Economically, the importance of *B.thuringiensis*-based bioinsecticides has grown tremendously over the last two decades. For instance, while the current market for *B.thuringiensis* products is estimated at approximately US $ 107 million, this is expected to reach US $ 300 million by 1999 (Feitelson *et al.*, 1992).

In spite of these attractive attributes and the realization that *B.thuringiensis* is a potentially useful insecticide, its use in Africa has so far been very limited. *B.t. israelensis* has been used for the control of blackflies (*Simulium* species) as part of the World Health Organization campaign in Central and West Africa with a measurable level of success (Guillet *et al.*, 1990).

A major problem in the use of *B.thuringiensis* is the narrow host range of any isolate. Moreover, many important crop pests are not susceptible to any of the known *B.thuringiensis* strains. Therefore, there is a continuing search for novel strains that can either be isolated or genetically created. Another limitation in field application of *B.thuringiensis* is its low resistance to environmental factors. Repeated applications are, therefore, necessary to achieve any reasonable level of control (Aronson *et al.*, 1986).

In addition, the general assumption has been that resistance to *B.thuringiensis* by susceptible insect species is unlikely to occur particularly because of its unique mode of action. However, increasing evidence shows that these assumptions may be false. Several studies now show that under laboratory conditions, major lepidopteran pest species have developed appreciable levels of resistance to *B.thuringiensis* toxins. These include *Plodia interpuctella* Hubner (McGaughey, 1985; McGaughey and Johnson, 1987), *Heliothis virescens* F. (Van Rie *et al.*, 1990b), *Leptinotarsa decemlineata* Say (Stone *et al.*, 1989) and *Plutella xylostella* Linnaeus which is also reported to have evolved high levels of resistance in the field (Kirsch and Schmutterer, 1988; Tabashnik *et al.*, 1990). At least in two species, *P.interpunctella* and *P.xylostella*, resistance is reportedly due to a reduction in the binding affinity of receptors or binding sites in the brush border membrane vesicles (BBMV) of insect midgut (Van Rie *et al.*, 1990a; Ferre *et al.*, 1991). Also, Gould *et al.* (1992) reported broad spectrum resistance to several strains of *B.thuringiensis*

that differ significantly in structure and function, contrary to earlier held view that resistance where it develops, is restricted to a single group of closely related *B.thuringiensis* toxins.

The mechanism involved in resistance appears to be the same as that involved in host specificity of *B. thuringiensis* δ-endotoxins. Consequently, the need to adequately understand the toxin-receptor interaction is extremely crucial. Furthermore, identification and possible characterization of a wide range of receptors from different insect species will provide much needed information. This will assist in the formulation of *B.thuringiensis*-based bioinsecticides, with the aim of eliminating most of the limiting factors in its use and especially guard against the development of resistance.

1.2 Rational for the study.

B.thuringiensis proves to be a potential and powerful tool in insect pest management, providing a safe alternative to chemical insecticides. Formulations of *B.thuringiensis* have been used for over two decades as bioinsecticides in the control of agricultural pests (Deacon, 1983) and insect vectors of a variety of human diseases (Service, 1980).

There is need to improve some areas in *B.thuringiensis*, use/application, especially the delivery system for either the *B.thuringiensis* or their toxins since traditional products are relatively unstable in the environment; increasing potency and designing future products that will prevent or at least delay the

development of resistance; and thirdly, broaden the host range. These can be achieved only when the mode of action and the underlying mechanisms for selective toxicity are fully studied and understood. This work will therefore add to current knowledge and help to further elucidate these underlying mechanisms.

Characterization of the toxic moiety in addition to being an essential step in understanding the mode of action, would give impetus to genetic manipulations which could enhance effectiveness. The cloning of insecticidal crystal protein genes (Whiteley Schnepf, 1986) and their expression in plant-associated organisms (Obukowicz *et al.*, 1986) or transgenic plants (Barton *et al.*, 1987; Vaeck *et al.*, 1987) have provided attractive strategies for the protection of crops against insect pests.

This research work was designed with the broad aim of determining the mechanisms for selective toxicity in locally isolated strains of *B.thuringiensis* that has entomopathogenic activities against the following species of insects: *Chilo partellus* Swinhoe (Lepidoptera:Pyralidae), *Spodoptera exempta* Walker (Lepidoptera:Noctuidae), *Glossina* species (Diptera:Glossinidae) and both Culicine and Anopheline mosquitoes. These insects were chosen to serve as model systems in which the interactive processes that occur between the insect and *B.thuringiensis* δ-endotoxin will be studied.

The specific objectives included the following:

1. To optimize the conditions for culturing *B. thuringiensis* and for the isolation, purification and solubilization of the δ-endotoxin.
2. To carry out studies on physical and immunological characterization of the δ-endotoxin.
3. To establish the presence of binding sites (receptors) for the δ-endotoxin in the insect midgut membrane.
4. To carry out studies on the effect of *B.thuringiensis* δ-endotoxin on the activity of insect midgut ATPase

CHAPTER 2.

LITERATURE REVIEW.

2.1 General properties of *B.thuringiensis*.

B.thuringiensis is an aerobic, gram positive, spore-forming, soil-dwelling bacterium. It is highly ubiquitous with thousands of isolates having been made. *B.thuringiensis* is characterized by the production of proteinaceous crystalline inclusions which appear concurrently with the spore during sporulation (Bechtel and Bulla, 1976; Tyrell *et al.*, 1979; Mikkola *et al.*, 1982). The crystal protein is synthesized *de novo* within the protein turnover that takes place during spore formation (Somerville, 1971). The inclusions are variously referred to as delta-endotoxin (δ-endotoxin), parasporal bodies and insecticidal crystal proteins (ICPs). *B.thuringiensis* produces in addition to the δ-endotoxins, α-exotoxin, β-exotoxin and a louse factor (Burges, 1981). The α- and β-exotoxins are water soluble. The host range covers not only invertebrates but vertebrates and even microorganisms (McConnell and Richards, 1959; Heimpel, 1967; Krieg, 1971). In contrast, the δ-endotoxin is water insoluble and exhibits a limited host range (Faust and Bulla, 1982).

2.2 Fermentation of *B.thuringiensis*.

Factors that affect bacterial growth and δ-endotoxin production during fermentation are related to the medium and other culture conditions mainly pH, temperature and aeration. Of these, the composition (quantitative and qualitative) of the culture medium is the most important. Several authors have been able to demonstrate a direct relationship between the growth of the organism and the medium. Scherrer et al.(1973) for instance, showed that a relationship exists between glucose concentration and δ-endotoxin production.

Irrespective of differences in the nutritional needs between various *B.thuringiensis* strains, the formulation of a fermentation medium must take into account the physiological requirements of the organism in order to achieve optimal biomass and more importantly, biological activity. For large scale production, cost obviously becomes an important issue.

Although the primary aim of growing *B.thuringiensis* is to get a good yield of the endotoxin, few studies have been done to specifically improve growth conditions to maximize crystal production. Under optimal conditions growth and sporulation (and by extension δ-endotoxin production) of several strains are reported to be maximized by high levels of aeration at a temperature of 20° C-32° C (Dulmage and Rhodes, 1971; Dulmage, 1981 and Luthy *et al.*, 1982). In addition, Dulmage (1981) reported that a balance of nitrogen and carbon levels is essential to prevent severe pH fluctuations during vegetative growth which may inhibit the process of fermentation.

2.3 Classification of *B.thuringiensis*

The classification of *B.thuringiensis* has been difficult due to the large number of strains that have been isolated over the years. A current and conservative estimate puts the figure at 40,000 (Lambert and Peferoen, 1992). Basing their classification on morphological and biochemical characteristics, Heimpel and Angus (1959) made the first substantial effort to classify *B.thuringiensis*. The classification was, however, revolutionalized by de Barjac and Bennefoi (1968), who developed a nomenclature system based on serological analysis of vegetative cell flagellar (H) antigens supplemented by biochemical characteristics such as the number of constituent polypeptides in the dissolved crystal protein. Using this system, current classification recognizes 36 *B.thuringiensis* subspecies (serovars) based on both serological, microbial (biochemical) characteristics and host range information (Dulmage and Aizawa, 1982; de Barjac and Frachon, 1990). As important as this system has been in the classification of *B.thuringiensis*, it has a major short-coming because it fails to consistently reflect the structure or vast diversity in insect specificity of the δ-endotoxin. Hofte and Whiteley, (1989) using information from the structure, antigenic properties and activity spectrum classified the *B.thuringiensis* crystal proteins and their genes into four major groups (comprising 14 distinct "Cry" genes): Cry 1 (Lepidoptera-specific), Cry 11 (Lepidoptera- and Diptera-specific), Cry 111 (Coleoptera-specific), and Cry 1V (Diptera-specific). Recently, two new classes of nematode-active toxins; Cry

V and Cry V1 have been added to the Hofte and Whiteley classification (Feitelson *et al.*,1992). In addition, a few *B.thuringiensis* crystal toxins display a broad spectrum cytolytic activity *in vitro* while still retaining specific insecticidal action *in vivo* towards dipteran insects. These are referred to as "Cyt" genes. This was based on their structure, antigenic properties, and activity spectrum. Attempts have also been made to classify the *B.thuringiensis* crystal into pathotypes based largely on insecticidal activity and to a lesser extent on shape and protein characteristics (Hofte *et al.*, 1988). No one classification system seems to be generally acceptable but for rapid identification of new isolates, the system of de Barjac and co-workers remains the major technique in use.

2.4 The δ-endotoxin *of B.thuringiensis*.

The δ-endotoxin accounts for 20-30% of the cell dry weight (Rogoft and Yousten, 1969) and accumulates as an inclusion either within (Aronson and Fitz-James, 1976) or outside the exosporium (Debros *et al.*, 1986). Considering the amount of protein that is utilized in δ-endotoxin production, it must represent an important physiological step for the *B.thuringiensis*, probably conferring a selective advantage over other spore-formers (Ellar, 1990). The midgut of susceptible insects usually has a high pH which prevents germination of ingested spores. The δ-endotoxin solves this problem because through its action the gut wall is broken down allowing blood to mix with gut contents thereby lowering the pH. The ingested spores can then germinate and

draw its nutritional needs from the body of the dead insect.

The crystal is characteristically bipyramidal in shape but other shapes, such as square, flat or amorphous have also been reported (Grigorova and Kaucheva, 1966; Ibarra and Federici, 1986). The shape of the crystal is strain-specific.

The δ-endotoxin crystals differ in their insecticidal spectra (Krywienczyk *et al.*, 1978). The majority of strains that have been described show insecticidal activities against Lepidoptera (Dulmage, 1981), with fewer strains being insecticidal to Diptera (Goldberg and Margalait, 1977; Drummond *et al.*, 1992), Coleoptera (Herrnstadt *et al.*, 1986; Krieg *et al.*, 1983) and very recently the Helminthic roundworms, Nematoda (Meadows, *et al.*, 1990). It can be assumed that with greater effort at screening for new isolates, *B.thuringiensis* strains that are effective against other insect species will be identified.

There is usually one insecticidal inclusion body per cell but two or more have been observed (Bechtel and Bulla, 1976). Ibarra and Federici (1986) revealed that the δ-endotoxin of *B.thuringiensis* subsp. *isrealensis* contained three major inclusions held together by a coat-like envelope (Huber and Luthy, 1981) and could be differentiated on the basis of electron opacity, size and, to some extent shape.

In its natural state, the crystal is bare of biological activity either *in vivo* as demonstrated by direct injection of crystal suspension into insect haemocoel

or *in vitro* when assayed in a sensitive tissue culture system (Huber and Luthy, 1981).

The natural *B. thuringiensis* crystal under the electron microscope reveals a regular fine structure consisting of a single protein unit held together by disulfide bridges (Dastidar and Nickerson, 1979; Huber *et al.*, 1981). The insolubility of the crystal is partly conferred by these disulfide bonds (Bietlot *et al.*, 1990). Although a similarity in structure seems to cut across the *B. thuringiensis* species even at the molecular level (Choma *et al.*, 1990), an exchange of any given set of regions between two crystal proteins often does not result in a functional hybrid (Convents *et al.*, 1990, 1991). The presence and amount of carbohydrate in *B. thuringiensis* δ-endotoxins has been reported by several authors. Estimates of the quantity of carbohydrate from various strains range between 5-12% and the primary sugars detected were glucose and mannose, with lesser amounts of xylose and arabinose. (Bateson and Stainsby, 1970; Bulla *et al.*, 1977; Nickerson, 1980; Tyrell *et al.*, 1981).

Within the alkaline environment of a susceptible insect midgut, the δ-endotoxin dissolves. The precise number and size of polypeptides (protoxin which is biologically inactive) released depend on the *B. thuringiensis* strain and the insect midgut environment, ranging from 130-140 Kilodalton (Kd) (Bulla *et al.*, 1981; Amstrong *et al.*, 1985; Herrnstadt *et al.*, 1986) . Generally one of the polypeptides is mostly responsible for the toxic activity. The solubilized protoxin molecule is further proteolytically activated by midgut and crystal

associated proteases into a stable protease resistant toxic core fragment (toxin) (Lecadet and Martouret, 1965; Fast and Martin, 1980; Hofte and Whiteley, 1989). The biochemical events that underline the activation of protoxin to an active toxin has received attention very recently. Choma *et al* (1991) studied possible conformational changes that could occur during the activation process. They observed that as no internal cleavages were generated during the process, the polypeptide chain of the toxic moiety in the protoxin is the same as in the activated toxin. The protease resistant toxic moiety which are smaller polypeptides are generated by the cleaving off of the C-terminal (structural) fragment of the protoxin (Choma *et al.*, 1990). The toxin which is therefore derived from the N-terminal half of the protoxin is described as having dual functional domains: a toxic and a binding domain (Hofte *et al.*, 1986; Choma *et al.*, 1990).

Although differences in the specific activities of the δ-endotoxin from various *B.thuringiensis* strains are well documented (Jaquet *et al.*, 1987; Lecadet and Martouret, 1987), the mechanisms for this selective toxicity is not adequately understood. Several reasons have been suggested to explain this but generally, factors that determine specificity would be expected to reside at different stages in the overall insect-toxin interaction. Firstly, quantitative and qualitative differences in the δ-endotoxin production between different strains could influence specificity (McGaughey and Whalon, 1992). Secondly, in certain cases the distinct host range of a given δ-endotoxin is accompanied by

a characteristic pattern of polypeptides in the native crystal, suggesting that in these cases specificity might reside in unique proteins (Yamamoto and McLaughlin, 1981; Aronson et al.,1991). Thirdly, specificity may reside in differences in the biochemistry of the midgut between various insect groups. The high pH (7.0 - 9.1) and gut proteases which characterize insect midgut environment (Berenbaum, 1980) and required for toxin activation, raises the possibility that variations in these midguts generate toxins of varying potency and specificity (Haider *et al.*, 1986, Oppert *et al.*, 1994). Cheung and Hammock (1986) are of the opinion that methods in activation and subsequent separation would therefore be important in studies using *in vitro* systems. Haider and Ellar (1986) for instance reported dual specificity of a single δ-endotoxin originating from a strain of the subsp *aizawai* and expressed by a cloned gene in *E.coli*. Trypsin-treated δ-endotoxin was toxic only towards lepidopteran cultured cells, whereas subsequent treatment of the trypsin digested δ-endotoxin with gut juice of *A. aegypti* Linnaeus (Diptera:Culicidae) generated a protein which was almost exclusively toxic against dipteran cell lines. In addition, the δ-endotoxin is known to bind to receptor in the midgut cells of susceptible insects. The importance of binding sites was highlighted by Huber and Luthy (1981). They reported that gut juices from *Pieris brassicae* and *Spodoptera littoralis* were able to digest the protoxin of *B.t.Kurstaki*, with the product of both digestion showing high specific activity against *P.brassicae*. This was not the case with *S.littoralis*, an insect that is not affected by

B.t.Kurstaki. This demonstrated that the ability to solubilize and even activate the protoxin confers no specificity even for closely related species.

2.5 Mode of Action of *B.thuringiensis* δ-endotoxin.

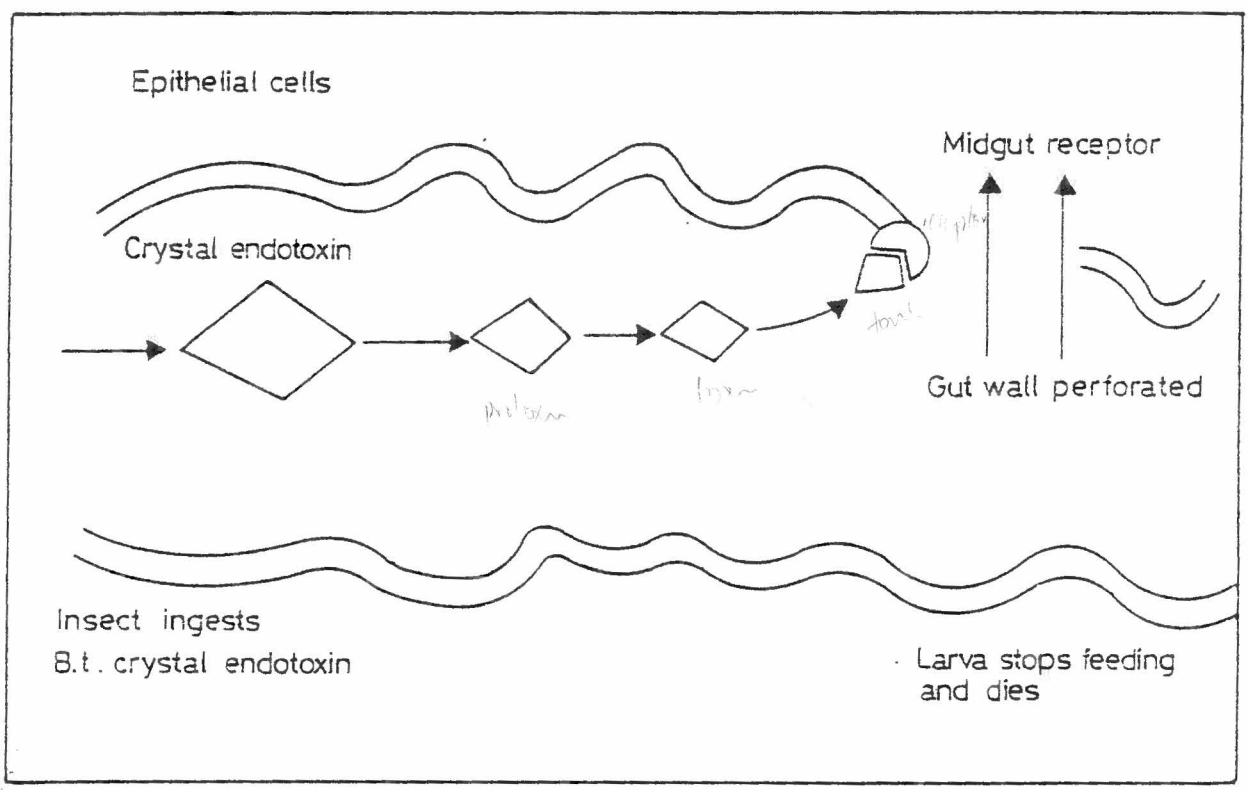
The δ-endotoxin of *B.thuringiensis* acts very quickly after it has been ingested by a susceptible insect (Ebersold *et al.*, 1977, 1978; de Barjac, 1978; Endo and Nishiitsutsuji-Uwo, 1980; Percy and Fast; 1983). The target of the activated toxin is the plasma membrane of midgut cells. Within a few minutes, these become leaky to small ions and show pathological changes that lead eventually, to the death of the insect (Fast and Donaghue, 1971). While the midgut columnar cells are damaged within minutes, structural changes in the goblet cells are usually visible only at a later stage.

The first external symptoms observed in affected insects are paralysis and cessation of feeding activities (Heimpel and Angus, 1959). Several studies show that damage to the midgut and the breakdown of permeability control occur as a consequence of the δ-endotoxin (Fast and Morrison, 1972; Fast and Donaghue, 1971; Ebersold *et al* 1980). The subsequent lowering of the gut pH allows *B.thuringiensis* spores to germinate and invade the haemocoel where

they rapidly multiply in the nutrient rich haemolymph. The ensuing septicemia has been suggested to contribute to insect mortality, especially in less sensitive species in which the toxin alone cannot bring about mortality (Heimpel and Angus, 1959; Ebersold *et al.*, 1978). *B.thuringiensis* spores are however not effective when administered alone without the crystals. Khawaled *et al.*(1992), were able to show, for instance, that the larvae of *A. aegypti* can in fact digest and utilize the products of digestion from *B.t.isrealensis* spores. Spores alone show limited toxicity which is attributed to some crystal protein that forms part of the spore coat during sporulation (Lecadet *et al.*, 1972).

Histopathological studies suggest that the δ-endotoxin acts on the surface of the brush border membrane and that penetration through the membrane is not a prerequisite for the expression of full toxicity. Fast *et al* (1978) demonstrated this by showing that labelled *B.t.Kurstaki* toxin attached to sephadex beads too large to enter lepidopteran cells in tissue culture still lowered ATP levels while acting from the surface of the cells. The sequence of events seems to suggest that the structural changes might not coincide with the first step of the molecular mode of action of the endotoxin (reported by Fast and Donaghue 1971). Ebersold *et al.* (1980) demonstrated that the morphological changes observed in toxin-treated *Choristoneura fumiferana* Clemens cultured cells occur simultaneously with the collapse of membrane permeability for a cytolytic enzyme, lactate dehydrogenase (LDH). They further showed that the amount of LDH detectable outside the cells was directly

proportional to time and the membrane becomes increasingly permeable even for high molecular weight compounds. Other authors (Knowles and Ellar, 1987), demonstrated toxin induced permeability changes for certain ions (Rh^+, H^+ and CrO_4^+) in *C.fumiferana* cells. They also suggested that the toxin first binds specifically to a membrane bound receptor and then subsequently generates or induces the formation of holes or pores (0.5-1.0 nm in diameter). The cellular osmotic balance is disturbed causing equilibration of ions which leads to a net influx of water, the cells then swell and lyse in what has been termed "colloid-osmotic lysis". They also showed that cell lysis was partially prevented by osmotic pressure induced by high amounts of sugars present in the culture medium. The entire process involved from the time of toxin ingestion to breakdown of the midgut membrane is illustrated in Scheme 1.



Scheme 1 Mode of action of *B. thuringiensis* through receptor mediated pore formation.

In addition to the receptor mediated events, the δ-endotoxin has been shown to demonstrate inhibitory action on midgut ATPase which sustains a potassium (K^+) ion electrogenic pump across the midgut membrane. This pump functions to effectively move K^+ ions into the cells against a concentration gradient and was probably first described in detail by Harvey and Nedergaard (1964). It is reportedly restricted to the goblet-cell apical membrane (Dow *et al.* 1983; Wieczorek *et al.* 1986). The potential difference generated by this pump across epithelia is also thought to be utilized for nutrient uptake (Giordana *et al.* 1985).

Experiments performed using isolated midguts of lepidopteran insect larvae showed that the net flux of K^+ ions across the midgut from the haemocoel- to the lumen-side is decreased by the δ-endotoxin (Harvey and Wolfersberger, 1979; Griego *et al.*, 1979; Gupta *et al.*, 1985). Two components are thought to contribute to the net flux of K^+ ions: an active ATP-dependant transport of K^+ ions from the haemocoel- to the lumen-side and the passive leak of K^+ ions back from the lumen to the haemocoel side.

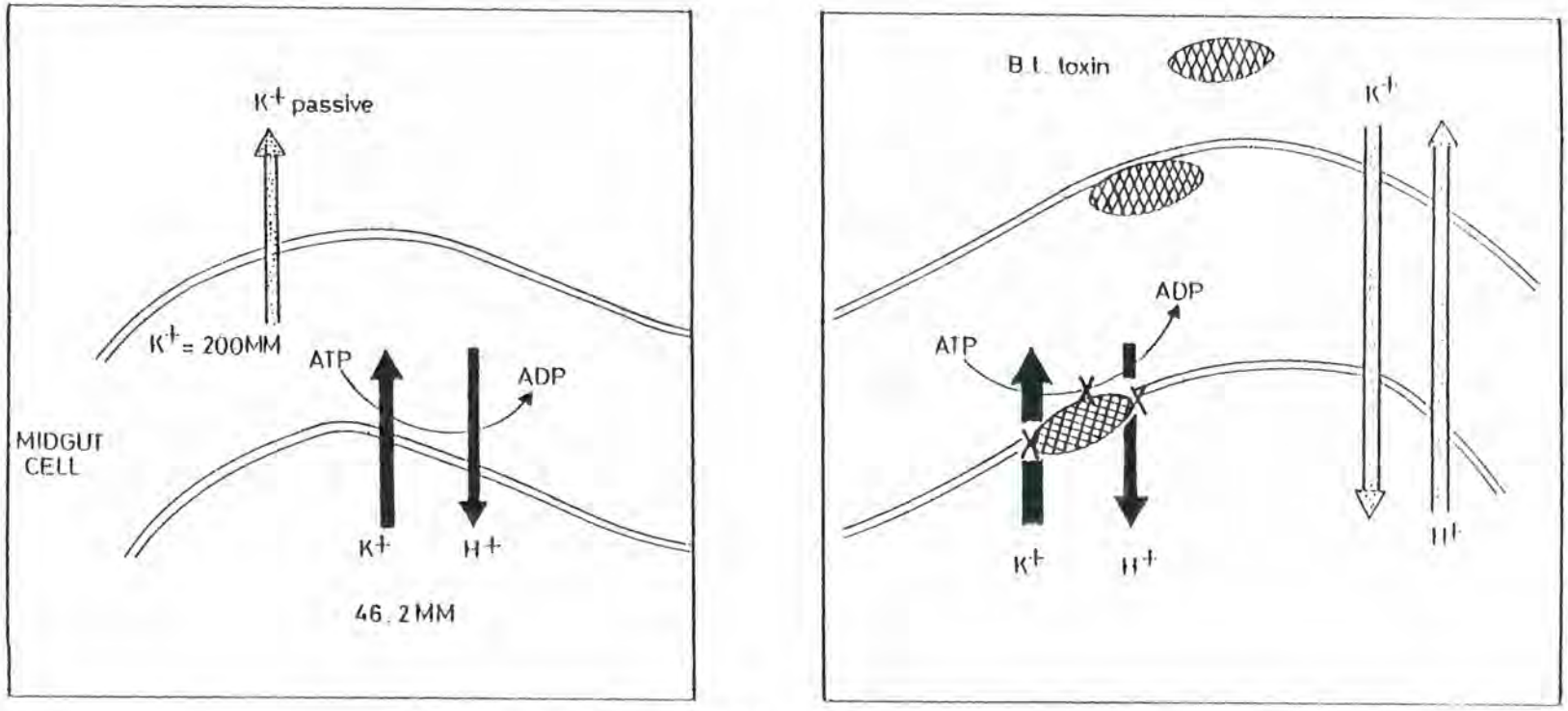
Harvey and Wolfersberger (1979) and Griego *et al.* (1979) confirmed an earlier observation by Fast and Morrison (1972) and concluded that the toxin enhances the K^+ ion permeability of the brush border membrane of columnar cells and, therefore, increases the passive leak of ions back from the lumen to the haemocoel side. Further evidence also comes from work done by Crawford and Harvey (1988), who were able to demonstrate the protective effect of

barium, a K^+ channel blocking agent, towards isolated *Manduca sexta* midguts from toxin effects. Knowles and Ellar (1987) found that the 27 Kd cytolytic toxin of *B.t.isrealensis* form cation-selective channels in planar lipid bilayers in the absence of specific receptors. In addition, it has been shown that the *B.t.isrealensis* toxin can intercalate into artificial phospholipid vesicles, inducing cation-selective leakage as well as leakage to water into the vesicles (English and Readdy, 1989). Slatin *et al.* (1990) made similar observations with activated 55 Kd Cry 1A(C) and the 67 Kd Cry 111A toxins. These results suggest very strongly that the endotoxin itself is creating the cation-selective K^+ channel. Since the maintenance of a steady state of the K^+ gradient across the insect midgut is a prerequisite for its normal physiological functions (Dow, 1986), changes in K^+ permeability might well contribute to the toxic effect of the δ-endotoxin (Scheme 2).

As an extension of this theory, Gupta *et al.* (1985) have suggested that the primary effect of the δ-endotoxin is actually on the K^+-ATPase. This is a transport ATPase located in the apical membrane of goblet cells and responsible for the primary coupling of ATP hydrolysis to the active transport of K^+ ions across several insect transporting epithelia. These include the midgut of lepidopterous larvae, salivary glands of flies and malpighian tubules of several insect species (Harvey *et. al.*, 1983). In the midgut of lepidopterous larvae, the transport ATPase sustains the movement of K^+ ions from the haemocoel- to the lumen-side. English and Cantley (1986) showed the inhibition of a purified dog

kidney [Na^+-K^+]- ATPase activity by the toxin of *B.t.Kurstaki* strain. There is, however, reasonable doubt as to whether the system used by English and Cantley (1986) represents an appropriate model as it is known that the insect ATPase is a K^+ ion dependent ATPase. This is demonstrated by its insensitivity to oligomycin or azide, vanadate or fluoride and ouabain (Wieczoreck, 1982; Wieczoreck and Gnatzy, 1985). These compounds inhibit the mitochondrial H^+-ATPase, the veterbrate [K^+-H^+]-ATPase and the [Na^+-K^+]-ATPase, respectively. English and Cantley (1986) also claimed that the inhibition of a K^+-ATPase present in cultured cells of *M. sexta* was due to toxin activity. However, subsequent trypsination of the toxin prevented this inhibitory action. Since it is well established that toxin is resistant to trypsin activity (Hofte and Whiteley, 1989), the effects they observed probably do not reflect a true toxin action. It might well be due to another factor present in the toxin preparation rather than the δ-endotoxin itself.

Sacchi *et al.* (1986) studied toxin activity on isolated brush border membrane vesicles of the larval midgut of *P.brassicae*. The inwardly directed and K^+ dependent amino acid transport across the membrane was inhibited by the toxin, whereas the Na^+-dependent transport was not. They presented further evidence to show that the K^+ ion permeability of the vesicles was affected rather than the amino acid transport itself. The inhibitory effect of *B.thuringiensis* on K^+ dependent ATPase is illustrated in Scheme 2.



Scheme 2 Mode of action of *B.thuringiensis* through the inhibition of midgut ATPase

2.6 Membrane binding sites for *B.thuringiensis* toxins.

Knowles and Ellar (1986) demonstrated endotoxin binding to *in vitro* cultured cells of non-midgut origin. However, proof of high affinity saturable binding was not presented. Using labelled toxin, Hofmann *et al.*(1988a) showed that the primary target of *B.thuringiensis* endotoxin is the brush border membrane vesicles (BBMV) of the midgut epithelial cells. Results from other studies suggest that there is a correlation between toxicity and binding specificity (Van Rie *et al.*, 1990a). Wolfersberger (1990) reported a case in which there was negative correlation between receptor affinity and toxicity which seemed to suggest that binding of the toxin is not sufficient for toxicity. However, Lee *et al.*(1991) presented quantitative data to show that toxicity was definitely related to binding affinity between *B.thuringiensis* HD1 toxin and BBMV from *Bombyx mori*. It has also been suggested that more than one type of receptor may exist in the midgut (McGaughney and Johnson, 1987; Van Rie *et al.*, 1990a). Competitive binding studies conducted on *P.brassicae* (Hofmann *et al.*, 1988b) and *Heliothis virescens* (Van Mellaert *et al.*, 1988) indicated the existence of a family of receptors in the midgut epithelium. These differ between insects and are recognized by different classes of ICPs (Hofmann *et al.*, 1988b).

The biochemical nature of the δ-endotoxin receptors is not adequately known but preliminary studies on the BBMV suggest that the receptors consist of a protein component and is more probably a glycoprotein (Knowles and Ellar,

1986; Van Mellaert *et al.*, 1988). Knowles *et al.* (1984) had earlier demonstrated that BBMV were sensitive to treatment with proteases but not to phospholipase C suggesting that the receptors were most probably glycosylated proteins. The receptors could also be glycolipids as reported by Thomas and Ellar (1983b) who described the binding of *B.t.israelensis* delta-endotoxin to specific phospholipids in the plasma membrane. This subsequently caused rapid cytolysis suggested to be a detergent-like rearrangement of these lipids. If the receptors are phospholipids or at least have a lipid component, this might well explain the finding that *B.t.israelensis* activated *in vitro* is cytolytic to a wide variety of cell types, including mammalian cells (Cheung *et al.* 1987), since phospholipids enjoy an ubiquitous presence in eukaryotic membranes.

Work by Haider and Ellar (1987) suggests also that D-glucose is a part of the receptor site or at least plays a significant role in the endotoxin-receptor interaction. Another sugar, N-acetyl galactosamine has also been implicated as part of the receptor that recognizes *B.thuringiensis* toxins (Knowles and Ellar, 1986; Knowles *et al.*,1991; Garczynski *et al.*, 1991). However, little is known about the carbohydrate moieties and their possible role in the toxin-receptor interaction. To date, no study has been carried out to conclusively demonstrate whether the carbohydrate moieties are important for the full expression of toxicity. Available information is largely controversial. However, results from an experiment performed on a different species of

bacillus, *B.sphaericus*, by Nielson-Leroux and Charles (1992) showed that high concentrations of sugars did not inhibit the binary toxin from this species from binding to its receptor. The authors therefore concluded that the sugar moieties are not likely to be involved in either the recognition or binding of the toxin in *Culex pipiens* larvae.

The binding of activated toxin to a receptor is not a simple reversible process. Wolfersberger *et al.* (1986) demonstrated that the initial reversible binding observed is quickly followed by an irreversible interaction between the toxin and the midgut membrane. They further suggested that this irreversible step involves the insertion of at least a portion of the toxin molecule into the membrane thereby initiating the formation of a pore.

Research work on receptor identification has progressed rather slowly. Haider and Ellar (1987) identified receptors (90-120 Kd) for *B.t.aizawai* ICI toxin in three different lepidopteran cell lines. Knowles and Ellar (1986) reported a receptor (146 Kd) they described as very specific to *B.t.kurstaki* HD1 PI toxin in *C.fumiferana* cultured cells. While using cultured cell lines to demonstrate specific binding of several toxins is encouraging, a system using proteins of midgut origin will be a more desirable model. In this direction, Hofmann, *et. al.* (1988a) reported the binding of a ^{125}I labelled 55 Kd toxin from *B.t.thuringiensis* to BBMV prepared from *P.brassicae*. From their data, the toxin-receptor complex co-migrated with the labelled toxin but they made no suggestions about the probable molecular size of the receptor. Binding was,

however, not observed with a 70 Kd protein subunit which was also labelled. Very recently, Sanchis and Ellar (1993) identified a 40 Kd and 65 Kd, and a 40 Kd protein in BBMV of *S.littoralis* which bound to Cry 1c and Cry 1A(c) toxins, respectively.

The composition of δ-endotoxin is encoded for by extrachromosomal genes located on bacterial plasmids. It has been suggested that differences in the features of these genes (and hence the endotoxins) of *B.thuringiensis* may be partly responsible for the different activity spectra of *B.thuringiensis* strains. For instance, a high degree of homology in the C-terminal amino-acid sequence has been observed although the endotoxins may exhibit different insect specificities (Chunjatupornchai *et al.*, 1988). However, the N-terminal sequences show less homology (Aronson, *et. al.*, 1986).

The mechanisms involved at the molecular level that determine specificity of different *B.thuringiensis* strains to different insect groups is not yet fully understood. While a few studies have suggested that specificity may reside in high affinity midgut membrane-bound receptors, conclusive evidence is lacking for a large number of insects. It is also not clear whether the relationship between toxicity and these receptors applies as a general rule.

CHAPTER 3.

MATERIALS AND METHODS.

3.1 Experimental insects.

Adult teneral males of *G. m. centralis*, 5th instar larvae of *C. partellus* and 1st to early 4th instar larvae of *Aedes aegypti* and *Culex quinquefasciatus*, were supplied by the Insect and Animal Breeding Unit of ICIPE.

3.2 *B. thuringiensis* isolates.

The three isolates used in this study: **TIKKI, MF4B/2** and **M37/2** which are effective against *Glossina* species; *Chilo partellus* and the mosquito species, respectively, were obtained from the ICIPE Microbial bank.

3.3 Selection of media for the culture of *B. thuringiensis.*

The media for growth of the *B. thuringiensis* under study were formulated as follows:

Table 1: Composition of test media.

	Media			
	M1*	M2**	M3	M4
Composition (g/l)				
Glucose (monohydrate)	3	1	3	3
Yeast extract	2	2	2	2
Peptone	-	-	-	5
$(NH_4)_2SO_4$	2	2	2	-
$K_2HPO_4.3H_2O$	0.5	0.5	0.5	0.5
KH_2PO_4	-	-	-	0.5
$MgSO_4.7H_2O$	0.2	0.2	0.2	-
$MnSO_4.H_2O$	0.05	0.05	0.05	-
$FeSO_4.H_2O$	-	-	0.05	-
$ZnSO_4.7H_2O$	-	-	0.05	-
$CaCl_2$	0.08	0.08	0.08	-

Legend
- ingredient is absent in media
* medium from Luthy *et. al.*, 1982
** medium from Yousten *et. al.*, 1962

A 100 ml of each medium were prepared in 250 ml Erlenmeyer flasks and pH was adjusted to about 7.2 using 1 N HCl/NaOH. The flasks were then autoclaved at 121° C for 15 min. These were inoculated with 1 ml of 15-20 h old starter culture of M37/2 and TIKKI. The starter culture was grown in Tryptone Soya Broth (Oxiod Ltd, Basingstoke, England). The cultures were incubated until sporulation (200 rpm, 30° C, Orbit Labline incubator-shaker). All flasks were incubated in duplicates and the fermentation runs repeated three times.

The cultures were observed microscopically at intervals of 0, 24, 48 and 72 h. At 48 and 72 h, Smirnoff stains (Smirnoff, 1962) were made to confirm production of δ-endotoxin in the media. Cell counts were carried out at 0 h to determine the number of starting cells and at the end of incubation, total cell and spore count were determined in each flask. Bioassays were carried out on each medium using 4th instar larvae of *A. aegypti* and teneral *G. m. centralis* for M37/2 and TIKKI isolates respectively.

From the results obtained for mosquitoes, M3 medium was studied further by varying the quantities of nitrogen and carbon. Carbon was varied between 0.3-1% (w/v) and nitrogen 0.2-0.5% (w/v) of medium. The concentration of all other components remained the same. From this variation, thirteen media were formulated and tested. Cell count, sporulation and toxin production, were determined from each experimental flask. Toxin production was determined *in vivo* through bioassays using 4th instar larvae of *A. aegypti*.

3.3.1 Determination of bacterial growth and sporulation.

Cell and spore counts were carried out to determine bacterial growth and sporulation as an assessment of the suitability of the different media formulations. Ten-fold serial dilutions were made from cultures at the end of the incubation period using sterile saline (0.85% NaCl, w/v). The samples for spore count were heat-shocked at 80° C for 10 min, then diluted as above. 1 ml from dilution 10^{-6} - 10^{-8} were transferred into separate sterile Petri dishes (disposable polystyrene, 10 x 15 mm), each in triplicate and approximately 10-15 ml of media was poured into each dish. These were then mixed well by making circular movements of the dish. The plates were left to settle and incubated at 30° C. After 24 h of incubation, bacterial colony counts were carried out. The numbers were expressed as cells/ml or spores/ml respectively.

3.3.2 Preparation of *B. thuringiensis* isolates for bioassays.

All bioassays were carried out using a crystal/spore suspension. A 72 h-old *B.thuringiensis* culture was centrifuged (3000 x g, 5 min, GSA rotor), and the supernatant solution discarded. The pellet was washed three times with ice-cold distilled water. Pellet from the last wash was suspended in 10 ml of sterile saline (0.85% NaCl, w/v). This formed the stock for the bioassays.

Before bioassays were done on *A. aegypti* with M37/2 isolate larvae were graded to confirm their instars.

3.3.2.1 Age grading of *A. aegypti* larvae.

Age grading of *A. aegypti* larvae was carried out to confirm the instar stages. Glass jars labelled A-D were half filled with dechlorinated tap water. About 200-250 eggs of *A. aegypti* were introduced into the jars and incubated at 30° C. After hatching, the mosquito larvae (1st instar) were fed with wheat germ (Country Style, Kenya). The larvae were maintained under laboratory rearing conditions (Temp 26-32° C; RH 70-80%; Photoperiod L:D 12:12). From day 2 up to day 8 after hatching, 20 larvae were randomly collected daily into small glass jars containing 70% ethanol. The alcohol was used to immobilize the larvae and preserve them over the period while being measured. With fine forceps, a larva was carefully aligned on a calibrated (1 mm divisions) glass slide and the total length recorded.

3.3.3 Screening assays.

3.3.3.1 Bioassay of TIKKI isolate against *G. m. centralis*

Blood was collected by cardiac puncture from albino rats (*Rattus norwegicus*) and defibrinated in a glass tube containing sterile glass beads. After defibrination, free-flowing blood was separated from the clot by pipetting. Nine ml of the defibrinated blood was aliquoted into sterilized tubes. To 4 of the tubes, bacterial suspensions (1 ml) from the different media were added. Blood for the control experiment was prepared in a similar manner with 1 ml of

sterile saline replacing the bacterial suspension. These were placed in a water bath at 30° C until required.

The artificial feeding apparatus was set up, with temperature maintained at 30° C. Blood with the test samples were placed at 8 spots on the metal template, to make replicates of about 2 ml each. Blood for the control assay was then placed at 2 spots, sufficiently apart, to avoid contamination from the test sample. These were covered with the artificial membrane and blood on each spot spread out gently within an area of about 8-10 mm in diameter. Ten cages with 10 teneral flies each, that had been starved for 24 h, to improve their feeding, were placed one cage per blood-covered area. The cages were then covered with a piece of black cloth. The numbers of fed flies were observed after 1 h and recorded as the numbers exposed and unexposed to the *B.thuringiensis* for the test and control experiments respectively. Mortality was recorded after 24 h.

3.3.3.2 Bioassay of M37/2 against *A. aegypti* and *C. quinquefasciatus*.

The bioassays performed with **M37/2** were carried out firstly, to determine the ability of the different test media to support a high growth rate of the bacterium and maximum production of the δ-endotoxin, secondly, to ascertain the efficacy of the isolate against different larval instars of the two mosquito species. The bioassays were performed in accordance with standard procedure (McLaughlin *et al.*1984; W.H.O. 1992). Graded larvae were used.

A 10-fold serial dilution of culture was made to a final volume of 50 ml by adding 5 ml of culture to 45 ml of tap water in a 250 ml plastic beaker. A dilution range of 10^{-5} - 10^{-10} was used in the assays. Control beakers were set up similarly but contained only 50 ml of water. Ten larvae of the test insect were added to each beaker and left at room temperature. Mortality was recorded after 24 h.

3.3.3.3 Bioassay of MF4B/2 isolate against *C. partellus*.

The bioassay was carried out according to procedure described by Brownbridge (1991). Molten (45° C) artificial diet (9 g) as modified by Ochieng, *et. al.* (1985), was mixed with 1 ml suspension of the *B.thuringiensis* and poured into plates. These plates were left aside to set before ten 5th instar larvae of *C. partellus* were introduced. Control plates were prepared in the same way but without the bacteria. The plates were incubated at room temperature. After 5 days, the contents of each plate were emptied into a small tray and the larvae separated from diet and counted. Mortality was then recorded.

3.4 Production of δ-endotoxin.

According to the results, M3 and M4 gave better results for M37/2 and TIKKI respectively. These media (M3 and M4) were used to produce δ-endotoxin for further studies. One thousand ml of the media were prepared and

200 ml aliquoted in five 500 ml Erlenmeyer flasks. The media were autoclaved and inoculated with 2 ml of starter culture. The cultures were then incubated for 72 h (200 rpm, 30° C). The medium used to culture MF4B/2 was adapted from formulations studied by Magoma and Osir (1993, unpublished). The medium was constituted using the following in g/l of distilled water; peptone, 5; glucose, 5 and supplemented with $CaCl_2$, 0.08; $MgSO_4.7H_2O$, 0.2 and 0.05 of $MnSO_4.4H_2O$, $FeSO_4.7H_2O$ and $ZnSO_4.7H_2O$ (pH 7.2).

Shake-flask cultures grown for 72 h were harvested and centrifuged at 10,000 x g, 5 min, in a GSA rotor (DuPont Sorvall Instruments Inc., California, U.S.A.). The pellet obtained was further resuspended in distilled water, vortexed vigorously and centrifuged as before. This procedure was repeated twice. After the final wash, the pellet was either resuspended in a small quantity (½ its volume) of distilled water to undergo separation of crystals from spores, or was frozen at -20° C and subsequently lyophilized into powder (Ibarra and Federici, 1986). The powder was stored at 4° C until used. This was resuspended in ice-cold distilled water prior to isolation of crystals.

3.5 Isolation of *δ*-endotoxin.

Crystals of MF4B/2 and M37/2 were separated from spores by use of gradient centrifugation employing a 40-70% (w/v) continuous sucrose gradient (modified from Thomas and Ellar, 1983a). Gradients were made using a gradient maker. Ten ml linear gradients were formed in polyallomer tubes(14

x 89 mm, Beckman Instruments Inc., California, U.S.A.). One ml of a thick slurry of pellet or resuspended powder was then layered onto the sucrose solution. The tubes were subsequently centrifuged in a SW 50.1 Ti rotor at 10,000 x g (4° C, 90 min, Beckman L8-M ultracentrifuge). Smear preparations were made from each phase as seen after centrifugation to determine the crystal phase. This phase was then pipetted out and pulled in a Falcon tube (Greiner labortechnik, GMBH, Nurtigen, Germany). The crystals were washed to remove the sucrose solution by addition of x10 its volume of distilled water and centrifuged (15 min, 27° C, 3,000 x g) in a Heraeus-Christ centrifuge. This washing process was repeated three times. The crystals were then resuspended in 1 ml of distilled water and stored at -20° C until required. For TIKKI, isolation involved centrifuging the culture (300 x g, 4° C) and washing in ice-cold distilled water three times. Pellet from the last wash was stored at -20° C until needed.

3.6 Solubilization of crystals.

Purified crystals were solubilized in alkaline sodium carbonate-bicarbonate buffer containing dithiothreitol (DTT) as previously described (Haider *et. al.*, 1986). The solubilization buffers were varied to achieve maximum dissolution of the crystals from the three *B.thuringiensis* isolates as shown in the table 2.

Table 2: Composition of solubilization buffer for the three *B.thuringiensis* isolates.

B.thuringiensis isolates	DTT	$Na_2CO_3/NaHCO_3$	pH
TIKKI	10 mM	50 mM	9.5
MF4B/2	30 mM	50 mM	10.5
M37/2	50 mM	10 mM	10.5

Solubilization was achieved by suspending 100 μg of crystal in 1 ml of buffer in eppendorf tubes (1.5 ml, Netheler-Hinz GMBH, Hamburg, Germany). Incubation was carried out at 37° C for 12 h. Insoluble material was removed by centrifugation at 30000 x g, 4° C (Eppendorf microcentrifuge, model 5451C, Netheler-Hinz GMBH, Hamburg, Germany). The supernatant solution containing the protoxin was pooled in a dialysis membrane (Spectrum Medical Industries Inc., U.S.A.) and concentrated to ¼ of its original volume using polyethylene glycol (PEG) at 4° C. Subsequently, it was dialyzed against PBS (10 mM, pH 7.2) or Tris-HCl (20 mM, pH 8.0). After dialysis, the sample was stored at -20° C.

3.7 Activation of protoxin.

Activation was achieved using commercial trypsin (Sigma chemicals Co., St Louis, U.S.A.). One hundred μg of protoxin were mixed with 500 μg of trypsin in an Eppendorf tube and incubated at 37° C for 30 min. This was

stored at -20° C until required.

3.8 Protein determination.

Protein estimations were carried out by bicinchoninic acid-binding protein analysis using Bovine Serum Albumen (BSA) (Sigma) as the standard (Smith, *et. al.*, 1986, Pierce Co., Rockford, Ill. USA).

3.9 Physical characterization of TIKKI isolate.

3.9.1 Electrophoresis.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (1970). Vertical slab gradient (4-15%) gels were cast using a gradient maker (BRL). Gels were calibrated using molecular weight standards (Pharmacia, low molecular weight standard): phosphorylase b, 94 Kd; albumin, 67 Kd; ovalbumin, 43 Kd; carbonic anhydrase, 30 Kd; trypsin inhibitor, 20 Kd; a-lactalbumin, 14 Kd; high molecular weight standards: thyroglobulin, 330 Kd; ferritin, 220 and 18 Kd; catalase, 60 Kd; lactate dehydrogenase, 36 Kd; albumen, 67 Kd). Dialyzed protein samples (30 μg) were dissolved in equal volumes of sample buffer (0.13 M Tris-HCl, 20% glycerol, 0.002% bromophenol blue, 4% SDS, 1% B-Mecaptoethanol, pH 6.8) and boiled for 3 min in a water bath before application onto the gel. Electrophoresis was carried out at a constant current of 25 mA at room temperature. After electrophoresis, gels were stained for

proteins according to the method of Reisner *et al.* (1975) using 0.6% Coomassie Brilliant Blue in a solution of acetic acid, methanol and distilled water (in ratios of 9.2:50:40.8, respectively), for 3 h at room temperature. The gels were subsequently destained with several changes of destaining solution (acetic acid, methanol, distilled water; 9.2:50:40.8) until the gel background was clear and protein bands distinct. Destained gels were stored in 7% acetic acid until photographed.

3.9.2 Molecular weight determination.

SDS-PAGE was used to determine the molecular masses of the major bands in the crystal, solubilized protoxin and activated toxin preparations. After staining and destaining the gels, molecular masses were estimated from plots of molecular weight versus the relative mobility (RF) of the standards using a 2-cycle semi-log graph paper.

3.9.3 Column chromatographic fractionation of protoxin samples.

Gel filtration and anion exchange chromatography were employed to fractionate the protoxin preparations. Samples of protoxin (500 μl) were subjected to gel permeation chromatography using a Superose 12 (10 x 300 mm) and Mono Q HR/5/5 columns (Pharmacia LKB Biotechnology, Sweden) attached to a Fast Protein Liquid Chromatography (FPLC System, Pharmacia Fine Chemicals, Sweden). The Superose 12 column was equilibrated in 20 mM

Tris-HCl (pH 8.5) prior to use. The buffer system for the Mono Q column was as follows: buffer A; 20 mM Tris/HCl, pH 8.5 and buffer B; 20 mM Tris/HCl in addition to 0.5 M NaCl, pH 8.5. The salt gradient was formed to cover a range of 0-100%. Fractions were eluted in both columns at a flow rate of 0.5 ml/min and were monitored by absorbance at 280 nm. Protoxin samples were routinely dialyzed against Tris-HCl (20 mM, pH 8.5, 12 h), prior to fractionation.

3.10 Immunological characterization.

3.10.1 Immunological procedures: 1. Antibody production.

Antibodies (Abs) against the activated toxin (M_r ~ 62 Kd, TIKKI isolate) were raised in New Zealand white rabbits according to previously described protocol (Osir *et. al.*, 1986). The band of interest was electro-eluted using a model 422 (Bio-Rad, Richmond, CA, USA.) following separation of the proteins on a 4-15% SDS-PAGE. After electro-elution, the resultant solution was concentrated using PEG and then dialyzed against PBS (pH 7.2, 12 h, 4° C). 1.0 mg of toxin was emulsified in Freund's complete adjuvant (Gibco Laboratories, New York, U.S.A.) before injecting intramuscularly into the rabbits. A booster injection of toxin (0.5 mg) in incomplete Freund's adjuvant was administered after four weeks. The rabbit was bled two weeks later. The fresh blood was left to stand at room temperature for 1 h and subsequently at 4° C for 12 h. The serum was decanted into another tube and centrifuged

(1000 x g, 30 min). Sodium azide (1%) was added to the resulting supernatant. Aliquots of 100 µl (serum containing Abs) were stored in Eppendorf tubes at -20° C. Prior to immunization, 5 ml of blood was collected, processed and the serum used as a control when testing the presence of Abs at the end of the immunization schedule.

3.10.2 Immunological procedures: 2. Antibody detection.

The presence of Abs from the rabbit antiserum was confirmed using double radial immunodiffusion technique (Ouchterlony, 1958). This was performed using 1% agarose (Serva, Feinbiochemica Heidelberg, New York) in PBS (with 0.2% NaN_3, pH 7.2,) on glass slides. A well was punched at the center of the glass slide and other wells made circumferentially around the central well. Another set of wells were made for the control experiment. Ten µl of pre-/post-immunization sera were placed in the appropriate center well for the control/test experiments, respectively. Ten µl of the crystal, suspended in PBS, protoxin and activated toxin were placed in separate peripheral wells. The glass slide was incubated in a moist chamber (27° C, 48 h). The slide was subsequently washed in several changes of PBS to remove excess protein. It was dried, stained with Coomassie Brilliant Blue R-250 and then destained.

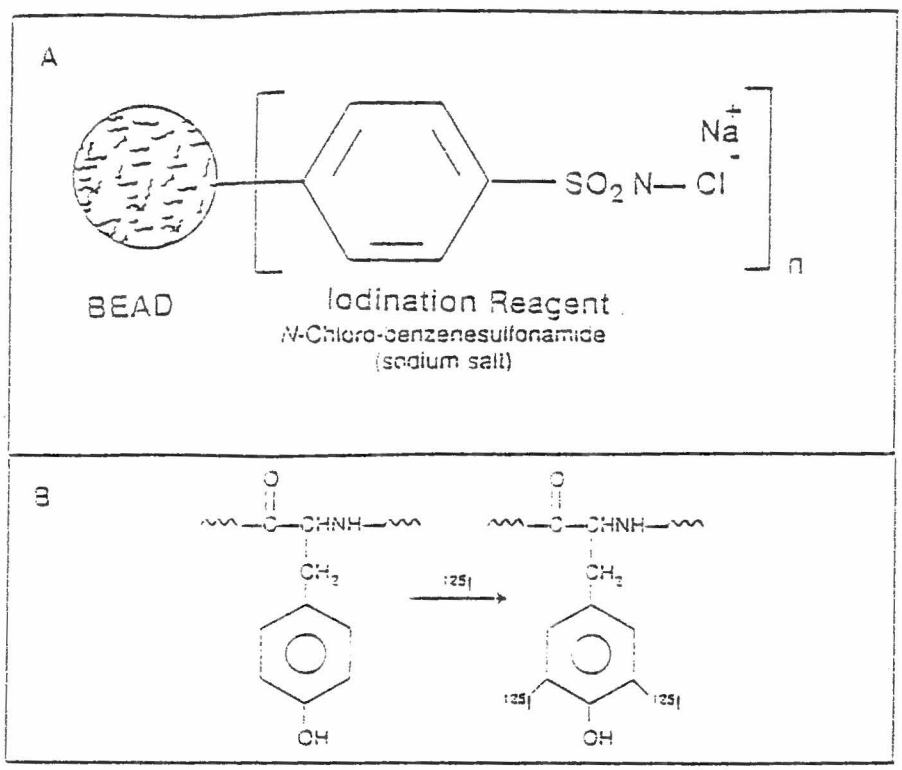
3.10.3 Determination of immunological relationship between the *B.thuringiensis* isolates.

To study the immunological relationship between the isolates, double radial immunodiffusion as already described was employed. Glass plates were prepared and 10 μl of serum (with Abs raised against **TIKKI** toxin) was placed in the center well. Ten μl of activated toxins from **TIKKI, MF4B/2** and **M37/2** were placed in separate peripheral wells. The pre-immunization serum was used to set up a control experiment.

3.11 Radiolabelling of delta-endotoxin.

The solubilized protoxin and activated toxin were radiolabelled with $Na^{125}I$ according to procedures described by Wolfersberger (1990). Slight modifications were made to this original protocol. Two Iodo-beads were added to a test tube containing 1 ml of sodium carbonate buffer (Na_2CO_3, 50 mM, pH 9.5). The Iodo-beads (pre-coated with iodination reagent, N-chloro-benzylsulfonamide, Pierce) was used per manufacturer's instruction and the details of the iodination reaction are illustrated in Scheme 3. Protoxin or activated toxin (150 μg) was then added to the tube and incubated (27° C, 25 min). The reaction was stopped by pipetting the reaction mixture away from the Iodo-beads. Unbound iodide was removed by loading the mixture onto a desalting column (Bio-Gel P6 DG, Bio-Rad). Several fractions (1 ml) were collected. At the end of fractionation, 10 μl of each fraction was aliquoted into

plastic tubes with snap-tops. The amounts of radioactivity in these were determined in a gamma counter (model 4/600 ICN Isomedic, ICN Biomedicals INC, Huntsville.). The fraction with the highest counts per min (cpm) of radioactivity was then stored at 4° C and used in the binding assay studies.



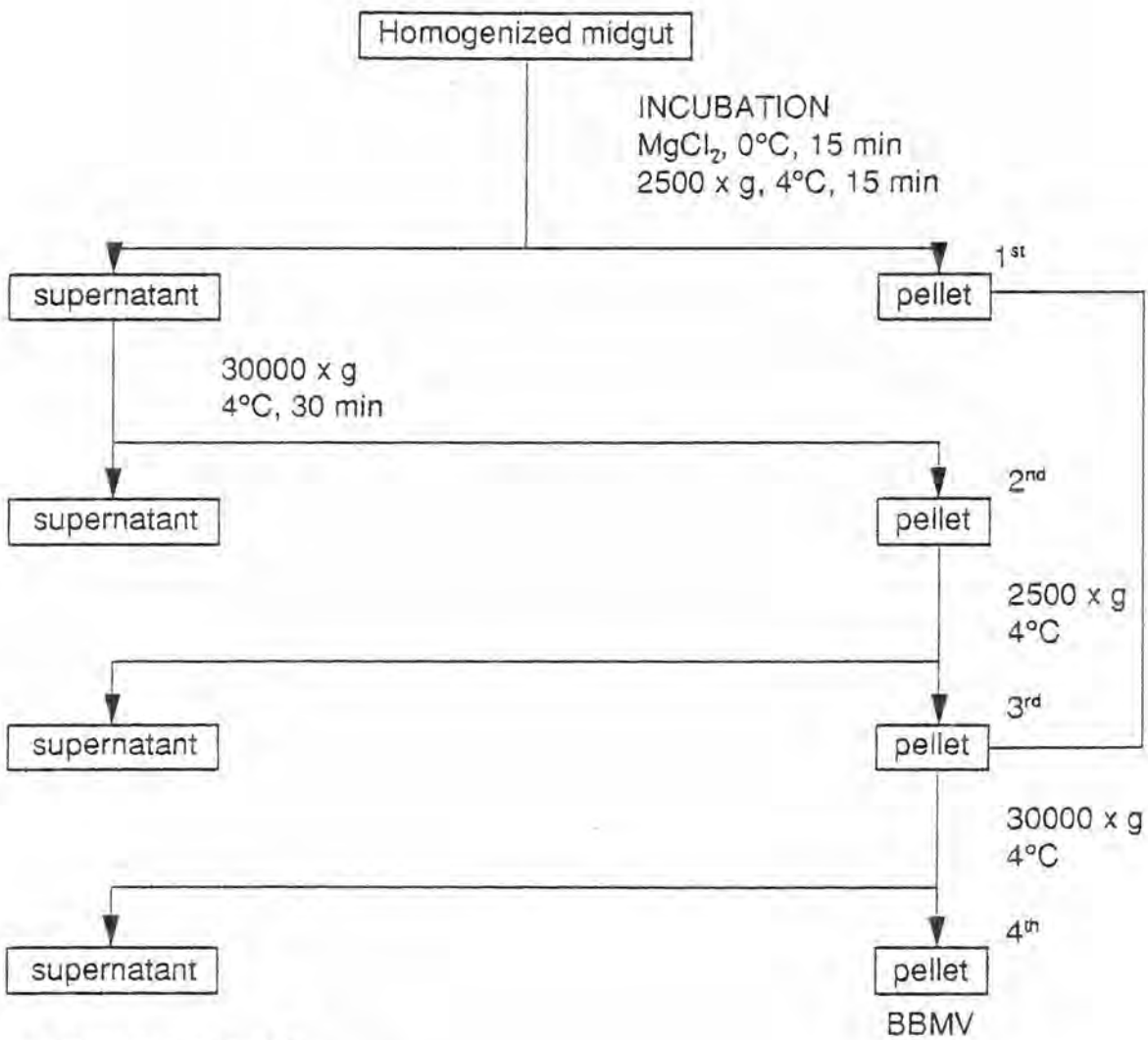
Scheme 3 Chemical radioiodination of proteins using ^{125}I. A:structure of iodobead; B:chemical reaction involved in the attachment of 125I to positions ortho to the hydroxyl group in an amino acid.

(Modified from PIERCE chemical company, 1992).

3.12 Preparation of brush border membrane vesicles (BBMV)

3.12.1 Purification of BBMV.

Brush border membrane vesicles were prepared from midgut cells of *C. partellus* and *G. m. centralis* using a method described by Biber *et. al.* (1981) and modified by Wolfersberger *et al.* (1987). The specific activity of a brush border membrane marker enzyme, alkaline phosphate (AP) was monitored during the purification procedure. Midguts dissected from either *G. morsitans centralis* or *C. partellus* were washed twice in buffer (mannitol; 300 mM, EDTA; 5 mM, Tris; 17 mM, pH 7.5) and homogenized in twice its volume of buffer (4° C, 1 min) in a plastic vial. The cells were treated with an equal volume of $MgCl_2$ (24 mM) and incubated for 15 min on ice. The sample was then pelleted (1st pellet) by centrifugation in a Sorvall SS-34 rotor (3000 x g, 15 min, 4° C). The supernatant was transferred to another tube and re-centrifuged (30,000 x g, 30 min, 4° C). The pellet (2nd pellet) was resuspended in buffer containing $MgCl_2$, vortexed thoroughly and pelleted again (3rd pellet). The 1st and 3rd pellets were combined, resuspended in ½ strength buffer and centrifuged (30,000 x g, 30 min, 4° C). The resultant pellet which contained the BBMV was suspended in 1 ml of ½ strength buffer and dialyzed against PBS (pH 7.2, 12 h). It was then stored at -20° C until used. Scheme 4 gives a diagrammatical representation of the major purification steps.



Scheme 4 A flow chart outlining the key steps in the preparation of brush border membrane vesicles from insect midgut cells.

3.12.2 Assay conditions for Alkaline phosphatase (AP).

The activity of AP was monitored during the purification of BBMV using a modified method from Bingham and Malin (1992). The activity of the enzyme was followed by incubating a known amount in the reaction buffer (0.1 M glycine, pH 10 with 1% Triton X-100) with 4-nitrophenol phosphate (4-NPP, 0.6 M). The production of 4-nitrophenol (4-NP) was measured spectrophotometrically (405 nm) at 1 min intervals for 3 min. A time course experiment was conducted to determine the optimal incubation time for the assay. 4-NPP (4 mM in distilled water) was added to 8 ml of reaction buffer and incubated at 30° C in a small conical flask. Six tubes were numbered and, to each, 3 ml of 0.1 M NaOH was added. The reaction in the flask was started by the addition of 0.4 mg of AP. At 10 min intervals, 2 ml sample was removed and added to the appropriate tube. The reading for zero time was taken immediately after the addition of the enzyme. Spectrophotometric readings were taken against a blank of 0.1 M NaOH. After the determination of optimal incubation time, a standard curve was prepared and used in all subsequent assays. Dilutions of 4-NP were made over a range of 0.005 mM to 0.04 mM. Absorbance in each concentration was determined at 405 nm against a blank of 0.1 M NaOH. A standard curve was then plotted using absorbance against the amount of 4-NP present and recorded in μmoles (1 mM = 1 μmole/ml). Midgut membrane preparations were assayed for AP activity by incubating 10 μl of midgut protein preparation (30 min, 37° C) in the

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reaction buffer and the quantity of 4-NP produced estimated using the standard curve. The specific activity of the enzyme was subsequently determined by the following formula;

$$\text{Enzyme activity} = \frac{\text{change in OD/min x V}}{\text{E x v}} \ \mu\text{moles/min.}$$

where,

change in OD	=	change in absorbency
V	=	volume of assay mixture
E	=	extinction coefficient (6.22 for NADH)
v	=	volume of enzyme.

$$\text{Specific enzyme activity} = \frac{\text{Activity}}{\text{Protein conc. mg of protein.}} \ \mu\text{moles/min/}$$

3.12.3 Incubation of labelled ^{125}I-Toxin with BBMV.

Incubation procedure were adopted with modifications from Wolsfersberger (1990), Haider and Ellar (1986). BBMV (100 μg) were incubated with ^{125}I-toxin (50 μl) in borosilicate glass culture tubes (6 x 50 mm) coated with 1% PEG. A final incubation volume of 200 μl was achieved using Tris saline buffer (10 mM Tris, 0.9% $NaCl_2$, pH 7.4). Incubation was allowed to proceed at room temperature with gentle agitation at regular intervals. After

90 min, the sample was centrifuged (30,000 x g, 5 min, 27° C) to separate the bound from free toxin. The pellet containing the BBMV-^{125}I-toxin complex was washed six times in TS buffer at 4° C. Pellet from the final wash was resuspended in 20 μl of buffer. For competition experiments, BBMV was first incubated with non-labelled toxin for 10 min before addition of ^{125}I-toxin. The competing toxin was usually two hundred times higher in concentration than the labelled toxin. The constituent proteins were then subjected to a 4-15% SDS-PAGE for separation. At the end of electrophoresis, the gel was stained in Coomassie Brilliant Blue, destained and dried completely. Autoradiography was carried out for varying lengths of time using 13 x 18 cm Kodak Diagnostic Film (X-OMAT, Eastman Kodak Company, Rochester, U.S.A.). The films were processed using Hi-Rendol developing solution (Fuji Photo FilmCo. Ltd, Tokyo, Japan) according to manufacturer's instructions.

3.13. Solubilization of insect midgut proteins.

Dissected tsetse midguts were washed six times in ice-cold PBS buffer (pH 7) to get rid of midgut contents. Mild sonication was carried out between the washes. The midgut membrane was then homogenized in the same buffer but containing protease inhibitors (1 mM PMSF and 5 μg/ml A protinin) and centrifuged in a Sorvall SS-34 rotor (16000 rpm, 1 h, 4° C). The resultant supernatant was stored at -70° C and it contained the soluble proteins. The pellet (1st) was then further processed by being resuspended in 1%

pre-condensed Triton X-114 containing protease inhibitors as above. This was incubated on ice for 1 h. The suspension was then sonicated mildly for 2 min and centrifuged (16,000 rpm, 1 h, 4° C) in the same rotor as above. The supernatant was pipetted away from the pellet immediately for further processing and the pellet stored on ice. Aliquots of 500 ml of 6% sucrose solution (w/v) were placed in Epperndorf tubes on ice. To each tube, 500 ml of the supernatant was gently layered on the sucrose solution which acted as a cushion (separating zone) between the aqueous and detergent phases after separation. The tubes were then incubated in a water bath (30° C, 5 min). After incubation, the tubes were centrifuged (14,0000 rpm, 5 min, 27° C) and the aqueous (top layer) phase pipetted out gently. The sucrose solution (2nd layer) was then removed from the tube. The 3rd layer which was the detergent phase was washed to get rid of sucrose by addition of x4 its volume of buffer, vortexed thoroughly, incubated for 5 min (30° C) and centrifuged as before. The supernatant which contained some amount of sucrose was discarded. The clean detergent phase was then stored at -70° C. The aqueous phase was then washed to remove all traces of the detergent phase proteins. Tx-114 was added to the aqueous proteins to a final concentration of 0.5%. 500 ml was then layered onto 500 ml 6% sucrose solution in Eppendorf tubes. Incubation was for 5 min at 30° C. The different phases were then separated by centrifugation (14,000 rpm, 5 min, 27° C). The supernatant was pipetted off, concentrated to about ½ its volume and stored at -70° C until needed. The 2nd

pellet was then solubilized as in the foregoing procedure. The three fractions: soluble proteins, aqueous phase proteins and detergent phase proteins were then subjected to SDS-PAGE (4-15%) to examine the protein subunits which they contained.

3.14 Preparation of histological sections for light microscopy

3.14.1 Dissection of midgut.

Teneral *G. m. centralis* were fed with varying amounts (10, 25, 50 and 100 μg/ml of defibrinated rabbit blood) of *B. thuringiensis* toxins (TIKKI, MF4B/2 (positive control) and M37/2 (negative control). The midguts were then dissected out in PBS (pH 7.4) after intervals of 1, 5 and 10 min. The midguts were cut into four sections comprising the anterior, mycetome, secretory and posterior components and placed in different vials for processing.

3.14.2 Tissue fixation, dehydration and embedding

Dissected tissues were immediately fixed at 4° C in 2.5% glutaraldehyde buffered to pH 7.4 with sodium cacodylate containing 5% sucrose and 0.01 M $CaCl_2$ and kept at 4° C. These were later washed three times in two changes of 0.05 M sodium cacodylate buffer then post-fixed for 1 h at room temperature in cacodylate-buffered 1% osmium tetroxide. The tissues were washed in 0.5 M cacodylate buffer again and then block-stained overnight in saturated aqueous uranyl acetate. Dehydration was carried out at room temperature

through an ethanol series from 30% - 96%, 10 min in each then into two changes of absolute ethanol for 15 min in each case. The tissues were cleared in two changes of propylene oxide for 10 min per change. They were then infiltrated in araldite made in a 1:1 ratio with propylene oxide overnight then infiltrated further for 24 h in fresh araldite. The tissues were finally embedded in araldite and left to polymerize at 60° C for 72 h. The blocks were trimmed using a block trimmer before sectioning.

3.14.3 Tissue sectioning and staining

Thick sections (1 μm) were cut for light microscopy using a glass knife mounted on an LKB ultramicrotome and collected on a drop of distilled water on a microscope slide. The sections were dried on a warm plate and then stained with toluidine blue with 1% borax on a warm plate. The stain was washed off with distilled water, the slide dried and mounted in DPX. These were examined under light microscope. Ultrathin sections for electron microscopy were also cut using a glass knife. Ultrathin sections of golden to silver colour (as viewed on the water surface) were collected on uncoated 300 -mesh copper grids and later stained in concentrated uranyl acetate made in 50% ethanol, for 30 min. Excess uranyl acetate was washed off with 50% ethanol and then with distilled water. The sections were counter stained in lead citrate (Reynolds, 1963) for 10 min, then excess lead citrate washed off with 0.02 M sodium hydroxide and then with distilled water, and dried ready for

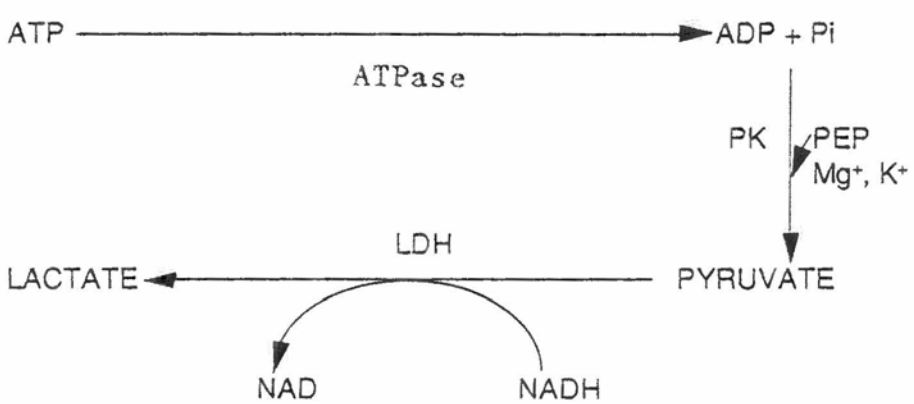
examination. The sections were examined on a Phillips 201-S transmission electron microscope.

3.15 Effect of *B.thuringiensis* delta-endotoxin on the midgut K^+-ATPase activity.

3.15.1 Establishment of an ATPase assay system.

The activity of ATPase was determined using the NADH-coupled system (Scheme 5). The assay medium contained 2 ml of buffer (80 mM K-acetate; 3 mM Mg-acetate; 30 mM Na-acetate; 25 mM Tris acetate, pH 8.0), 10 µl NADH (30 mM), 30 µl Phosphoenol pyruvate (0.1 M), 5 µl Lactate dehydrogenase, 5 µl Pyruvate kinase, 30 µl ATP (0.3 M), 5 µl Rotenone (1 mg/ml). *G. m. centralis* midgut homogenate (either crude or partially purified) was the source of ATPase.

Ten μg of the midgut preparation was used. Incubation was carried out for 15 min at 37° C. The level of NADH was monitored spectrophotometrically at 340 nm wavelength.



Scheme 5 The reaction steps in the NADH coupled system.

3.15.2 Optimization of assay conditions.

The assay conditions for ATPase activity was optimized by varying the concentrations of Na^+ and K^+ salts in the assay buffer. The salt concentrations were varied between 0 mM and 100 mM. As one salt content was varied, the other was kept constant at 30 mM.

3.15.3 Isolation and partial purification of midgut ATPase.

The procedure used for the partial purification of ATPase was modified from Guerrieri and Scarfo, (1990). Dissected *G. m. centralis* midguts were washed thoroughly in PBS (pH 7.2) and then homogenized in the same buffer. Sodium deoxycholate was added to a level of 0.3 mg/ml of protein. This was followed by the addition of 72 g/l solid Potassium chloride. The KCl was allowed to dissolve completely and the suspension centrifuged in a 60 Ti rotor (Beckman ultra-centrifuge) at 50,000 x g (30 min, 4° C). The supernatant was pipetted off and its volume determined. It was then diluted with 0.25 volumes of distilled water. The centrifugation step was repeated under the same conditions. The supernatant was dialyzed against 10 mM Tris-HCl (pH 8.0) overnight. In the second phase of this purification method, the dialyzed material was again centrifuged (50,000 x g,1 h, 4° C). The supernatant was separated from the pellet and the pellet resuspended in 1 ml of PBS buffer. Both fractions were then assayed for ATPase activity following the method already described in section 3.15.1.

3.15.4 Assay conditions for studying the effect of oligomycin and *B.thuringiensis* δ-endotoxin on midgut ATPase activity.

An assay was established, by measuring a constant and known amount of enzyme (10 μg of partially purified midgut ATPase) against varying concentrations of substrate (commercial ATP, Sigma Chemicals) ranging between 0.5 to 6 mM. All other assay conditions remained the same as described in section 3.15.1. The plot obtained formed the baseline and subsequent assays done with either oligomycin or *B.thuringiensis* δ-endotoxin were compared to this.

CHAPTER 4.

RESULTS AND DISCUSSION.

4.1 Selection of growth media using four formulations.

4.1.1 Microscopic examination of culture during fermentation .

During fermentation, the cultures were examined microscopically, firstly, to observe for contamination and secondly, to assess bacterial growth.

The four test media types used in this initial media selection studies covered a range of media already in use. Table 3 shows the characteristics as observed under the microscope. The important parameters that were monitored during growth were amount of growth (i.e. biomass) and at the end of the growth phase, the number of spores and crystals (i.e. biological activity).

Table 3: Biomass and Bioactivity of M37/2 and TIKKI during fermentation in four media formulations.

Medium	Time (h)	M37/2 Cells	M37/2 Spores	M37/2 Crystals	TIKKI Cells	TIKKI Spores	TIKKI Crystals
M1	24	+ +	-	-	+ +	-	-
	48	+	+ + +	+	+	+ + +	+
M2	24	+	-	-	+	+ +	+ +
	48	+	-	-	+ +	+ +	+ +
M3	24	+	+ +	+	+ +	+ +	+ + +
	48	+	+ + + +	+ + + +	+ +	+ + +	+ + +
M4	24	+ + +	-	-	+ + +	-	-
	48	+ + +	+	+ +	+ + + +	+ +	+ +
	72	+ +	+ +	+ +	+	+ + + +	+ + + +

Legend

-	none
+	few
+ +	moderate
+ + +	many
+ + + +	very many

M37/2 in M1 had high sporulation, with lysis occurring within 48 h of growth but the final crystal numbers were low. In M2 the cells did not attain the sporulation stage even after 96 h. With TIKKI, although sporulation did occur, the final number of spores and crystals were comparatively low in this medium. M3 gave the best result for M37/2 with sporulation and crystal being observed in the growth medium within 48 h. M4 contained in addition to 3 g (w/v) of glucose, 5 g of peptone. This made the medium very rich and resulted in prolongation of the logarithmic phase of growth in both isolates. It was the medium in which high cell numbers and spores were recorded for TIKKI. Sporulation in this medium commenced 6-12 h later than was observed in M1 and M3 for both M37/2 and TIKKI isolates. On the average, sporulating cells were observed after 24 h and cells began to lyse at about 48 h with total lysis occurring at 72 h in M4. No differences were observed in crystal size, shape or their staining intensities in these media. Fig 1 shows sporulating cells and lysed culture of *B. thuringiensis*.

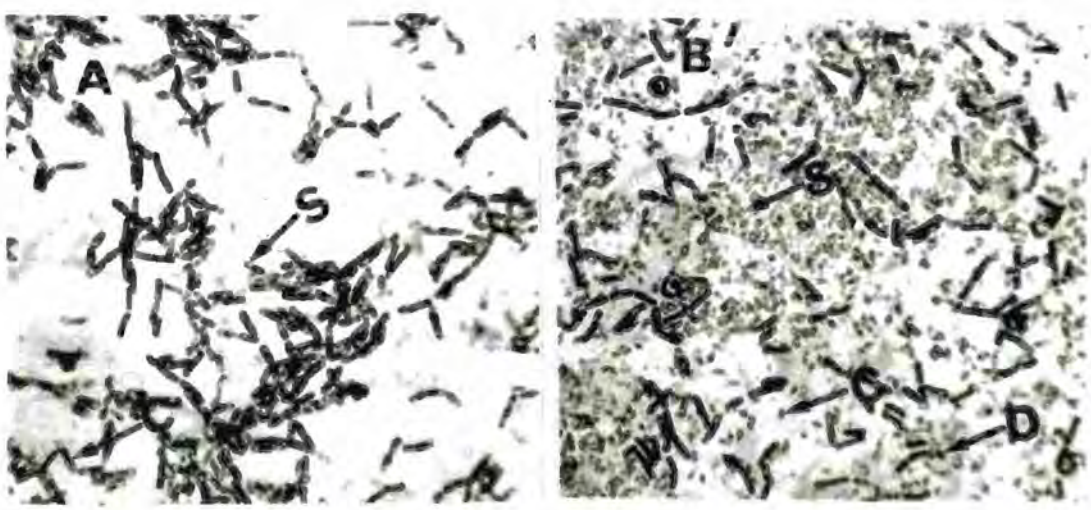


Fig 1 Smirnoff stained smear of B.thuringiensis culture showing A:sporulating cells; B:lysed culture, arrows indicate S = spores, C = crystals, D = cell debris.

4.1.2 Bacterial growth and sporulation.

The results as shown in Table 4 a and b were consistent with the microscopic estimations that were made simultaneously. In M4 cell and spore counts were carried out at 72 h.

Table 4a: Total cell and spore counts for M37/2.

Time (h)	Cell count/ml of culture $x10^{-8}$				Spore count/ml of culture $x10^{-8}$			
	M1	M2	M3	M4	M1	M2	M3	M4
48	3.2	6.0	3.2	1.5	2.8	0	3.2	-
72				1.1				2.5

Count at start of fermentation i.e. 0 h $= 1.8 \times 10^6$ cells/ml

Table 4b: Total cell and spore counts for TIKKI.

Time (h)	Cell count/ml of culture $x10^{-8}$				Spore count/ml of culture $x10^{-8}$			
	M1	M2	M3	M4	M1	M2	M3	M4
48	1.6	1.5	2.1	2.4	1.4	1.3	1.6	-
72				2.9				2.3

Count at start of fermentation i.e. 0 h $= 1.5 \times 10^6$ cells/ml

4.1.3 Biological activity.

4.1.3.1 Age grading of *A. aegypti* larvae.

The results of the study on age grading of A.aegypti larvae are shown in Table 5 and reveals an increase in length as larvae age.

Table 5: Age grading of *A. aegypti* larvae: mean of daily length measurements.

Day	Mean length ± S.E.(mm)
2 (1st instar)	1.32 ± 0.03
3 (2nd instar)	2.23 ± 0.06
4 (3rd instar)	3.16 ± 0.1
5 (Early 4th instar)	3.42 ± 0.1
6 (Early 4th instar)	3.85 ± 0.08
7 (Early 4th instar)	4.94 ± 0.1
8 (late 4th instar)	5.13 ± 0.12

For each instar n = 20.

4.1.3.2 Efficacy of M37/2 against different larval instars of *A. aegypti* and *C. quinquefasciatus*.

Table 6 shows the efficacy of M37/2 against different instars of *A. aegypti* and *C. quinquefasciatus*. M37/2 isolate was found to be effective against the two mosquito species tested. Comparison of the data shows that larvae of *C. quinquefasciatus* were more susceptible to the toxin having higher LC_{50} values, especially, at the late developmental stages (instar 3 and 4). In Fig 2 the larval LD_{50}s and growth curve for *A. aegypti* larvae were compared. Larval growth and LC_{50} of the *B.thuringiensis* toxin increased directly proportional to age ($r = 0.9$). All instars were susceptible and the tradition of using early 4^{th} instar larvae of *A. aegypti* for bioassays was justified. For practical purposes, the 4^{th} instar larva is large in size (3-4 mm in length) and can, therefore, easily be handled. Table 7 shows that the highest mortality observed in *A. aegypti* was in M4.

Table 6: Efficacy expressed as LC_{50} of M37/2 isolate against different larval instars of ***A. aegypti*** and ***C. quinquefasciatus.***

Larval instar	LC_{50} (spores/ml of broth)	
	C.quinquefasciatus	*A.aegypti*
1	0.0079	0.034
2	0.028	0.226
3	5.290	1.042
4	6.240	1.227

For each instar n = 30.

Table 7: Mean mortality in ***A. aegypti*** exposed to M37/2 grown in different media.

Medium	No.exposed	No. dead after 24 h	% mortality
M1	20	14	70 b
M3	20	13	65 c
M4	20	18	90 a
Control	20	2	10 d

Values significantly different at P=0.05, Duncan's Multiple Range Test.

The mortalities observed with different concentrations of test media (M3) was also considered in order to determine the best working range for use in bioassays. Fig 3 shows a dose-response relationship between 4th instar larvae of *A. aegypti* and varying concentrations levels of M37/2.

This test showed that bioassay concentrations of 10^{-1} to 10^{-4} were consistently higher than that required to kill 90% of the test insects in all the media. The difference became significant between concentrations of 10^{-5}-10^{-6}, indicating that the LC_{50} lies between these two concentrations.

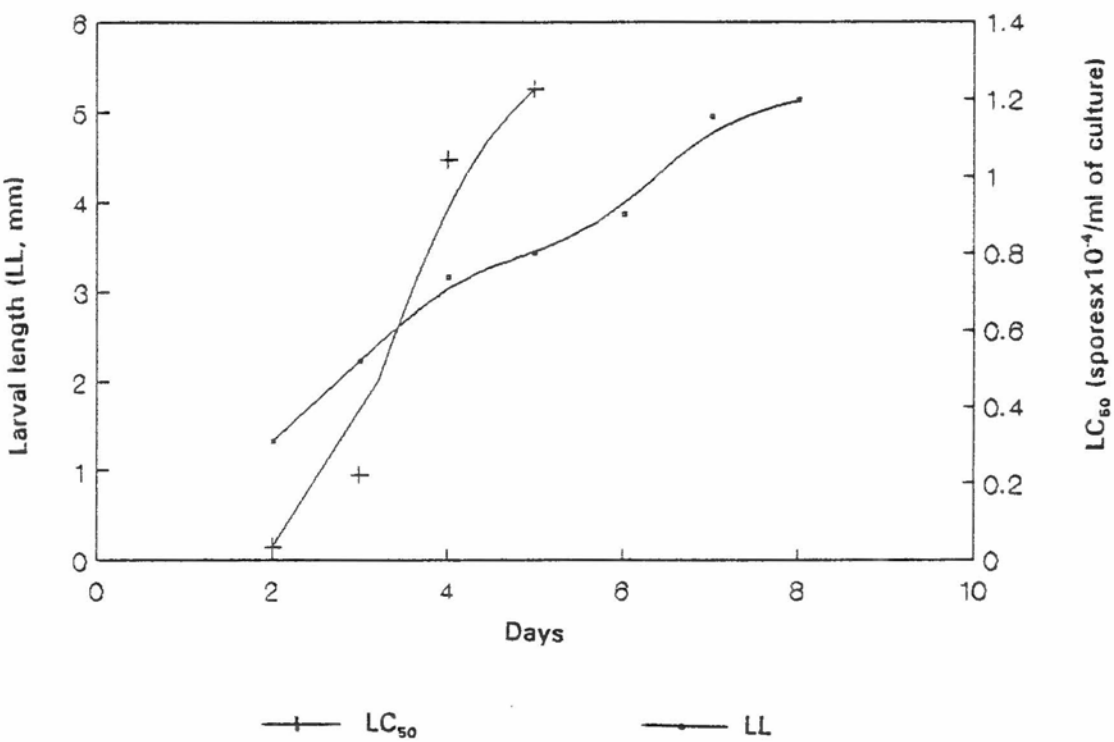


Fig 2 Comparison of the efficacy of *B.thuringiensis* against different larval instars and growth curve of *A.aegypti*. Correlation coefficient r = 0.9.

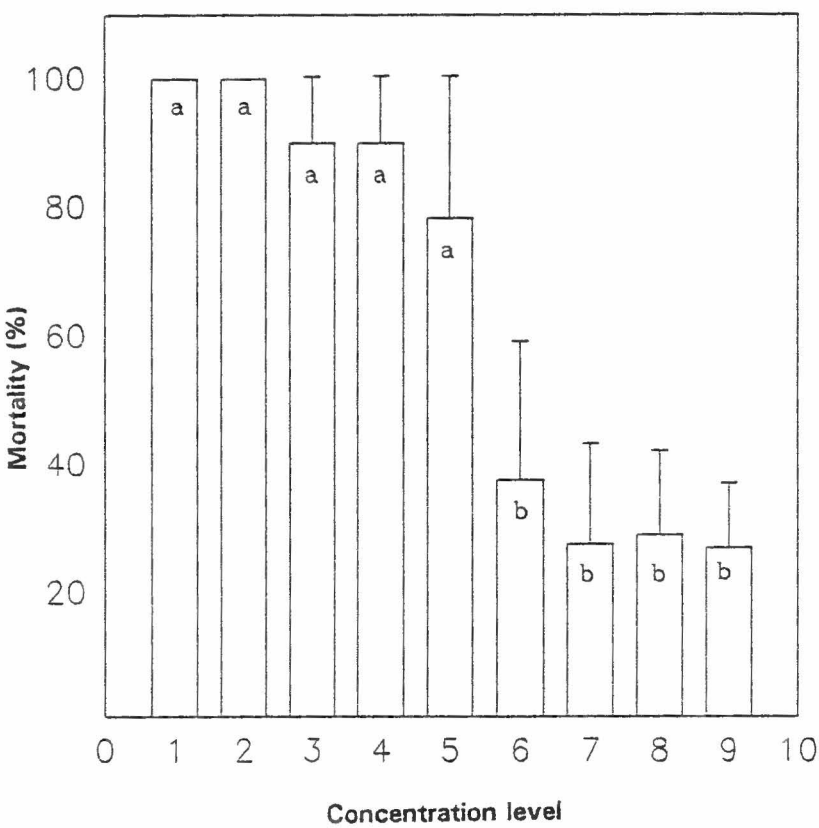


Fig 3 A dose-response histogram *A.aegypti* larvae against varying concentration levels of M37/2. a & b indicate significant difference at P = 0.05.

4.1.3 Efficacy of TIKKI against *G.m.centralis*.

From Table 8, the highest mortality (90%) was observed in M4 which also recorded the highest spore count for this isolate.

Table 8: Mean mortality in *G.m.centralis* exposed to TIKKI grown in different media.

Medium	No of fed flies	No dead after 24 h	% Mortality
M1	17	10	58.8 b
M2	17	8	47 c
M3	16	10	62.5 b
M4	20	18	90 a
Control	20	3	15 d

Means with the same letter are not significantly different. Duncan's Multiple Range test, P=0.05.

These studies formed the basis for a more detailed study involving thirteen media formulations with M3 as the basic formulation. The time of maximum biomass and LC_{50} range already determined, formed an important baseline data.

4.1.4 Further optimization of fermentation medium using thirteen media formulations.

4.1.4.1 Estimation of bacterial growth.

Bacterial growth was monitored in the different media formulations by cell and spore counts carried out at the end of the growth phase. Table 9 gives the mean of three replicate counts done with different batches of culture. Medium 2 maintained the bacterial cells in the vegetative stage for 6.8 days without sporulation.

Table 9: Cell and spore counts for thirteen media formulations.

Medium	Cell count/ml Mean ± S.E. ($x10^8$)	Spore count/ml Mean ± S.E. ($x10^8$)
1	2.1 ± 0.01 b	1.9 ± 0.01 cd
2	2.2 ± 0.05 a	1.68 ± 0.05 e
3	1.9 ± 0.05 c	1.99 ± 0.03 cd
4	2.1 ± 0.05 b	2.0 ± 0.005 c
5	2.0 ± 0.02 bc	1.98 ± 0.02 cd
6	2.0 ± 0.1 bc	1.99 ± 0.005 cd
7	2.0 ± 0.1 bc	1.99 ± 0.01 cd
8	2.2 ± 0.05 a	2.2 ± 0.01 a
9	2.2 ± 0.05 a	2.1 ± 0.05 b
10	2.2 ± 0.05 a	2.1 ± 0.02 b
11	2.1 ± 0.02 b	2.0 ± 0.03 c
12	2.2 ± 0.05 a	1.6 ± 0.05 e
13	2.2 ± 0.02 a	2.15 ± 0.008 bc

Starter culture = 1.9 $x10^6$ cells/m Means with the same letter are not significantly different. Duncan's Multiple Range Test, P = 0.05.

The pattern of growth was the same as already observed in the first series with four media formulations. The growth pattern did not however correlate in a definite manner to the amounts of glucose and nitrogen in each medium since the variation in these components formed the major difference between the media formulations. M8, M9, M10, M12 and M13 performed similarly in their ability to sustain bacterial growth. This picture was repeated for spore counts.

4.1.4.2 Biological activity.

This was estimated by measuring the killing ability of each formulation using 4th instar larvae of *A. aegypti*. Fig 4 and Table 10 show bacterial numbers and LC_{50} values. LC_{50}s were calculated through probit analysis. Results are the means of three replicate bioassays performed with different batches of culture. Biological activity was found to be highest in M4 in this series. This did not correspond with any of the media which sustained high bacterial numbers.

Table 10: LC_{50} for M37/2 from 12 media formulations.

Medium	LC_{50} (Sporesx10^{-4})
8	17.1
9	0.1
13	686.9
10	18.7
12	46.0
4	0.0
11	42.9
1	341.6
5	0.1
7	0.9
6	2.3
3	106.7

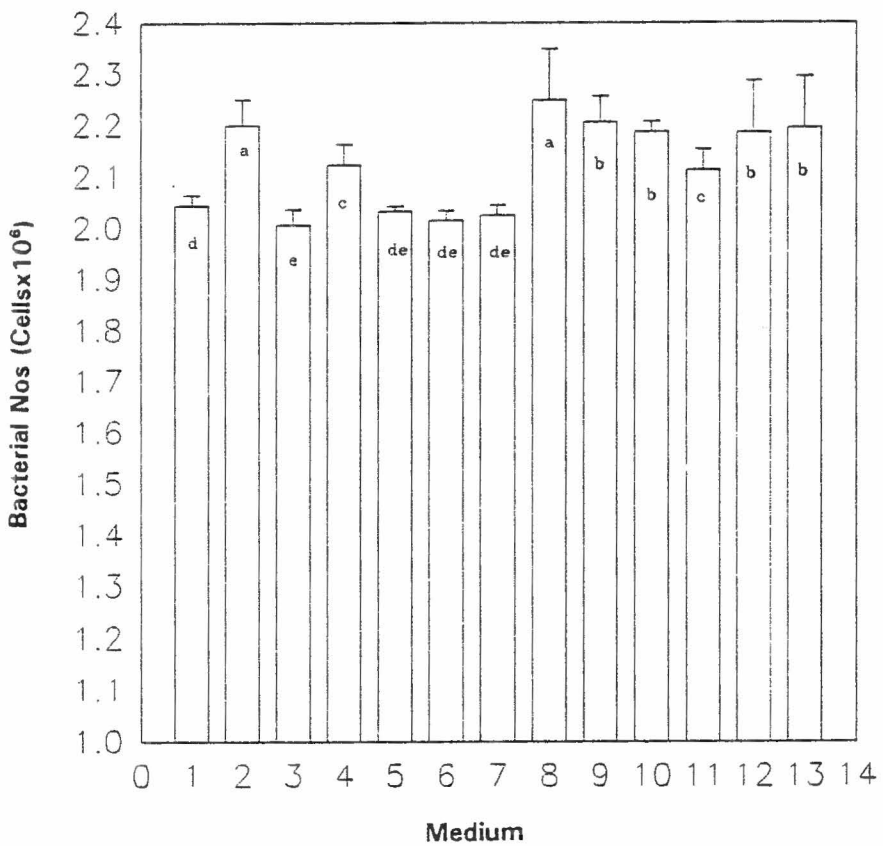


Fig 4 Total cell counts for thirteen media formulations.

4.1.5 Bioassay of MF4B/2 isolate against *C. partellus*

Bioassay was carried out on *C. partellus* to confirm its susceptibility to this isolate. Percentage mortality recorded after 5 days was approximately 73.3% in the test as compared to the control (16.6) (Table 11).

Table 11: Efficacy of MF4B/2 isolate against *C. partellus*.

	Test	Control
No of larvae used	30	30
Mortality (%) after 5 days	73.3	16.6

4.1.6 Discussion

Investigations were carried out to assess bacterial growth and δ-endotoxin production in a number of media formulations.

Considering that the main aim of the fermentation study was to select a medium good enough to grow reasonable quantities of the various *B.thuringiensis* isolates under study, using semi-defined synthetic media composed of materials available in the laboratory was considered a good starting point. Several media with varying ingredients in shake-flask cultures were assessed for their ability to support bacterial growth and δ-endotoxin production. Other media formulations have been used and are currently in use. These are usually tailored to meet specific needs, for example, to reduce costs in large production fermenters by using industrial wastes or locally available materials (Hertlen *et al.*, 1981; Obeta and Okafor, 1984).

The observations made on *B.thuringiensis* cells grown in M2 draws attention to the fundamental requirements in any growth medium. This medium being low in glucose led to a situation where the cells either failed to sporulate (M37/2) or very poor growth was recorded (TIKKI). Glucose as a medium component also affects *B.thuringiensis* growth by exerting an effect on the pH of the medium. All known *B.thuringiensis* strains produce acid from glucose, therefore, if the concentration is too high, the subsequent drop in pH can affect or even inhibit growth. Since nitrogen which is another important component, produces an alkaline environment, a balance in both carbon and nitrogen is also

a recommended standard procedure for any fermentation medium (Abdel-Hameed *et al.*, 1991). The variations of the amount of glucose and nitrogen incorporated in the 2nd fermentation series did not affect either the growth of the bacteria or the biological activity expressed by the broth. Although an improvement in these parameters was expected, the result showed that M3 in the first series was optimally constituted and therefore, an improvement over this could not have been achieved with the components being used.

Shake-flask cultures have two major disadvantages as a fermentation technique. There is limitation in air supply and therefore, best growth would be achieved when the nutrient content of the medium is kept low. This was put into consideration when the media used in this study were being formulated. Secondly, it is necessary to maintain a favourable pH level (6.5-7.5) throughout the fermentation period . However, this cannot be achieved in a shake-flask culture and therefore a careful balance between nitrogen and carbon becomes even more important. Despite the limitations, shake-flask culture is a useful method for laboratory-based *B. thuringiensis* small scale production.

The growth phases of *B. thuringiensis* have been routinely monitored by assessment of such parameters as biomass, with particular attention on the time of onset of sporulation and quantity of δ-endotoxin produced and the time required for complete lysis of the cells. These parameters are important

because the ultimate aim of any fermentation is to grow the *B.thuringiensis* of interest within the shortest time period possible. The size and stage of bacterial growth of the inoculum used as starter cultures have a direct effect on the parameters mentioned above. Starter cultures are important in order to reduce the lag phase that occurs at the beginning of a fermentation cycle before the logarithmic division of cells starts. The advantage is obvious as the parameters being investigated are timed events. The size of the inoculum used for seeding the broth at all stages of this study was 0.25% of the broth volume. Although this was low in comparison to other inoculum levels described by other workers (Dulmage, 1970; Goldberg *et al.*, 1980; Margalait *et al.*, 1983), it produced final biomass of comparable cell and spore counts. This implies that the source of the starter culture cells had vigorous and well maintained cells. The complete growth phase as observed from vegetative phase to lysis lasted 48-72 h. This is consistent with observations made in a similar study (Pearson and Ward, 1988).

Microscopic examination as a method of assessing growth proved to be comparable to actual cell and spore counts. Thus it can be used as a quick and easy technique, for monitoring bacterial growth and development during fermentation.

The commitment of *B.thuringiensis* to sporulate is known to be triggered by the exhaustion of a rapidly metabolizable carbon or nitrogen source or the presence of slowly metabolizable nutrients (Freese, 1977). On the other hand,

delay of sporulation can be attributed almost entirely to a medium too rich in nutrients. Under such a condition, the cells are maintained for much longer in the logarithmic (vegetative) growth phase. The observation that cells grown in M2 (in both series of experiments) did not sporulate means that while the medium was sufficient to support vegetative growth, it was insufficient for germination and outgrowth of spores. As observed by Bulla *et al.* (1980) and Nickerson and Bulla (1974), it is more difficult to determine the nutritional requirements for sporulation as a medium can no longer be defined chemically following vegetative growth. The absence of spores can also be remotely attributed to their having been defective and therefore lysed prematurely while trying to germinate (Nickerson and Bulla, 1974). A good medium should therefore produce dormant spores as an indication of its nutritionally superior formulation. In addition to this it was an important observation that crystals were also not formed. This is in agreement with numerous studies that have shown that sporulation and crystal formation are events that are not only linked, but that occur simultaneously in nature. Asporogenic (spo^-) but crystalliferous (Cry^+) forms can only be induced and are usually highly unstable, reverting to the sporogenic (Spo^+) and crystalliferous wild type (Bhattacharya 1993)

Any differences that might occur in size or staining intensity due to differences in media components were not observable probably due to the narrow range of the media formulations used or the less sensitivity of light

microscopy as a technique. Scherrer et al., 1973 reported that low glucose concentration (< 0.8% w/v) resulted in amorphously shaped crystals. Although in the second set of media formulation ten were constituted with < 1% glucose some having as low as 0.15% w/v, no variation in shape was observed for TIKKI isolate. Since the crystal shape of M37/2 isolate is amorphous in nature this effect would not be observable. Variations in the size of crystals is another morphological characteristic that has been shown to be affected by the growth medium. It is probable that the range of media used did not provide enough variation in nutritional contents for such an observation to be made. Distinctive staining intensities have only been reported for *B.t.israelensis* (Ibarra and Federici, 1986) in which the crystal consists of three parasporal bodies that have differential staining.

While total cell count would be a good parameter for determining bacterial mass, spore counts are a better indicator of potential bioactivity of any *B.thuringiensis* preparation (Brand *et al.*, 1976), although it must be pointed out that they do not always correlate with insecticidal activity (Dulmage, 1970; Dulmage and Rhodes, 1971). Attempts to use spore counts/ml of culture as a measure of activity has been based on the assumption that each *B.thuringiensis* cell produces one spore and one crystal. This indirect method has, however, failed repeatedly and can be attributed to the fact that spore counts do not quantify the amount of δ-endotoxin present. The cell and spore counts obtained from the fermentation series while being comparable to those

of Pearson and Ward (1988), did not vary as much as that recorded by Dulmage (1972) in three media formulations for 18 *B.thuringiensis* strains. Smith (1982) reported spore counts of $2x10^5$ - $9x10^8$ after 72 h in *B.t.israelensis* The media tested thus generally sustained an acceptable growth level for the isolate.

Clumping of *B.thuringiensis* cells during growth is a well recognized phenomenon and vegetative cells reportedly have a higher tendency to clump than spores dispersed in growth medium (Meenakshi and Jayaraman, 1979). Fully lysed cultures that are mixed well before aliquots are taken for dilution and subsequent counts offer the best condition for obtaining an unbiased assessment of bacterial numbers in a broth. Unclumped vegetative cells recommended for use as inoculum in fermentation runs also give fairly reproducible results. Experience gained from this study was that a whole range of dilutions needs to be studied before a suitable dilution factor can be obtained. In this study, 10^{-6} - 10^{-7} dilution factor formed the working dilution range. The determination of biological activity forms a fundamental aspect of any insecticidal material. Currently, potency assessments are made on measurable activity on the target insect through bioassays conducted *in vivo* (WHO, 1992). It is worth noting that the lethal concentration which kills 50% of the test insects (LC_{50}), a parameter used to determine biological activity, gives only an estimate of potency and not an accurate or exact measurement. This is basically because of the inherent limitations of testing a population of

living organisms that is not homogeneous. The efficacy of M37/2 isolate against both *A.aegypti* and *C.quinquefasciatus* was high and the added observation that *A.aegypti* was more susceptible agrees with other reports (Goldberg and Margalait, 1977; Hall *et al.*, 1977; Tyrell *et al.*, 1979) which shows that the order of decreasing efficacy of *B.t.israelensis* on three mosquito species was; *A. aegypti*, *C.quinquefasciatus* and *C.pipiens pipiens*. In a dose-response curve, the leveling off of the percentage mortality observed for the different instars as larval age increased can be explained by the fact that as the larvae increased in size, a certain percentage of the population being assayed became committed to pupation (Beegle *et al.*, 1981). This results in the cessation of feeding effectively shielding them from the lethal effects of the bacteria.

The general observation made from this study showing the non-correlation of bioactivity to cell/ spore count has been previously reported (Dulmage, 1970; Dulmage and Rhodes, 1971; Pearson and Ward, 1988; Abdel-Hameed *et al.*, 1991). Pearson and Ward (1988) demonstrated protease activity in culture supernatant fluids which could lead to proteolysis of the crystals and suggested that this might explain, at least in part, why a decline in biological activity was observed after maximum bioactivity levels were attained. In addition, the δ-endotoxin of *B.thuringiensis* is known to have proteases associated with them and this can lead to a disintegration of the crystals after its formation as found by Chestukhina *et al.*(1980).

The results obtained in this study were not compared to a standard *B.thuringiensis* preparation so comparison of the data generated to those of other workers was limited. However, the effective LC_{50} value of a dilution factor of 10^{-5} - 10^{-6} containing on the average 2-3 x 10^4 spores/ml of diluent compares closely to the range of 10^{-4} - 10^{-5} observed by Smith (1982) but were higher than those of Pearson and Ward (1988). They, however, used whole unwashed cultures and mortality could have been contributed by other culture contents other than the bacteria and its products (that is crystals, spores) unlike what was obtainable in the present report. Results obtained from the thirteen media formulations did not improve on those obtained for the basic four media already studied. The observations made, however, served in a large part to reconfirm that bacterial counts cannot be used to ascertain biological activity.

The fact that *B.thuringiensis* crystals can and do disintegrate after their formation means that the processing and storage of harvested cultures must be considered vital in the entire production process. The experience gained from this study is that lyophilized *B.thuringiensis* samples maintained both protein constitution and biological activity for several months (>9 months). In addition, sucrose gradient separation of the powder proceeded as efficiently as when freshly harvested cultures were used since it is recommended that the

moisture content of *B.thuringiensis* should be <4% for it to remain stable (WHO. 1992). Lyophilization is, therefore, appropriate for *B.thuringiensis* cultures.

4.2 Isolation, purification, solubilization and activation of *B.thuringiensis* crystals

At the end of the growth phase (72 h), *B.thuringiensis* crystals were harvested from the broth by centrifugation and subsequently purified using a 40-70% sucrose gradient. Four distinct phases were observed as shown in Fig 5.

This gradient was efficient in the separation of crystals from spores and cell debris. Solubilization of the purified crystals was not very efficient because total solubilization was not achieved. However, for the purposes of this study sufficient crystal protein went into solution and achieving complete solubilization was therefore never a target. Activation of the solubilized protoxin to active toxin was effectively achieved with commercial trypsin. Fig 6 shows a typical band pattern obtained from the δ-endotoxin of the three isolates after SDS-PAGE (4-15%) separation. MF4B/2 had a major band of M_r ~ 130 Kd, TIKKI had two bands of M_r ~ 120 and 66 Kd, while M37/2 had three bands of M_r ~ 140, 66 and 25 Kd.

Fig 5 Phases of purified *B. thuringiensis* crystals on a 40-70% linear sucrose gradient. 1 & 2, cell debris; 3, crystal; 4, spores.

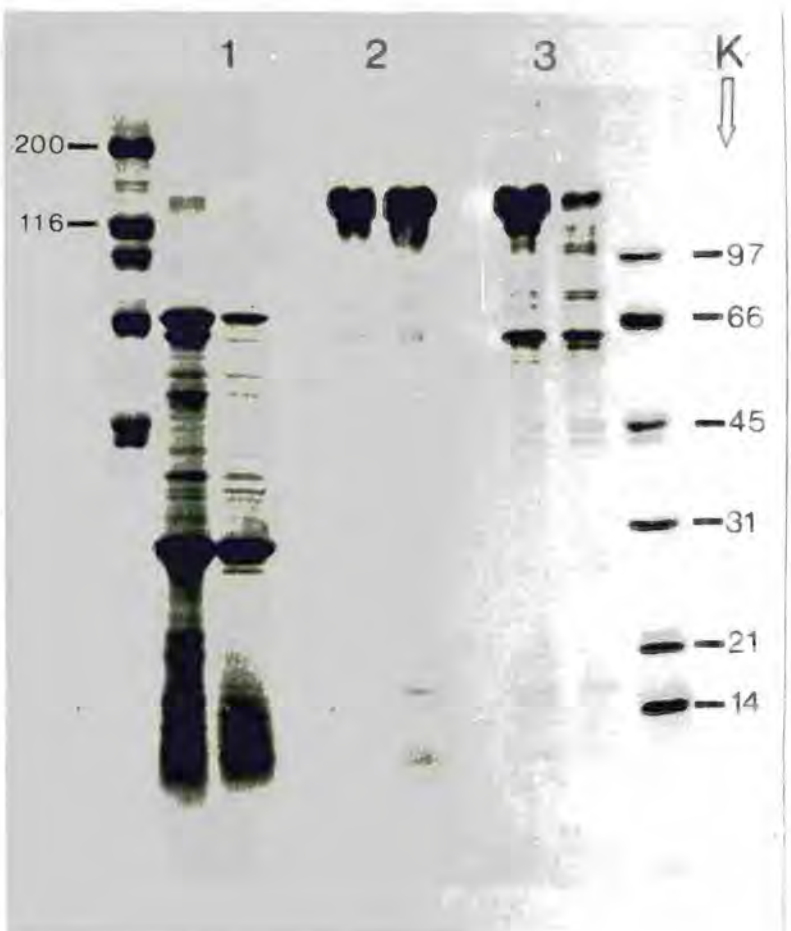


Fig 6 4-15% SDS-PAGE of δ-endotoxin of the three *B.thuringiensis* isolates. 1, M37/2; 2, MF4B/2; 3, TIKKI. High and low molecular weight standards are indicated on the left and right of protein bands respectively.

4.2.1 Physical characterization of TIKKI isolate.

4.2.1.1 Electrophoretic separation of constituent proteins and molecular weight determination.

The band patterns obtained of the crystal, protoxin and toxin are shown in Fig 7. Estimation of molecular weights for the observed proteins was made from a standard curve plotted from four determinations of relative mobility (RF) values against standard molecular weights as shown in Fig 8. The crystal had two major bands with a molecular weight of M_r ~ 120 Kd and M_r ~ 66 Kd, respectively. Upon solubilization, the M_r ~ 120 Kd band disintegrates while the M_r ~ 66 Kd protein is reduced to approximately M_r ~ 64 Kd. This is further proteolytically cleaved to give a toxin of M_r ~ 62 Kd upon activation with commercial trypsin.

4.2.1.2 Separation of crystal proteins using Fast Protein Liquid Chromatography (FPLC).

The solubilized crystal (protoxin) was separated by FPLC using two columns, gel filtration Superose 12 column and an ion exchange Mono Q column. The elution profiles obtained and the corresponding band patterns observed after electrophoresis (4-15% SDS-PAGE) are shown in Figs 9-11. The elution profile obtained after gel filtration of TIKKI protoxin is shown in Fig 9. A major peak (fraction 8-10) in addition to other less well defined peaks was obtained. When the protoxin was separated on an anion exchange column, the

profile was different (Fig 10), with four distinct peaks observed (fraction 12-13, 14-15, 16-17, and 18-20). The major peak (2) eluted at 375-400 mM NaCl concentration. The corresponding SDS-PAGE of the fractions containing the peaks is shown in Fig 11. The major band (M_r ~ 62 Kd) is prominent in all the fractions. This corresponds to the major band observed after trypsination of the protoxin (Fig 7). The differences in the band pattern of the peaks appear to be quantitative rather than qualitative.

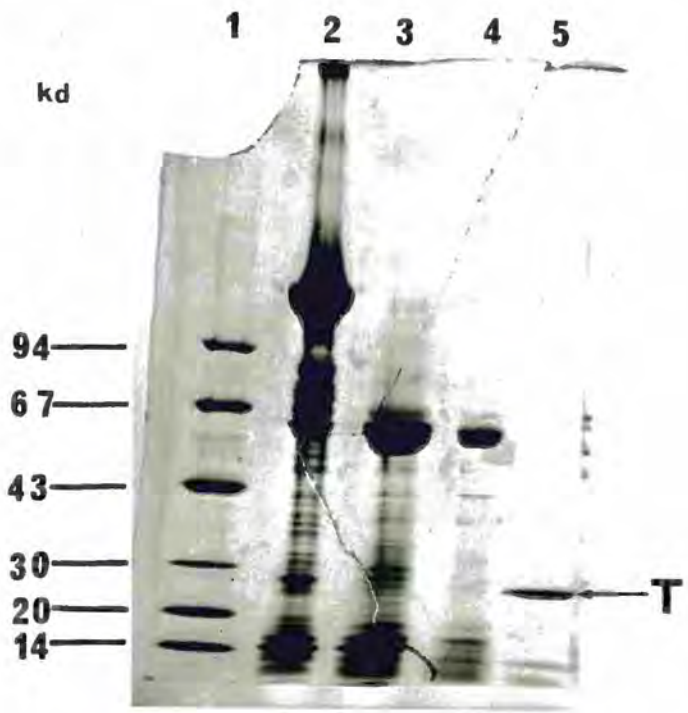


Fig 7 4-15% SDS-PAGE of TIKKI isolate. 1, low molecular weight standards; 2, crystal; 3, protoxin; 4, toxin; 5, trypsin (T).

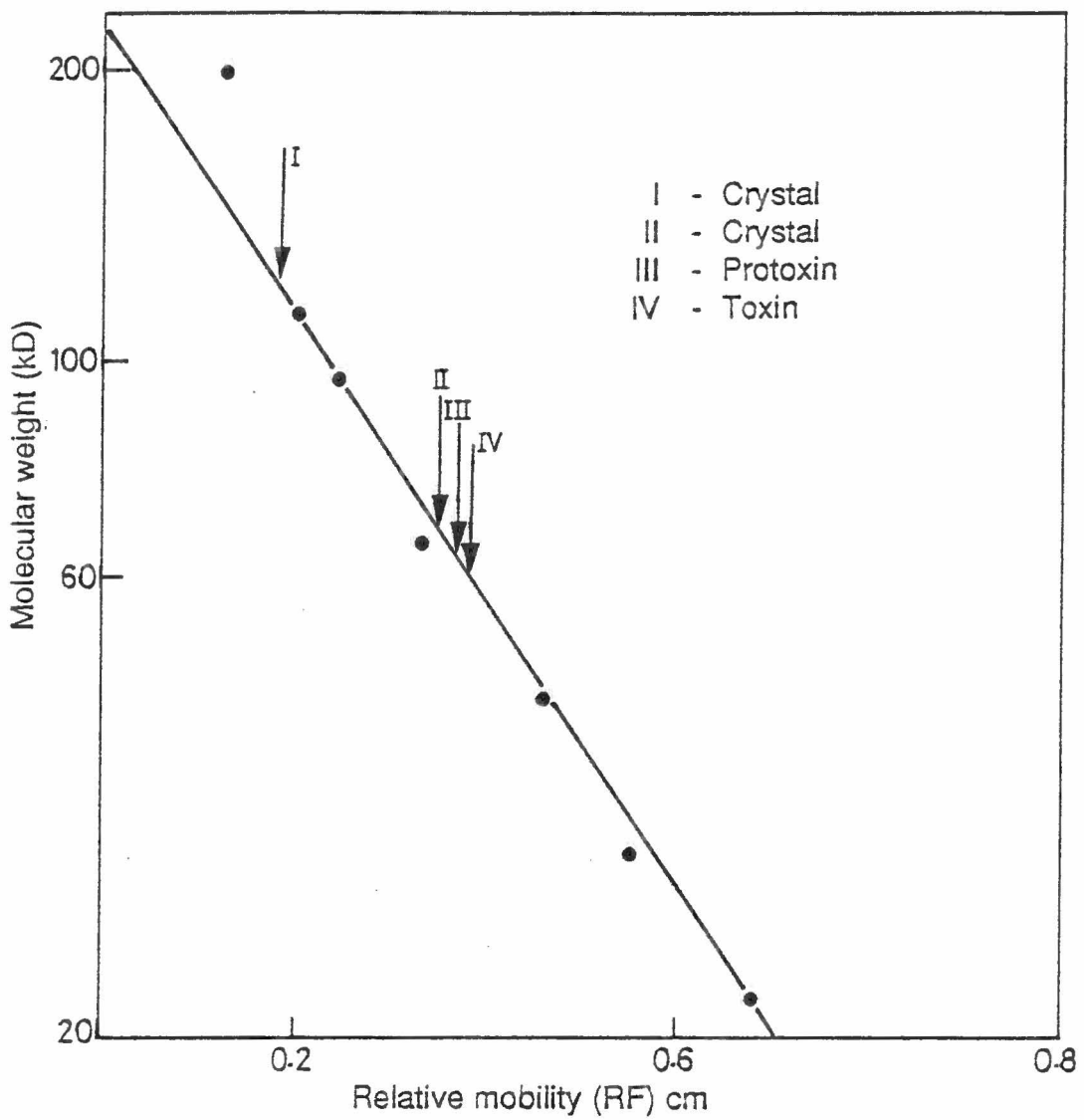


Fig 8 Standard curve (TIKKI) for protein molecular weight determination. Molecular weight versus the RF of the resultant subunits in the δ-endotoxin.

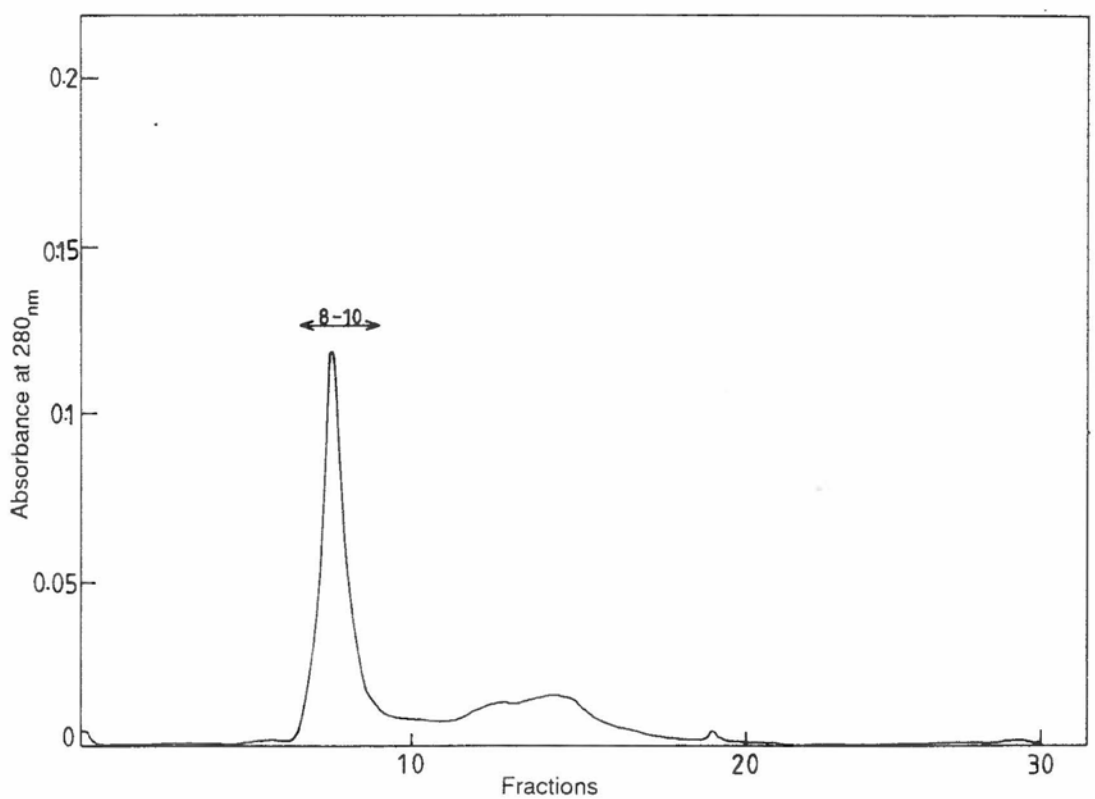


Fig 9 Separation of TIKKI protoxin by gel permeation chromatography. Flow rate was 0.5 ml/min. 1 ml fractions were collected, horizontal bar indicates combined fractions.

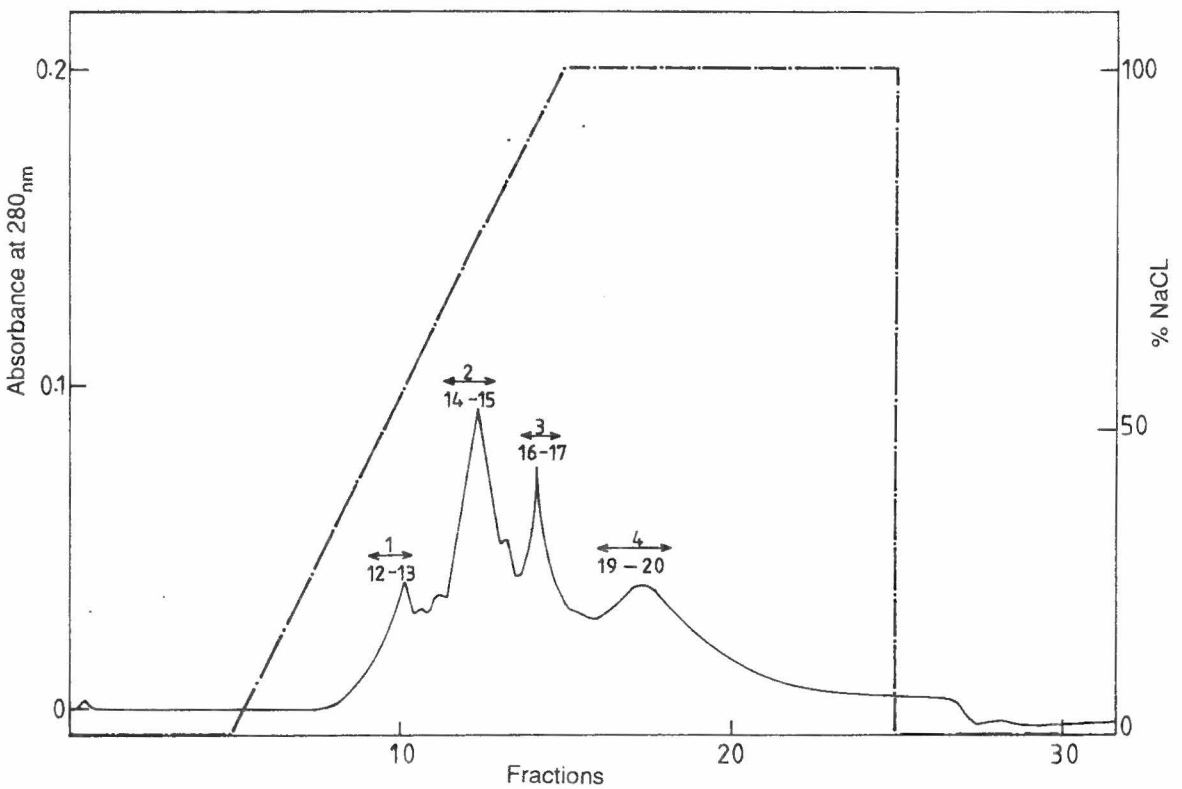


Fig 10 Separation of TIKKI protoxin by anion exchange chromatography. Flow rate was 0.5 ml/min and elution was by a linear NaCl gradient, 0-500 mM. 1 ml fractions were collected, combined fractions indicated by horizontal bars.

Fig 11 4-15% SDS-PAGE of the major peaks after fractionation of TIKKI protoxin by anion exchange chromatography. 1, low molecular weight standards; 2-6, fractions 13-17 as indicated in Fig 10.

Both purification methods were efficient although the anion exchange column was used in a 2-step purification procedure for preparing toxins used in midgut receptor studies. The major peak (2 in Fig 10) was concentrated and run through a modified salt gradient. The result obtained is shown in Fig 12. One major peak which eluted at 350-375 mM Nacl concentration was obtained (fractions 12-14).

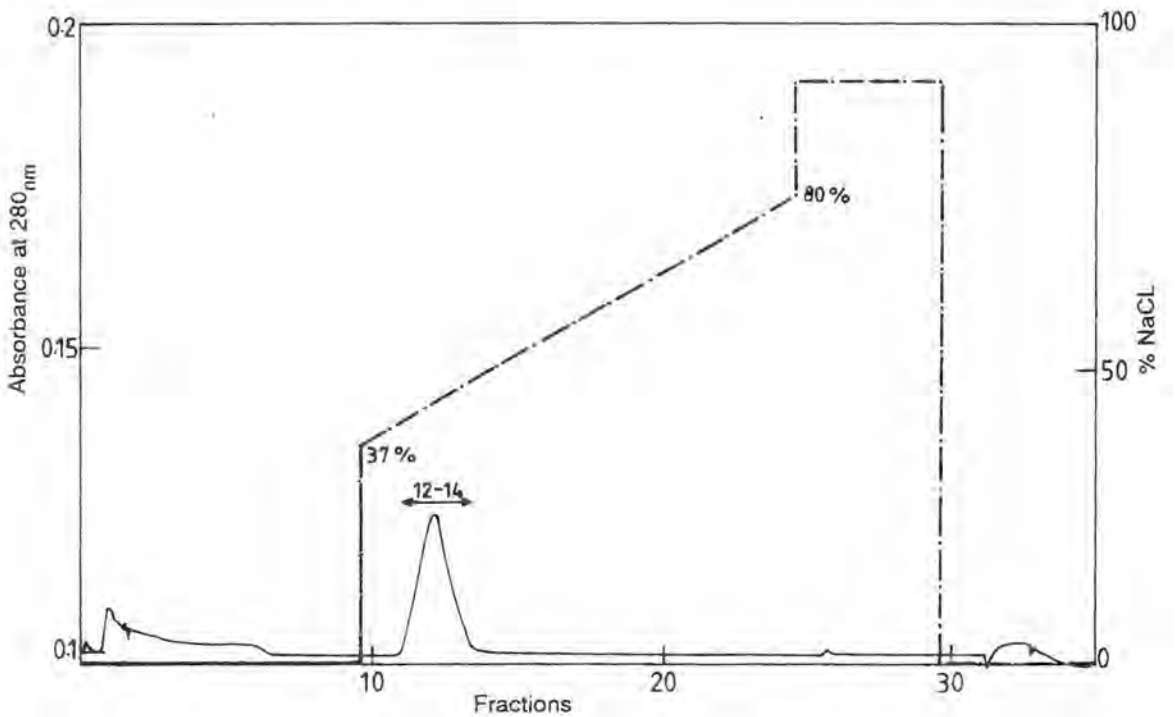


Fig 12 Purification of TIKKI toxin by anion exchange chromatography. Flow rate was 0.5 ml/min and elution was by a NaCl gradient, 185-400 mM. 1 ml fractions were collected, 12-14 were combined.

4.2.2 Discussion.

The modified sucrose gradient (40-70%) was able to separate the crystals of MF4B/2 and M37/2 from other cell components producing crystals with minimal contamination with spores. This technique was more efficient and comparatively simpler than other systems that have been used (Pearson and Ward, 1988), but would not be appropriate for a large scale purification.

Although total solubilization was not achieved in the current study, some reports exist in which total dissolution of the crystal occurred. Bioassay must of necessity be performed after processing the δ-endotoxin to confirm that the smaller molecular weight proteins produced still retained their biologically active component. Solubilization was carried out *in vitro* using 10 mM DTT in Na_2CO_3 buffer at pH 9.5-10. This closely mimics the conditions in insect midgut (Daad, 1975; Huber *et al.*, 1981; Dow, 1986). Results from several solubilization studies indicate that the majority of the crystals derived from different *B.thuringiensis* strains show a structural uniformity based on the protoxin subunit (Calabrese and Nickerson,1980). The higher molecular weight protoxins (M_r~ 30-80 Kd) produced upon solubilization has been demonstrated to have the biologically active molecule (Aronson *et al* ., 1991; Huber *et al.*, 1981), while low molecular weight polypeptides that appear as a by product usually do not show significant activity. Huber and Luthy (1981) reported that these proteins from *B.t.thuringiensis* were at least 500x less active against *P. brassicae* than the protoxin.

After solubilization, the protoxin was found to be stable (in terms of protein subunits as observed following electrophoresis) for up to two weeks at 4° C and 4 months at -20° C, when compared to the toxin which was less stable. This apparent stability in the protoxin has been attributed to the role played by the C-terminal fragment in the formation of parasporal inclusions (Gill *et al.*, 1992). The toxic fragment loses this C-terminal structural fragment during the proteolytic activation of the protoxin to the toxin and the consequent conformational change that occurs during this process has been reported (Choma *et al.*, 1991). Results from this study which show a very narrow range in molecular weight for the active moiety of the isolates goes to further strengthen the argument that the structural conformation and size of these toxins are strikingly similar (Huber and Luthy, 1981). While investigating further into the physical characteristics of the TIKKI isolate, a toxic moiety of M_r ~ 62 Kd eluted from both gel permeation and anion exchange chromatography appeared in more than one fraction. No explanation can be conceived to explain why several peaks that eluted at different time intervals would contain the same constituent proteins after electrophoresis. Similar results have, however, been reported by Ishii and Ohba, 1994, after the purification of *B.t.kyushensis* toxin on a DEAD-Toyopearl column.

4.3 Immunological relationship between the three *B.thuringiensis* isolates.

The immunological relationship between the three isolates was determined to give additional information about the possible biological activity range of the isolates. Results from immunodiffusion studies are shown in Fig 13.

There was a strong reaction observed between Abs raised against the toxin of the TIKKI isolate and the toxin of MF4B/2. However, no precipitating band was observed with M37/2 isolate. The cross-reactivity observed between TIKKI and MF4B/2 isolates shows that these two isolates are immunologically related and probably have dual specificity towards each other's target insect species.

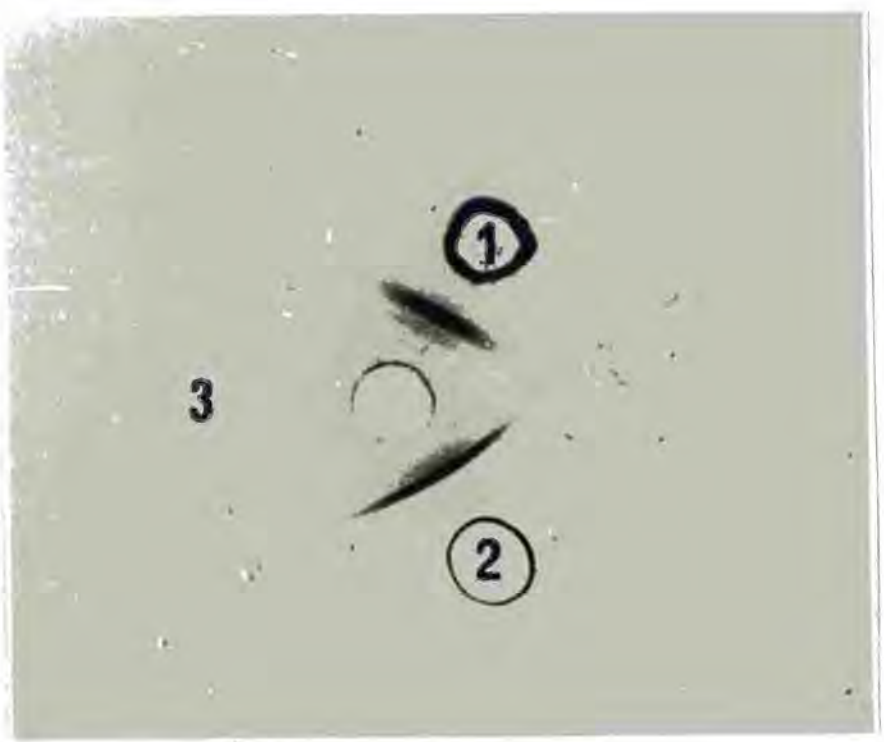


Fig 13 Double radial immunodiffusion showing the relationship between the three *B. thuringiensis* isolates: The center well had antibodies against the TIKKI toxin. 1, TIKKI toxin; 2, MF4B/2 toxin; 3, M37/2 toxin.

4.3.1 Discussion.

The implication of cross-reactivity observed between TIKKI and MF4B/2isolates is that they are immunologically related. Using the primary biological activity spectrum of the two isolates, they would be classified in different classes [TIKKI: Dipteran-active (Cry 111); MF4B/2: Lepidopteran-active (Cry 1)] but immunological studies show that cross-reactivity can occur within a class of *B.thuringiensis* proteins but not between classes. For instance, antisera raised against Cry 1 proteins do not recognize Cry 11 proteins (Hofte *et al.*, 1988; Knoweles *et al.*, 1986). It has however been reported that cross-reactivity does not necessarily imply a similar toxicity spectrum whether *in vitro* or *in vivo*. (Gill *et al.*, 1992). Therefore, the interpretation of immunological studies should be done cautiously and any cross-reactions observed must of necessity be supported by biological activity demonstrated through appropriate *in vivo* assays. Bioassay results using MF4B/2 and TIKKI isolates showed cross-biological activity (Vundla, personal communication.) further strengthening the position that these two proteins are related immunologically, displaying identical biological activity. They probably share common protoxin molecules that are digested and differentially activated within the insect midguts as has been reported for other strains (Debros *et al.*, 1986; Haider *et al.*, 1986).

Following the current and widely acceptable classification for *B.thuringiensis* toxins (Hofte and Whiteley, 1989), one might propose that

MF4B/2 and TIKKI both may possess the same class of *B.thuringiensis* protein, Cry II while M37/2 isolate which displays no similarity either serologically or in terms of biological activity belongs to a different class, Cry IV. The observations made on M37/2 were not surprising as other studies have shown that the mosquitocidal strain *B.t.israelensis* is serologically different from several other strains of *B.thuringiensis* that were studied (Krywienczyk and Fast, 1980; Ishii and Ohba, 1992). It would, therefore, be appropriate especially in the light of bioassay results that show no activity of TIKKI against mosquitoes and M37/2 against either *C.partellus* or tsetse flies (Vundla, personal communication), that M37/2 is not related to TIKKI or MF4B/2 and, therefore, shares no proteins that contribute to toxicity.

4.4 Interaction of midgut proteins with *B. thuringiensis* δ-endotoxin.

4.4.1 Radiolabelling of δ-endotoxin with ^{125}I.

Solubilized δ-endotoxin (protoxin) was labelled and the level of iodination achieved was high, providing a good detection system for studying the interaction between the δ-endotoxin and insect midgut proteins. A typical elution profile after iodination and subsequent gel filtration in a desalting column is shown in Fig 14. Over a million counts per minute were recorded in the fraction containing the labelled protein.

4.4.2 Purification of brush border membrane vesicles (BBMV).

The purification of BBMV was achieved through differential magnesium centrifugation. Fig 15 shows the band pattern obtained when BBMV is compared to homogenized crude midgut proteins separated on 4-15% SDS-PAGE. A comparison between the band patterns in both the crude and BBMV preparation revealed only minor quantitative differences. The range of proteins observed was $M_r \sim <$ 14 - 70 Kd.

4.4.3 Specific activity of the marker enzyme : Alkaline phosphatase.

The level of purification achieved during BBMV preparation was monitored using a marker enzyme, alkaline phosphatase. Optimal assay conditions for this enzyme was first established. Fig 16 shows the graph of a

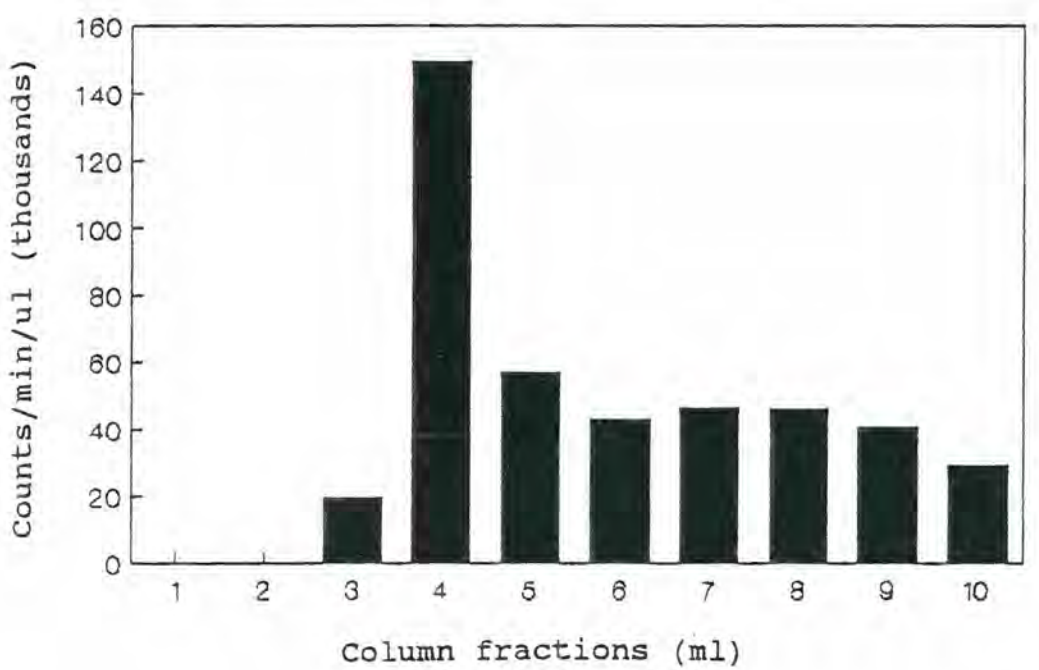


Fig 14 ^{125}I-iodination profile for TIKKI protoxin after separation in a desalting column.

Fig 15 4-15% SDS-PAGE of BBMV preparation.
A: G.m.centralis 1, crude midgut homogenate;
2, BBMV; 3, supernatant from last separation step
B: C.partellus 4, crude midgut homogenate; 5, BBMV;
6, supernatant from last separation step; 7, low
molecular weight standards.

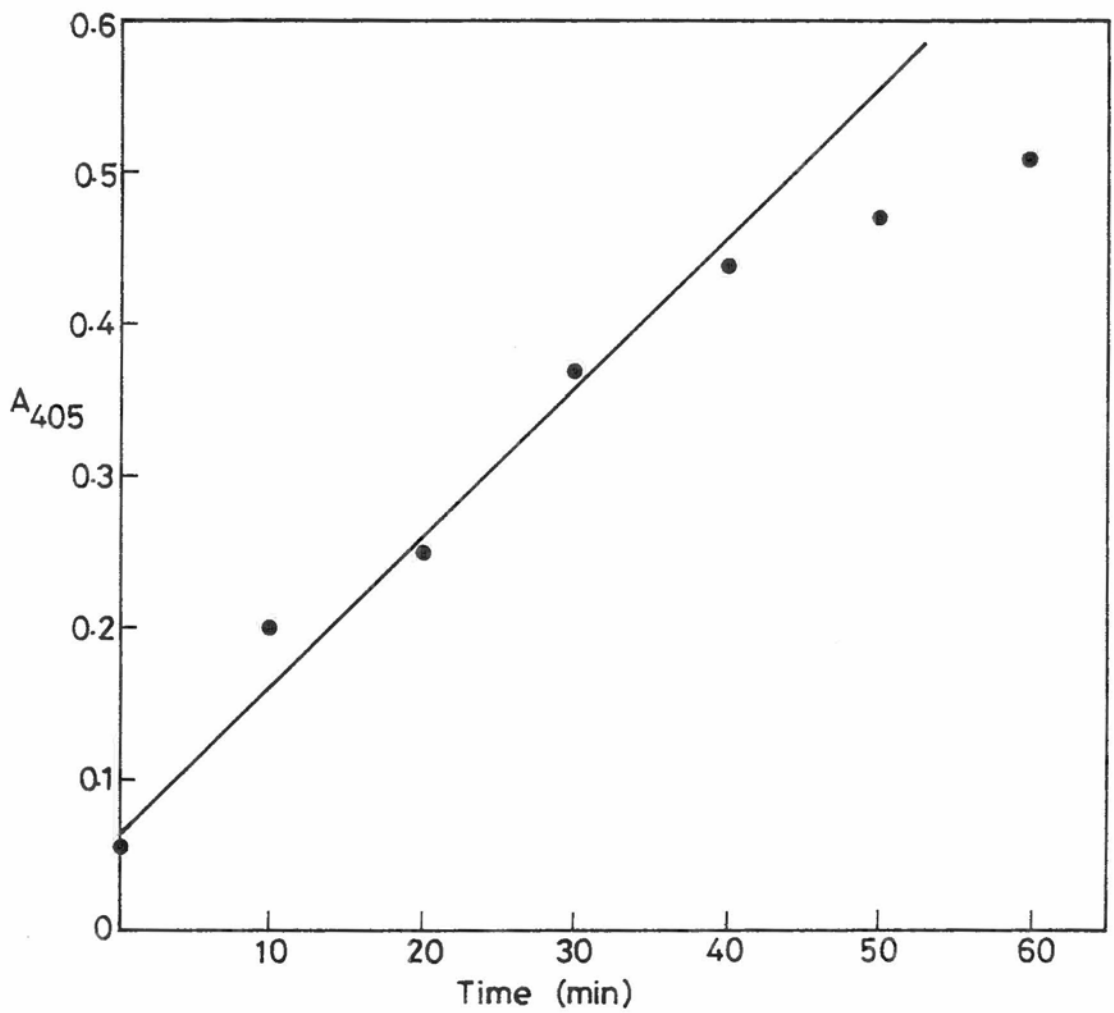


Fig 16 Time course assay for alkaline phosphatase enzyme reaction.

time course experiment from which the incubation period in the assay was set at 30 min. A standard reaction curve (Fig 17) was constructed and used in all subsequent assays. The activity of alkaline phosphatase increased six-fold in the final BBMV preparation as compared to the crude midgut homogenate.

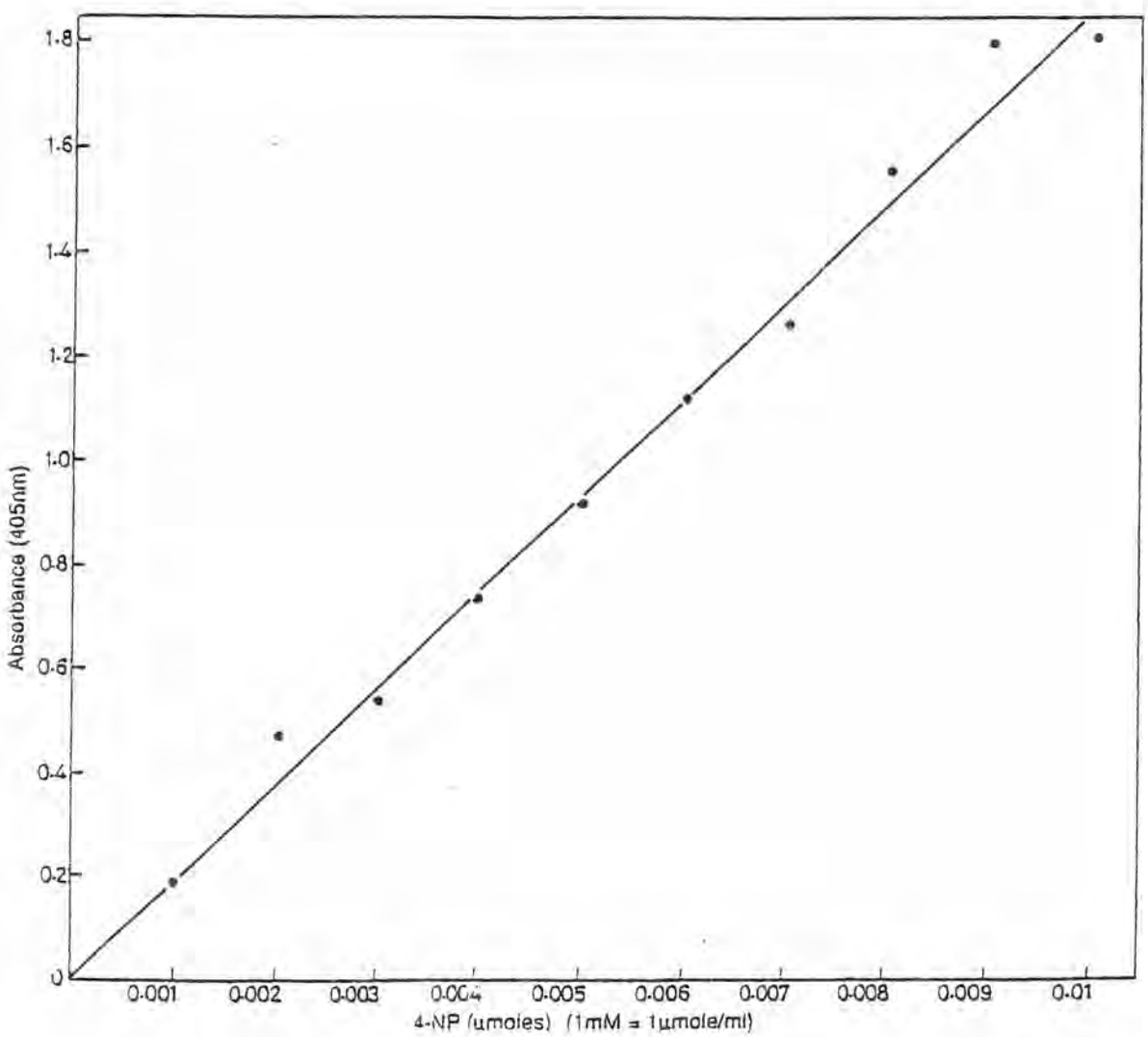


Fig 17 Standard curve for alkaline phosphatase enzyme reaction.

4.4.4 Solubilized midgut proteins.

Fig. 18 shows the separation of constituent proteins in the different phases of tsetse midgut proteins solubilized in Triton X-114. The aqueous phase obtained after extraction contained more protein subunits (lane 1) as compared to the detergent phase (lane 3). Of significance is the fact that re-extraction of the pellet after the first solubilization step revealed very little protein content (lanes 11 and 12), indicating that solubilization and subsequent extraction of proteins using this detergent was efficient. The electrophoretic profiles of the proteins range between $M_r \sim$ 30-200 Kd.

4.4.5 Midgut membrane proteins interacting with δ-endotoxin.

4.4.5.1 Brush border membrane vesicles (BBMV).

Brush border membrane vesicles prepared from both *G. m. centralis* and *C. partellus* were used to study the interaction of these with labelled protoxin from TIKKI and MF4B/2, respectively. The results obtained were distinctively different. As shown in Fig 19 which is an autoradiogram obtained after incubation of TIKKI with BBMV of *G. m. centralis*, one band of $M_r \sim$ 68 Kd was observed. Fig 20 shows the corresponding SDS-PAGE separation of proteins after incubation with labelled toxin. In the case of MF4B/2, two bands of $M_r \sim$ 68 and 64 Kd was observed on the autoradiogram (Fig 21). In addition, a third band of much smaller molecular weight of $M_r \sim$ 28 was also observed.

Fig 18 4-15% SDS-PAGE of *G.m.centralis* midgut membrane proteins solubilized with Triton X-114. 1, detergent aqueous phase; 2, buffer soluble; 3, detergent phase; 4, re-extracted pellet of detergent phase; 5, low molecular weight standards; 6, high molecular weight standards.

Fig 19 Autoradiogram of ^{125}I-BBMV (*G.m.centralis*) complex. 4-15% SDS-PAGE separation. 30 min exposure. 1, low molecular weight standards; 2, ^{125}I-BBMV complex; 3, ^{125}I-BBMV complex (+ unlabelled toxin).

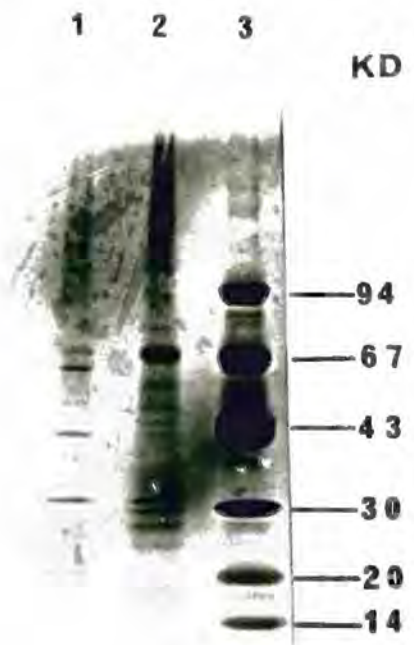


Fig 20 4-15% SDS-PAGE of ^{125}I (*G.m.centralis*) complex. 1, low molecular weight standards; 2, ^{125}I-BBMV complex; 3, ^{125}I-BBMV complex (+ unlabelled toxin).

Fig 21 Autoradiogram of ^{125}I-BBMV complex (*C.partellus*). 30 min exposure. 1, low molecular weight standards; 2, ^{125}I-BBMV complex; 3, ^{125}I-BBMV complex (+ unlabelled toxin).

Challenging the labelled protoxin with 100 fold higher concentration of unlabelled protoxin in a homologous competition experiment apparently did not affect its ability to interact with the BBMV proteins (lane 3, Figs 19 and 21 for TIKKI and MF4B/2 respectively).

4.4.5.2 Solubilized midgut proteins.

Both buffer soluble and detergent extracted aqueous phase proteins from *G.m.centralis* were employed to investigate the interaction with labelled TIKKI toxin. Fig 22 shows protein profiles from Superose 12 (FPLC) and radioactivity counts for labelled toxin. Both protein and radioactivity profiles peaked identically (fractions 19-20). This can be interpreted to mean that the ^{125}I-toxin complex eluted at this point. From Fig 23, it can be seen that the radioactivity profile obtained after incubation of ^{125}I-toxin with soluble midgut proteins was very similar to that of ^{125}I-toxin complex alone. The single prominent peak (fraction 20) indicates the elution of the ^{125}I-toxin in the incubation with the soluble proteins. On the other hand, the protein profile showed a main peak at fraction 7, a point at which very little radioactivity was recorded. Fig 24 shows the results for the ^{125}I-toxin-aqueous protein profiles. Radioactivity had two prominent peaks (fraction 5-6 and 18-20) which was distinctively different from the single peaks observed for the ^{125}I-toxin and

^{125}I-toxin-soluble protein complexes. The second peak again is presumably the excess ^{125}I-toxin that did not interact with the midgut proteins while the first could well represent the ^{125}I-toxin-aqueous protein complex.

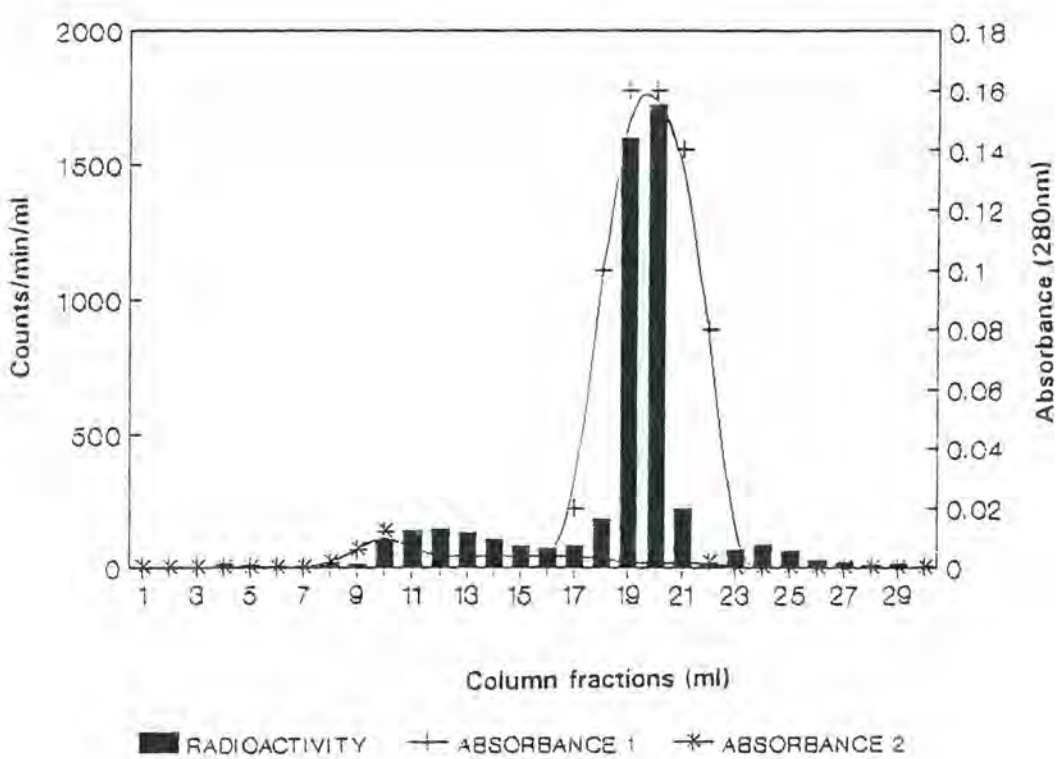


Fig 22 Measurement of radioactivity (cpm) in eluted fractions ^{125}I-toxin complex after gel permeation on Superose 12 column attached to the FPLC. Absorbance 1 & 2 are protein profiles before and after incubation with labelled TIKKI toxin.

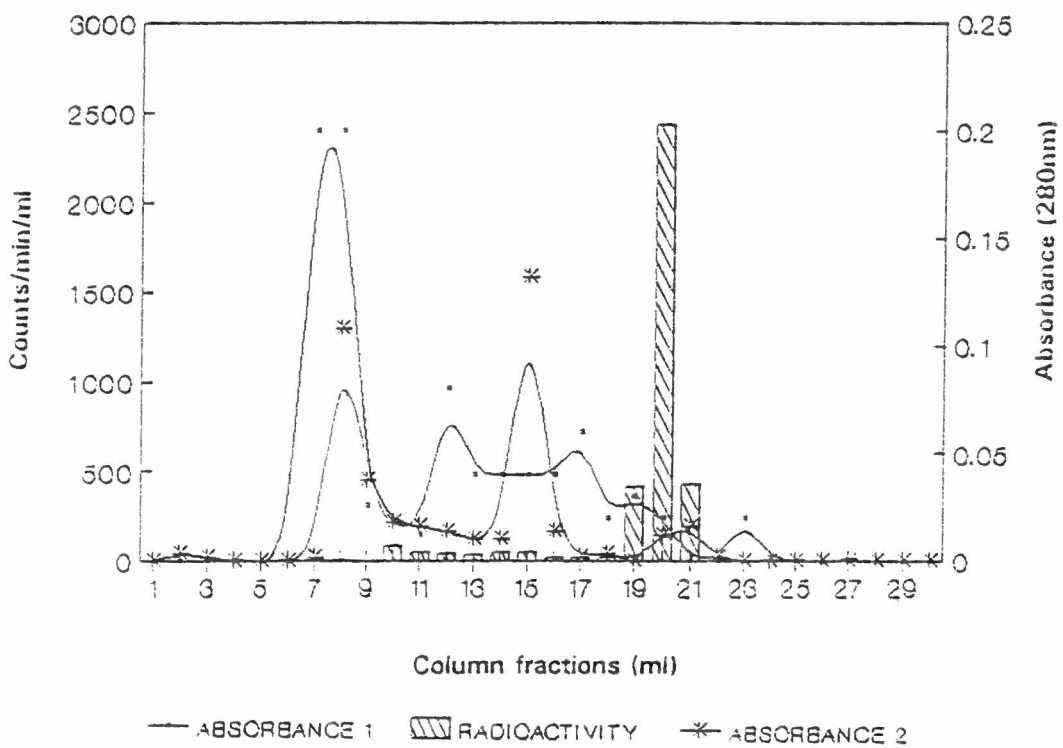


Fig 23 Measurement of radioactivity (cpm) in eluted fractions of ^{125}I-toxin-soluble protein complex after gel filtration on a Superose 12 column attached to the FPLC. Absorbance 1 & 2 are protein profiles before and after incubation with labelled TIKKI toxin.

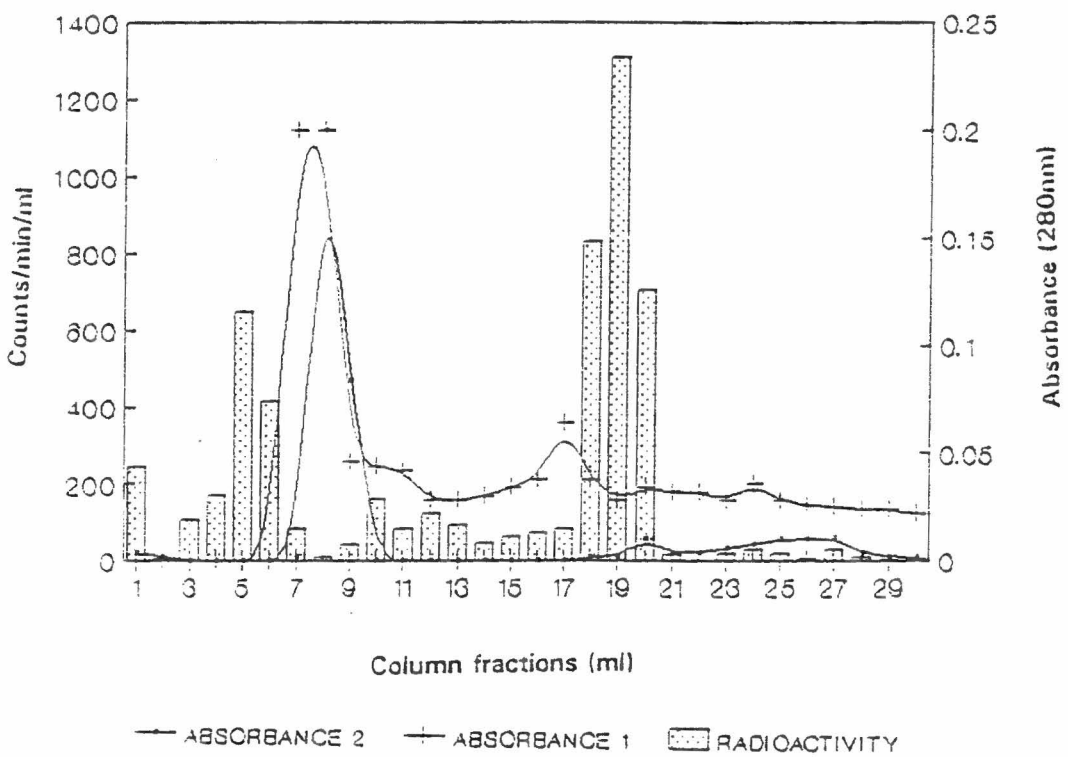


Fig 24 Measurement of radioactivity (cpm) in eluted fractions of ^{125}I-toxin-aqueous protein complex applied to a Superose 12 column attached to the FPLC. Absorbance 1 & 2 are protein profiles before and after incubation with labelled toxin.

4.4.6 Discussion.

The radiolabelling of *B.thuringiensis* protoxin and toxin utilizing ^{125}I was simple and provided a detection system sensitive enough for the current study. The stability of labelled toxin has previously been reported by other workers and found to be the same in this study. However, it was not possible to ascertain the biological activity of the labelled material. Hofmann et al. (1988a) performed bioassays using toxin labelled with a non-radioactive iodide, ^{131}I, to directly confirm that labelling did not affect the overall biological activity of the protein. Activity was, however, reduced by up to 50%. From the present study, constituent protein from labelled and unlabelled material showed the same molecular weight range after electrophoresis. Although this does not provide any direct information about the retention of its biological activity, it at least confirms that the protein still maintains some of its important physical characteristics.

Brush border membrane vesicles which consists primarily of the apical midgut columnar cells has now been employed by several workers (Wolfersberger *et al.*, 1987; Indrasith and Hori, 1992; Parenti *et al.*, 1993). It provides a potent tool for studies on the interaction of *B.thuringiensis* toxin and target insect tissue. It has an added advantage over established cell lines that are usually derived from non-midgut cell origins. Having an *in vitro* system means that the interaction between the vesicles and toxin can be studied without interference from often subtle but uncontrollable *in vivo* conditions. In

addition, because of the nature and origin of the vesicles, results obtained using this system can be assumed to be closer to what might be obtainable within the insect midgut environment.

Alkaline phosphatase being a major marker enzyme in BBMV preparation increased in activity six-fold in the present study. This level of purification compares closely with that of Wolfersberger *et al* (1987) and Indrasith and Hori (1992), who reported a seven- and six-fold increase in the activity of AP, respectively, and also with BBMV prepared by other methods (Hanozet *et al.*, 1980; Ferreira *et al.*, 1981). Although other enzymes have been used as markers, monitoring a major enzyme such as AP served the purpose of following the purity of each BBMV preparation for the duration of this study.

The constituent proteins of the BBMV prepared from both *G.m.centralis* and *C.partellus* had similarity with those reported for *P. brassicae* (Wolfersberger *et al.*, 1987) and mammalian small intestine (Kessler *et al.*, 1978).

The impetus to investigate insect midgut membrane proteins with the aim of identifying proteins which interact with *B.thuringiensis* toxins was derived from results that indicate that the attachment of the toxin to the membrane of the midgut epithelium is an important prerequisite for insecticidal activity (Hofmann *et al.*, 1988a and b; Van Rie *et al.*, 1989, 1990a).

The observation that TIKKI and MF4B/2 toxins interact with a 68 Kd protein in the respective target insects raises the possibility that they might

possess a population of binding proteins that are common to both insects. Oddou *et el* (1993), made similar observation when they demonstrated that Cry 1A(a) and Cry 1A(b) toxins bound to the same protein in *Heliothis* and *Spodoptera* insects. However, the results from the present study has greater implication for a broad-based *B. thuringiensis* strain that is toxic to insects belonging to different orders rather than to insects of different species. In addition, tests of immunological relationships which showed cross-reactivity further strengthen the position that these two proteins are similar.

The observed similarity in radioactivity profiles of ^{125}I-toxin and ^{125}I-toxin-soluble proteins can be interpreted in two ways. Firstly, no interaction took place. This is supported by the protein profile having a peak at a point of very low radioactivity. This showed that whatever eluted was the protein only. Secondly, interaction could have occurred with a small portion of the soluble proteins which subsequently eluted at the same point as the labelled toxin.

Finally, the observation that detergent extracted (aqueous) proteins show interaction with labelled toxin but not with soluble is in agreement with the already established fact that the "receptors" are membrane bound (Knowles, 1994).

4.5 Histopathological studies.

4.5.1 Effect of toxins on midgut cells.

Fig 25 shows the toxic effect of TIKKI toxin on anterior midgut cells. When compared to the control (A), large vacuoles appear in damaged cells with complete destruction of cell organelles. The microvilli are also damaged and are no longer observable. The basal membrane, longitudinal and circular muscles also showed signs of pathology. Fig 26 is a section of the mycetome showing both columnar and giant cells. The columnar cells are affected to an extent comparable to the cells of the anterior midgut (Fig 25) but the giant cells showed no apparent pathology. From Fig 27, the pathological signs of large vacuoles and a breakdown in muscular tissues were observed although to a lesser extent when compared to Fig 25. Fig 28 shows that damage to the posterior section of the midgut was largely on the basal membrane. The microvilli still appeared intact with minimal signs of pathological damage.

4.5.2 Effect of different toxins on midgut cells.

The result of exposing the anterior midgut to the three B.thuringiensis toxin is shown in Fig 29. The damage observed was most severe in B (MF4B/2) in which the muscular tissue layer was disrupted extensively, as compared to A (TIKKI) in which only large vacuoles were observed with an intact muscular tissue. No pathology was seen in C, with the entire cell components remaining intact.

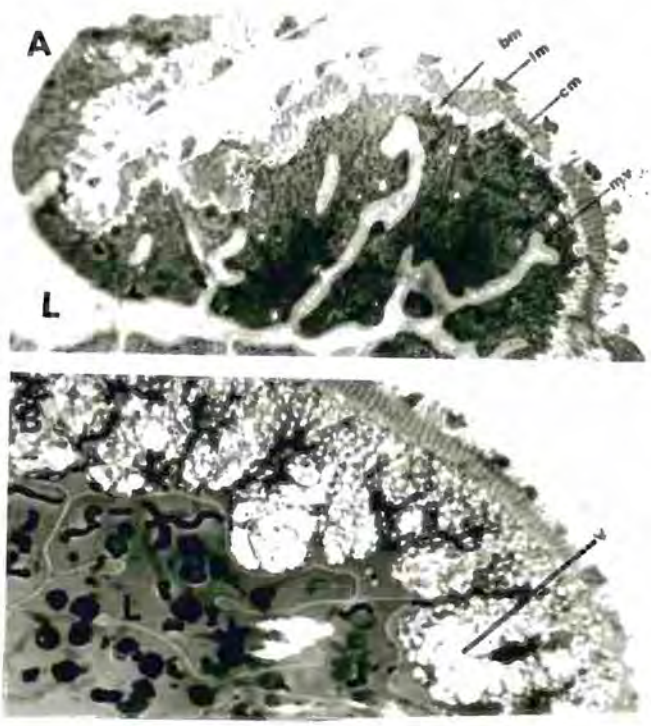


Fig 25 Photomicrograph of a section of the anterior midgut. A, control; B, cells exposed to TIKKI toxin (25 μg, 5 min). L, lumen; bm, basement membrane; lm, longitudinal muscle; cm, circular muscle; mv, microvilli; v, vacuole. Mg x100.

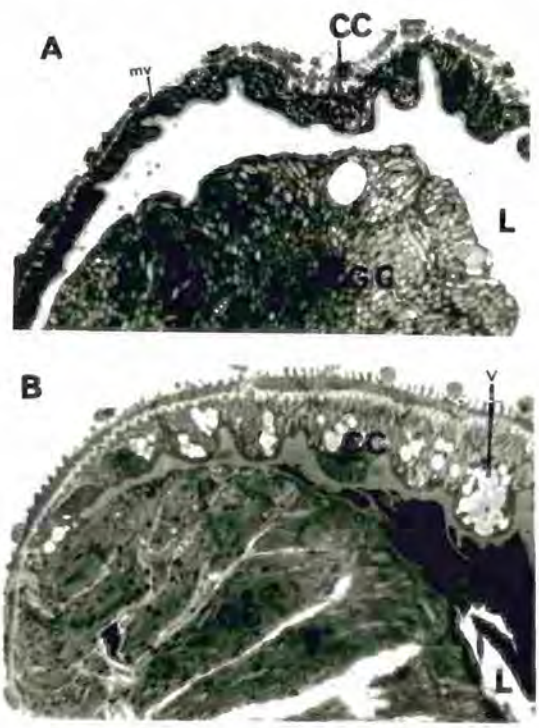


Fig 26 Photomicrograph of a section of the mycetome. A, control; B, cells exposed to TIKKI toxin (25 μg, 5 min). L, lumen; mv, microvilli; CC, columnar cells; GC, giant cells; v, vacuoles. Mg x100.

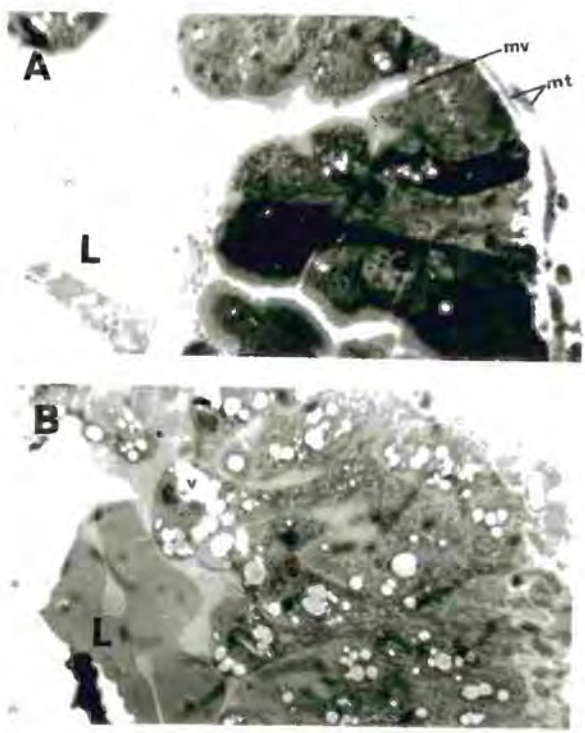


Fig 27 Photomicrograph of a section of secretory midgut. A, control; B, cells exposed to TIKKI toxin (25 μg, 5 min). L, lumen; mv, microvilli; mt, muscular tissue; v, vacuole. Mg x100.

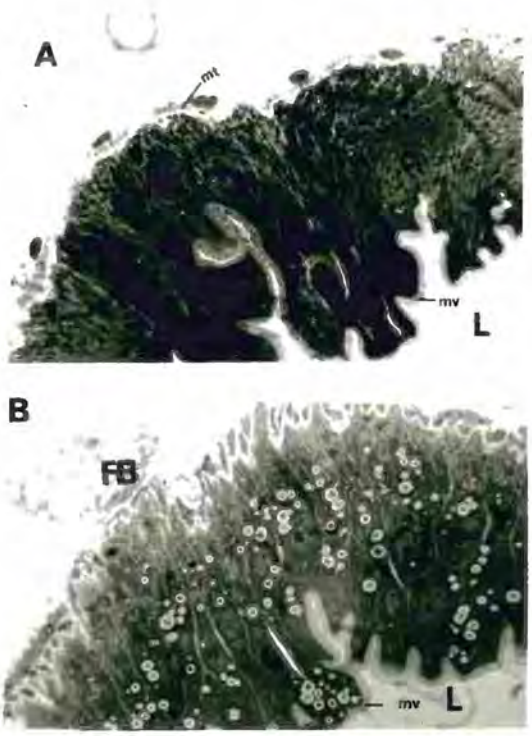


Fig 28 Photomicrograph of a section of the posterior midgut. A, control; B, cells exposed to TIKKI toxin (25 μg, 5 min). L, lumen; mt, muscular tissue; mv, microvilli. Mg x100.

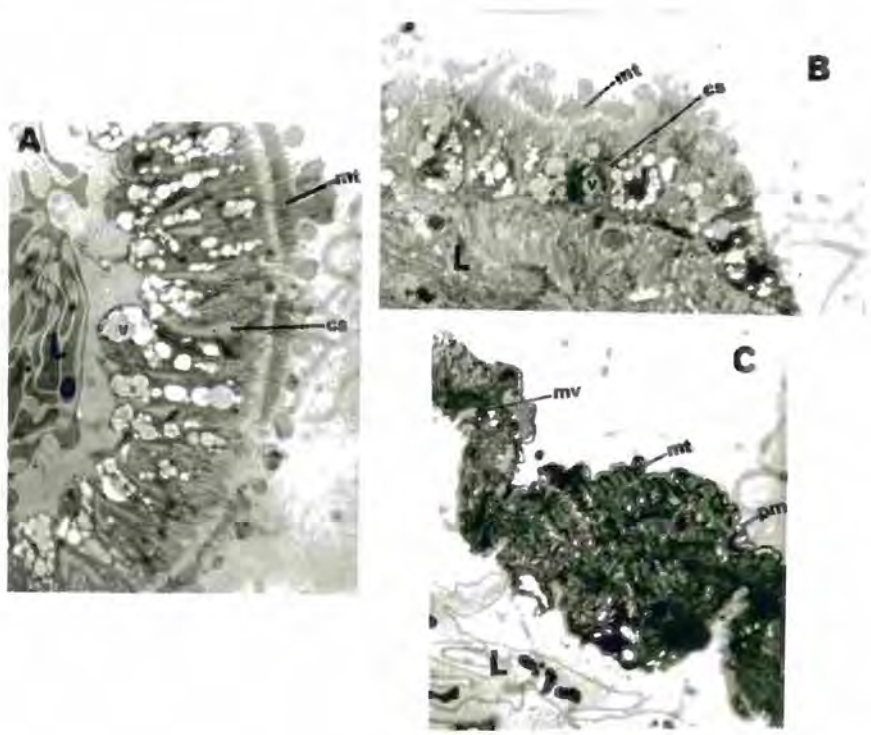


Fig 29 Photomicrograph of sections of the anterior midgut exposed to different toxins (10 μg toxin, 5 min). A, TIKKI toxin; B, MF4B/2 toxin; C, M37/2 toxin. L, lumen; mt, muscular tissue; cs, intercellular spaces; mv, microvilli; pm, peritrophic membrane. Mg x100.

4.5.3 Discussion.

The observation of high destruction of the anterior midgut is in conformity with the fact that this is the part of the midgut that first comes in contact with a blood meal. The apparent non-susceptibility of the giant cells in the mycetome was not expected. These cells contain numerous bacteroides (Wigglesworth, 1929) but their possible role in conferring resistance to the toxin is not known. The low level of pathology seen in the posterior section can be attributed to the shorter exposure to the toxin as compared to the other section.

TIKKI toxin affects all cells of the four sections of *G.m.centralis* studied except the giant cells of the mycetome. Since the mechanism of action implicates a midgut receptor(s), such a protein would appear to exist indiscriminately all along the cells of the entire midgut (Ryerse *et al.*, 1990; Bravo *et al.*, 1990). The microvilli are practically the only part of the gut epithelium exposed to the toxin *in vivo* and are, therefore, the structure of the cell in which toxic action is first seen. The breakdown of the basal membrane has been attributed largely to the disruption of the electrogenic pump that maintains ionic balance in the epithelial cells (English and Cantley, 1986).

4.6 Effect of *B.thuringiensis* δ-endotoxin on the midgut K^+ ATPase activity.

4.6.1 Optimal assay conditions.

The results obtained after assaying K^+ ATPase activity in buffer containing varying amounts of K^+ and Na^+ concentrations are shown in Fig 30 and 31. The highest activity was recorded at 80 mM and 30 mM salt concentrations for K^+ and Na^+, respectively. Subsequently, the assay buffer was prepared with these concentrations levels.

4.6.2 Partial purification of K^+ ATPase.

The specific activity of K^+ ATPase in the partially purified form was higher in the supernatant fraction when compared to the pellet fraction after the purification steps. An increase of approximately 6-fold was recorded.

4.6.3 Inhibition by oligomycin.

Table 12 below shows the activity recorded for ATPase and the inhibitory effect of oligomycin. The 51.4% reduction in activity was considered quite significant.

Table 12: Inhibition of midgut K+ ATPase by oligomycin.

Enzyme	Activity μmoles/min
ATPase	23.75
ATPase + oligomycin	11.53

% reduction in activity = 51.4%.

4.6.4 Inhibition by *B.thuringiensis* δ-endotoxin.

Fig 32 shows the depression observed in ATPase activity in the presence of oligomycin and *B.thuringiensis* toxin. The V_{max} was effectively reduced by approximately 50% by both oligomycin and *B.thuringiensis* toxin. From the double-reciprocal (Lineweaver-Burk) plot, it can be deduced that the inhibition exhibited by these two compounds was of the uncompetitive type. By comparison, the effect of M37/2 toxin on ATPase prepared from tsetse showed a non consistent result.

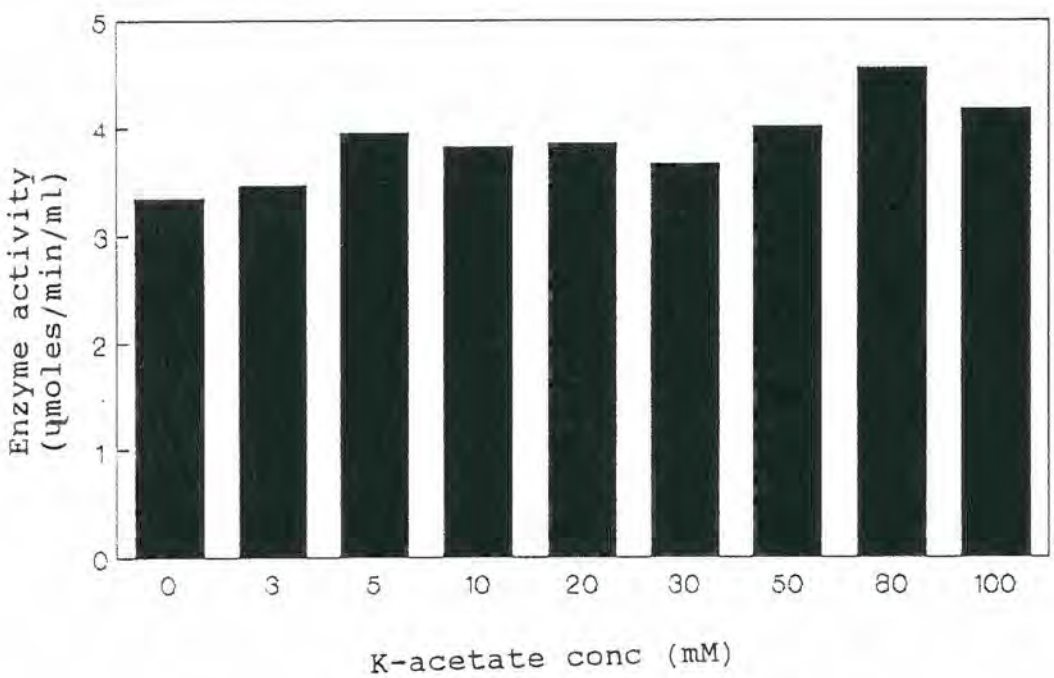


Fig 30 Midgut ATPase activity: effect of K^+ ion concentration.

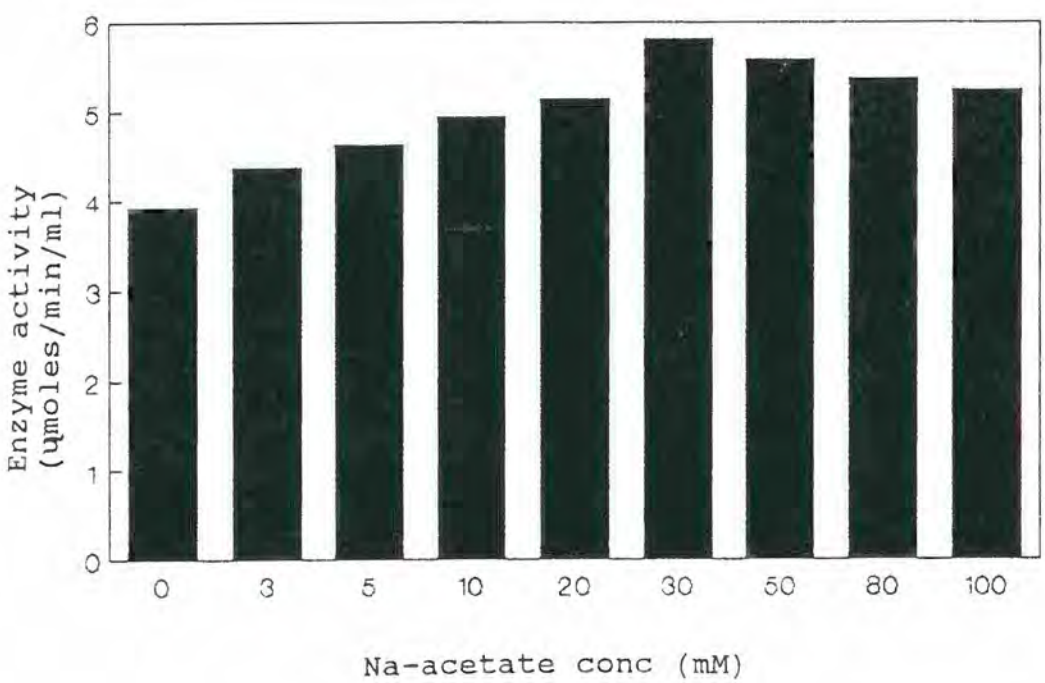


Fig 31 Midgut ATPase activity: effect of Na^+ ion concentration.

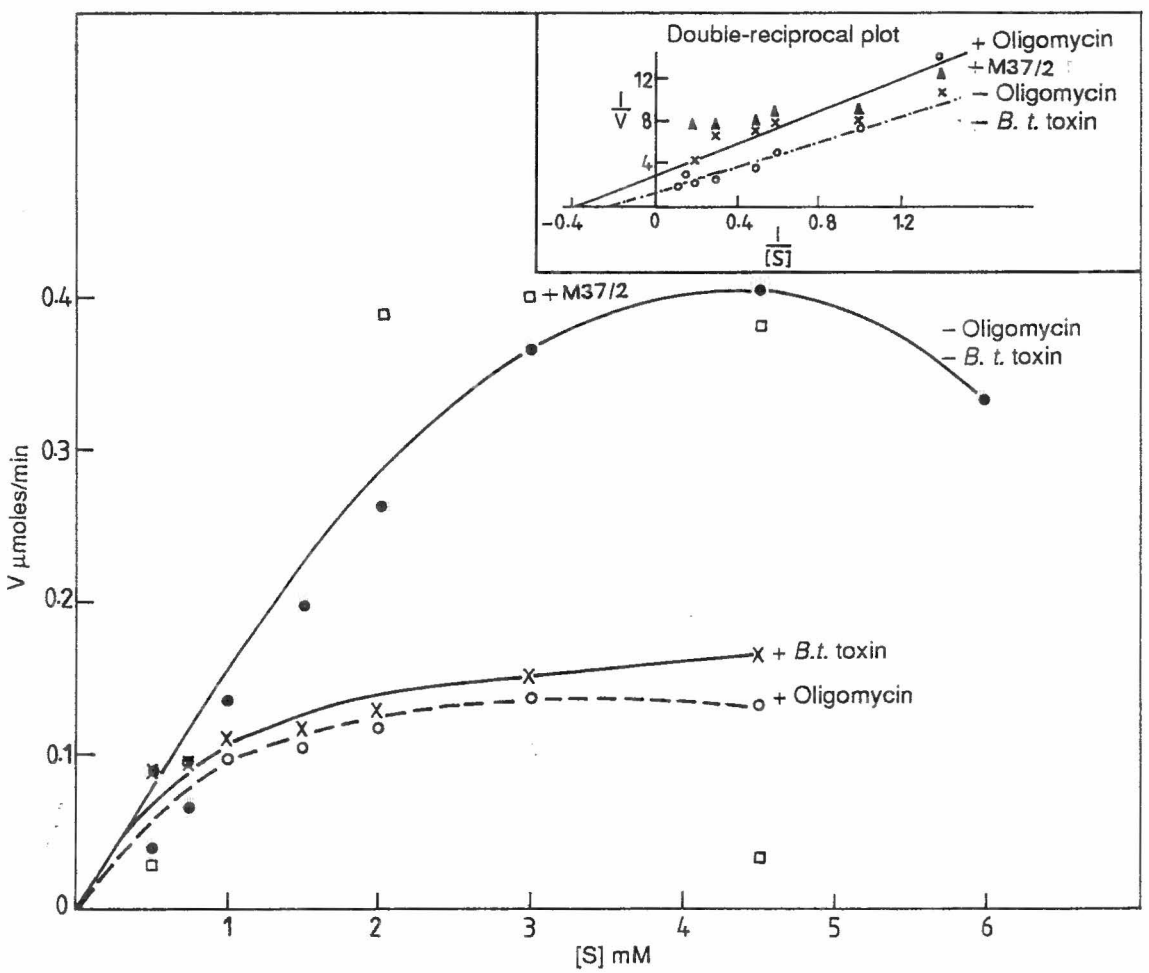


Fig 32 A plot of ATPase activity versus ATP (substrate concentration. Inset - double reciprocal plot of the initial velocity (V) versus increasing substrate concentration.

4.6.5 Discussion.

Due to the draw backs experienced in *in vivo* bioassay techniques, there has been an increasing search for a rapid and reliable way of investigating the mode of action of *B.thuringiensis* δ-endotoxin. K^+ and Na^+ salts as already reported (Giordana *et al.*, 1985; Wieczorek and Gnatzy, 1985) provide a strong driving force as compared to other salts in the electrogenic pump of insect midgut. The optimization of these salt concentrations in the development of an assay system for measuring ATPase activity *in vitro* was of primary importance. The experience gained from this study is that K^+ was definitely a greater stimulator for ATPase activity when compared to Na^+. This attribute of K^+ has been explained by the fact that *in vivo*, it has a luminal concentration as compared to Na^+ (Giordana and Sacchi, 1978; Giordana *et al.*, 1982). The optimal concentration of 80 mM observed for K^+ was comparable to that of Wieczorek (1982), who reported that the ½ maximal activity by K^+ was approximately 70 mM while Giordana *et al.*(1982) reported a lower value of 24 mM in BBMVs. On the other hand, the activity recorded in the absence of either K^+ or Na^+ confirms that ATPase maintains a certain amount of activity with either salt but the optimal situation is as already stated. In addition, some workers have also shown that under low salt conditions (< 50 mM) Na^+ acts as a better stimulator than K^+ (Weiczorek and Gnatzy, 1985).

Oligomycin is recognized as one of the inhibitors for the ATPase system under study (Harvey *et al.*, 1983). The inhibition observed with this substance

served to amply show that the purified ATPase had demonstrable and inhibitable activity before the effect of *B.thuringiensis* δ-endotoxin was studied. In investigating the effect of δ-endotoxin using an *in vitro* system, one presupposes that this will demonstrate, although indirectly, the effect this protein would have *in vivo*. The higher effect observed on ATPase activity by oligomycin as compared to the δ-endotoxin could have well been due to the purer form of the commercial oligomycin. The only other reported work in which a purified form of the enzyme was assayed is that of English and Cantley (1986) who reported a 30% reduction in the activity of a $[Na^+-K^+]$-ATPase purified from dog kidney in CHE cells using the NADH coupled system. The $[Na^+-K^+]$-ATPase, however, does not exist in insect epithelia and this result cannot be compared directly with those obtained from this study. Gupta *et al.* (1985) reported a very high percentage inhibition (78%) of active transport of K^+ in *B.t.kurstaki* treated midguts of *M.sexta*. However, this high inhibition may be attributable to the short circuit current method they used which records K+ transport very precisely. While in the present study, the ATPase activity was observed directly, others have done this indirectly by monitoring the inhibition the enzyme would have on K^+-dependent intravesicular accumulation of amino acids (Giordana *et al.*, 1982; Hendrickx *et al.*, 1990; Reuveni and Dunn, 1991; Parenti *et al.*, 1993). In the present investigation, the level of inhibition observed (50%) was not directly related to lethal concentrations *in vivo*. Other workers have however looked at this aspect with several

demonstrating that the toxin concentration which determines 50% of the effect on K^+ conductance of columnar cell apical (Sacchi *et al.*, 1986) or on K^+-dependent uptake of histidine into BBMV (Parenti *et al.*, 1993) correlated well with the LD_{50}s calculated *in vivo*. In addition, the fact that *B.thuringiensis* toxins have their effect *in vitro* against target insects only if the toxin is effective *in vivo* will be expected as a natural cause of events. This has been demonstrated in the present study using M37/2 isolate on ATPase of *C.partellus*. Others have reported similar results studying K^+ dependent transport across BBMVs (Uemura *et al.*, 1992; Parenti *et al.*, 1993)

Data deduced from this study showed that the inhibition by both oligomycin and *B.thuringiensis* δ-endotoxin was of the uncompetitive type, a situation in which the inhibitor does not combine with the free enzyme but rather with the enzyme-substrate complex yielding an inactive complex which cannot undergo further reaction to give the usual product.

It must, however, be emphasized at this point that the observation of an inhibitory effect on ATPase does not suggest that this forms the exclusive site of action for the δ-endotoxin. However, results obtained from this study partially satisfy the need to separate the ATPase from the midgut system and demonstrate that the K^+-stimulated ATPase activity is produced by a discrete enzyme.

CHAPTER 5

SUMMARY

1. The inoculum size of 0.25% of broth volume used during fermentation studies on M37/2 and TIKKI isolates were adequate to initiate satisfactory bacterial growth. spores/ml respectively.

2. M3 and M4 sustained highest bioamss of the organism for M37/2 and TIKKI respectively.

3. Biological activity did not correlate with either cell or spore counts. Bioassay was therefore the valid method used to assess biological activity.

4. M37/2 isolate was more toxic to *A.aegypti* when compared to *C.quinquefasciatus* larvae

5. Age of *A.eagypti* larvae showed a positive correlation with increasing amounts of M37/2 stock culture.

6. Linear sucrose gradient technique employed for crystal purification was efficient, yielding crystal suspensions that were virtually free of spores when examined microscopically.

7. The three *B.thuringiensis* strains could be differentiated on the basis of the band patterns obtained after SDS-PAGE separation.

8. The crystal of TIKKI isolate after electrophoresis revealed two major bands of $M_r \sim 120$ kd and 66 kd. The 66 kd protein solubilizes to yield the protoxin of $M_r \sim 64$ kd which is then further activated to a toxin of $M_r \sim 62$ kd.

9. TIKKI toxin was successfully purified in a 2-step procedure using an anion exchange column (Mono Q).

10. Immunological studies using antibodies raised against the TIKKI toxin showed a high cross reactivity between two strains; TIKKI and MF4B/2. No reaction was observed with M37/2 isolate.

11. ^{125}I-radioiodination of TIKKI toxin apparently did not affect either the physical (SDS-PAGE separation) or biochemical properties (binding studies).

12. ^{125}I-toxin-receptor interaction studies with BBMV showed a toxin-protein complex of $M_r \sim 68$, 64 and 28 kd for MF4B/2 isolate and $M_r \sim$ 64 kd for TIKKI isolate. The 64 kd protein is most probably a membrane receptor occurring in both isolates.

13. Incubation of both buffer soluble and detergent extracted aqueous midgut proteins with ^{125}I-toxin, and subsequent electrophoresis and autoradiography revealed a band of Mr $\sim$ 62-64 kd which seemed to be co-migrating with the labelled toxin.

14. Gel filtration of labelled toxin showed that protein and radioactivity profiles peaked at the same point indicating that labelling of toxin was achieved even after a 2-step purification procedure.

15. Different radioactivity profiles were obtained for soluble and aqueous proteins having one and two distinct peaks respectively. The peak of the soluble protein corresponds to the second peak of the aqueous protein, with both representing the elution point of excess ^{125}I-toxin in the incubation mixture. The first peak in the aqueous protein is thought to represent the toxin-protein complex.

16 All cells of the midgut were affected by TIKKI and MF4B/2 toxins. The giant cells of the mycetome were not susceptible. MF4B/2 was more pathogenic to the cells when compared to TIKKI.

17. Partial purification of a membrane ATPase from midgut of *G. m. centralis* was achieved through a potassium salt differential centrifugation technique.

18. Increasing the concentration of K^+ and Na^+ ions by a 100-fold in the ATPase assay medium did not proportionately increase the rate of ATPase activity. A small increase was observed at K^+ ion concentration of ~ 80 mM. Overall, K^+ ion affected ATPase activity when compared to Na^+ ion.

19. The δ-endotoxin of TIKKI isolate showed a non-competitive inhibition on ATPase activity.

20. V_{max} of the reaction was lowered by >50% by both oligomycin and *B.thuringiensis* δ-endotoxin

This study would be concluded with the following suggestions for further research,

Investigations should be encouraged into the development of other bioassay procedures that will eliminate inconsistencies in bioassay results. These are due to the heterogenic nature of living organisms used during the bioassays, and batch differences during the culturing of the bacteria.

The isolation and characterization of the $M_r \sim 64$ Kd midgut protein-toxin complex should be done to determine whether this is a membrane receptor for the toxin in both *C. partellus* and *G. m. centralis*.

Since TIKKI recognizes one protein in tsetse flies BBMV and MF4B/2, three proteins in *C. partellus* BBMV, the question of whether MF4B/2 is more toxic to *C. partellus* than TIKKI is to *G. m. centralis* can be investigated. Techniques to be used would be bioassays *in vivo* or through established cell lines *in vitro*.

Lastly, isolation, purification and reconstitution of the K^+ ATPase to show that it is a discrete enzyme catalyzing the hydrolysis of ATP within the insect midgut cells should be attempted.

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Appendix 1: Compositon of artificial diet for C.partellus.

Ingredient	Quantity
Fraction A	
Distilled water	1250 ml
Rosecoco bean powder	274 g
Brewers yeast	20 g
Sorbic acid	2.5 g
Ascorbic acid	6.6 g
methyl-P-hydroxybenzoate	4 g
Vitamin E	2.6 g
Benlate	100 g
Maize leaf powder	30 g
Fraction B	
Agar Agar	1000 g
Formaldehyde (40%)	5 g
Distilled water	1000 ml

Appendix 2: Total cell count for 12 media formulation.

Medium	Time (h)	Conc	Counts			
			1	2	3	Mean
1	S=48	1	128	127	118	124.3
	L=54	2	22.6	20.6	21.3	21.5
3	S=47	1	109	103	102.3	104.7
	L=53	2	12.3	12.3	13.3	12.6
4	S=28	1	137.6	179	169.3	108.2
	L=45	2	24	26.3	29	26.4
5	S=47	1	49.6	69	70	62.8
	L=54	2	29.6	29.6	30.3	29.8
6	S=17	1	118.3	114.6	113.3	115.4
	L=40	2	13.6	13	15.3	13.9
7	S=72	1	122.6	131	135	129
	L=78	2	13.3	15.3	14.3	14.5
8	S=48	1	190.6	222.3	219.6	210.8
	L=55	2	49	46.3	51.6	48.6
9	S=39	1	186.3	179	184	183.1
	L=45	2	44.3	44.3	45.6	44.7
10	S=46	1	181.6	185	191	185.8
	L=52	2	36.3	37.6	37.6	37.2
11	S=45	1	155	133.3	149.6	145.9
	L=49	2	29.3	27.6	28.6	28.5
12	S=46	1	213	216	213.6	214.2
	L=52	2	36.3	33.3	40.3	36.6
13	S=46	1	232.6	251.3	228.6	237.6
	L=52	2	34	39.6	32.6	35.4

S, Time at sporulation; L, Time at lysis; Conc., 1 = 10^{-6} and 2 = 10^{-7}.

Appendix 3: Spore count and bioassay for 12 media formulation.

Medium	Time (h)	Conc	Counts 1	2	3	Mean	LC_{50}
1	S=48	1	62	67	75	68	
	L=5	2	15	17	15.3	15.7	$10^{-6.6}$
3	S=47	1	92	92.6	101.3	95.3	
	L=53	2	13.6	9.6	12.3	11.8	$10^{-5.5}$
4	S=28	1	159	155	154.5	154.5	
	L=45	2	23.3	22.3	22.3	22.6	$10^{-8.7}$
5	S=47	1	35.6	40.6	23.3	33.1	
	L=54	2	24.3	25.3	23.3	24.3	$10^{-8.3}$
6	S=17	1	88.6	90.6	91.6	90.2	
	L=40	2	11.6	12.3	11.6	11.8	$10^{-7.5}$
7	S=72	1	85.6	78.3	74	79.3	
	L=78	2	12.6	14.6	14.3	13.8	$10^{-6.4}$
8	S=48	1	187.6	185	197.3	189.9	
	L=55	2	45.3	50.6	41	45	$10^{-6.6}$
9	S=39	1	132	136.6	113.6	127.4	
	L=45	2	37.6	42.6	36.3	38.8	$10^{-8.1}$
10	S=46	1	133	139.6	139.6	137.4	
	L=52	2	31	31	32.6	31.5	$10^{-6.5}$
11	S=45	1	101.6	106.3	107	104.9	
	L=49	2	26	24.3	29.3	26.5	$10^{-6.3}$
12	S=46	1	154.5	156.5	156.5	155.8	
	L=52	2	27	32.3	36.3	31.3	$10^{-6.4}$
13	S=46	1	128	133.6	130.3	130.6	
	L=52	2	30.6	37	29.6	32.4	$10^{-6.5}$

S, Time at sporulation; L, Time at lysis; Conc., 1 = 10^{-6} and 2 = 10^{-7}.

Appendix 4: Mortality achieved with different dilutions of bacterial culture (M3).

Conc (/ml of culture)	Mean mortality ± S.E. (%)
1	100 ± 0.0 a
2	100 ± 0.0 a
3	90.0 ± 10.4 a
4	90.0 ± 10.5 a
5	78.3 ± 22.2 a
6	37.7 ± 21.6 a
7	27.7 ± 15.7 b
8	29.1 ± 13.3 b
9	27.1 ± 10.1 b

Appendix 5: Age grading of *Aedes aegypti* larvae: Daily length measurements as a parameter for estimating larval growth and stage of development.

Day	Length (mm) Replicates				
	A	B	C	D	Mean (SE)
2	1.15	1.4	1.1	1.3	1.32 ± 0 03
3	1.98	2.3	2.5	2.25	2.23 ± 0.06
4	2.78	3.16	3.5	3.2	3.16 ± 0.1
5	3.33	3.43	3.7	3.24	3.42 ± 0.1
6	3.62	4.35	3.63	3.82	3.85 ± 0.08
7	4.81	5.12	5.02	4.81	4.94 ± 0.1
8	4.80	5.15	5.32	5.25	5.13 ± 0.12

Appendix 6: Specific activity of Alkaline phosphatase in crude midgut homogenate and final BBMV preparation.

A. Crude midgut homogenate

Activity	=	0.0008 μmoles/min/ml
Protein conc.	=	3.08 mg/ml
Specific activity	=	2.5×10^{-4} μmoles/min/mg of protein

B. Final BBMV preparation

Activity	=	0.0035 μmoles/min/ml
Protein conc.	=	2.35 mg/ml
Specific activity	=	1.4×10^{-3} μmoles/min/mg of protein

Appendix 7: ^{125}I-iodinated *B.thuringiensis* toxin: a typical count (cpm) obtained after iodination.

Radioactivity counts/min/μl	Column fractions ml
3.4	1
9.6	2
19452.2	3
149329.6	4
57154.5	5
42933.7	6
46228.3	7
46045.2	8
40404.6	9
29282.1	10

Appendix 8: ATPase activity: effect of K^+ and Na^+ ion concentration.

K- and Na- acetate conc. mM	Enzyme activity μmoles/min	
	K^+ ion	Na^+ ion
0	3.344	3.922
3	3.469	4.372
5	3.954	4.630
10	3.826	4.951
20	3.858	5.144
30	3.665	5.819
50	4.019	5.594
80	4.565	5.369
100	4.180	5.241

Appendix 9: Specific activity of partially purified K^+-ATPase from the midgut of *G. m. centralis*.

A. Supernatant fraction

change OD	=	0.032
V	=	2
E	=	6.22
v	=	0.02
Protein conc.	=	30 μg
Specific activity	=	17.15 μmoles/min/mg of protein.

B Pellet fraction

change OD	=	0.022
V	=	2
E	=	6.22
v	=	0.02
Protein conc.	=	132 μg
Specific activity	=	2.67 μmoles/min/mg of protein.

Appendix 10: ATPase assay using partially purified K^+ ATPase from *G.m.centralis* midgut and oligomycin, δ-endotoxin of TIKKI and M37/2 isolates.

[S] mM	1/[S]	No inhibition		Oligomycin V = μmoles/min/ml		δ-endotoxin	
		V	1/V	V	1/V	V	1/V
0.5	2	0.08	12.5	0.08	12.5	0.08	12.5
0.7	1.4	0.08	12.5	0.09	11.0	0.09	11.1
1.0	1.0	0.13	7.2	0.122	8.1	0.1	9.0
1.5	0.6	0.21	4.6	0.125	8.0	0.11	9.0
2	0.5	0.25	3.9	0.137	7.2	0.13	7.6
3	0.3	0.31	3.1	0.15	6.6	0.14	7.1
4.5	0.2	0.36	1.5	0.16	4.5	0.14	7.1
6	0.1	0.38	2.5	0.09	10.2	0.12	8.3