Insect Sci. Applic. Vol. 14, No. 3, pp. 377–381, 1993
Printed in Kenya.

0191-9040/93 $3.00 + 0.00

INTEGRITY OF THE 130 KDA POLYPEPTIDE OF *BACILLUS THURINGIENSIS* SSP. *ISRAELENSIS* δ-ENDOTOXIN IN K-S SPORULATION MEDIUM

Z. NGALO OTIENO-AYAYO*, DAVID M. CHIPMAN,
ARIEH ZARITSKY AND KAMAL KHAWALED
Department of Life Sciences, Ben Gurion University of the Negev
P.O. Box 653, Beer Sheva 84105, Israel

(*Received* 28 *January* 1992; *accepted* 18 *September* 1992)

Abstract—The 130 kDa polypeptide appears to be the most susceptible of the polypeptides constituting the *Bacillus thuringiensis* ssp. *israelensis* δ-endotoxin to the proteases associated with sporulation. Stabilization of this polypeptide in the recently described K-S sporulation medium has been achieved through limiting its cleavage by removing contaminating proteases from the culture by washing. In this way, the complete δ-endotoxin profile is retained for studies on all of the constituent polypeptide fractions.

Key Words: *Bacillus thuringiensis israelensis*, 130 kDa polypeptide, disappearance and stabilization, K-S medium

Résumé—Parmi les polypeptides constituant δ-endotoxine de *Bacillus thuringiensis israelensis*, le polypeptide de poids moléculaire 130 kDa semble étre le plus vulnérable aux protéases associées á la sporulation. La stabilisation de ce polypeptide par le procéde recémment décrit, "milieu de sporulation K-S", a été obtenue en limitant sa dégradation par lavage des protéase ayant contaminées de milieu de culture. Ainsi, le profile complet de δ-endotoxine a été retenu par études sur toutes les composantes des polypeptides.

Mots Clés: *Bacillus* ssp. *thuringiensis israelensis*, polypeptide deolypeptide de poids moléculaire 130 kDa, disparition et stabilisation, milieu K-S

INTRODUCTION

Bacillus thuringiensis israelensis is a Gram positive bacteruim that synthesises a parasporal proteinaceous crystal, the δ-endotoxin, during sporulation (Bulla et al., 1980; Somerville, 1978). The crystals consist of major polypeptides of approximate molecular weights 28, 65 and 130 kDa (Thomas and Ellar, 1983; Chilcott et al., 1983). Among other factors, the actual number of the polypeptides depends on the culture medium and culture conditions, the *B.t. israelensis* strain, the crystal purification procedure and the method of separation of the constituent polypeptides.

Stability of the δ-endotoxin polypeptide product depends, in part, on bacterial proteases which can digest the crystal toxin. Production of some of the proteases is induced by the onset of a nutrient-limited growth phase (Hanson et al., 1970). To prevent degradation of the endotoxin, Nickerson and Swanson (1981) suggested that contaminating proteases be removed during separation of crystals from spores. Other methods of separation (Mahillon and Delcour, 1984; Ang and Nickerson, 1978; Goodman et al., 1967; Pendleton and Morrison, 1966), likewise removed these proteases, but the success of the operation was dependent on the nature of the proteases and time of their production and activity.

The reported studies were necessitated by the need to use a growth and sporulation medium to

*Present address: The International Centre of Insect Physiology and Ecology, P.O. Box 30, Mbita, South Nyanza, Kenya.

produce *B.t. israelensis* labelled with [^{35}S]-Methionine, which, under normal culture conditions completely disrupted the integrity of the 130 kDa polypeptide. Using undefined medium for such studies would result in dilution of the labelled isotope, which may in turn require that more of the labelled amino acid be used to get the same quantity of radioactivity incorporated in the target object of the study.

MATERIALS AND METHODS

Sporulation and autolysis of the sporangia of *B.t. israelensis* were achieved by culturing in K-S sporulation medium as described elsewhere (Khawaled et al., unpublished data; Otieno-Ayayo et al., 1992), which had methionine as the only source of sulphur in chloride salts: NH_4Cl_2, 0.163g; $MgCl_2$, 0.012g; $CaCl_2.6H_2O$, 0.80 mg; MnCl, $6H_2O$, 6.17mg; $ZnCl_2$, 0.24 mg; $CuCl_2$. $2H_2O$, 0.34lmg; $FeCl_2$, 0.5g, and K_2HPO_4, 0.5g, supplemented with 1g/l glucose and 100 µg/ml of each (of the 18) amino acids except cysteine, for a litre of medium. K_2HPO_4 stock solution was autoclaved separately and added to the rest of the solution when cool to avoid precipitation. In radiolabelling experiments, [^{35}S]-Methionine of radioactivity ranging between 8.2 µCi and 635 µCi was added to 20 ml of medium before introducing the inoculum. *B.t. israelensis* was normally cultured in K-S medium for 120 hr. In our experiments the bacterium was cultured in the medium for only 30 hr until sporulation was completed. The biomass of the sporangia was washed twice with deionised distilled water by vortex shaking. The complex was spun down at 30°C 8000 X g for 10 min, and the supernatant decanted. The pelleted culture was washed by vortex shaking in sterile deionized distilled water. The above procedure was repeated twice and the washed biomass pelleted and resuspended in sterile deionized distilled water equivalent in amount to the original culture medium and shaken in an orbital shaker at 250 rpm, 30°C for an additional 42–90 hours. The spore-crystal complex was then harvested and washed as described earlier in ice-cold distilled water at 0°C and stored at - 20°C until used. To determine the best time to carry out the washing of the sporangia mass, this procedure was carried out at different culture stages, corresponding to end of growth phase, onset of sporulation, and end of sporulation. Two comparative control cultures were also produced, one in LB medium and the other in K-S medium, in which the spore crystal complex was harvested only after an incubation period of 120 hr [It should be noted that growth of cells and autolysis of the sporangia in LB medium was completed within 72 hr as opposed to "continuous" culture in K-S which required an additional 48 hr. It was not possible to purify crystals from K-S culture within 30 hr as most of the sporangia were still intact at this time.].

The spore crystal complex produced under each of the different culture regimes was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using Laemmli's method (Laemmli UK 1970) in a continuous buffer system. Gels were stained with 0.1% Coomassie blue in 25% methanol and 10% acetic acid.

RESULTS AND DISCUSSION

Using crystals from LB medium cultures standing as a check, crystals obtained from K-S medium, washed or unwashed during the 120 hr culture period, exhibited varying degrees of degradation. LB-produced crystals were the least affected while the unwashed K-S-produced crystals were degraded most. The 130 kDa polypeptide had been completely lost from crystals obtained from the 120 hr unwashed K-S culture (Fig. 1). This loss, however, persisted in radioactive crystals, especially with high specific radioactivity (SRA≈10^8 cpm/mg crystals), (Otieno-Ayayo et al., 1992), suggesting that high amount of radioactivity also affects the stability of the 130 kDa polypeptide.

A number of proteases are released at the onset of sporulation in *B. thuringiensis* (Hanson et al., 1970) and their activities appear to differ according to the culture medium used. If activated, they can partially digest the δ-endotoxin produced by the bacterium if culture conditions are maintained for an extended period. In the K-S medium for example, these proteases appear to be present and are activated in the post-sporulation/pre-autolysis culture solution, as evidenced by the different polypeptide profiles obtained following removal of this solution and washing of the sporangia mass when compared to those produced from the unwashed K-S culture. The washing procedure also served to remove excess salts in the medium that had caused a spreading of the polypeptide bands on the gel.

The timing of the washing procedure, however, is very important. Following inoculation of a growth medium with bacteria there is a lag phase before the cells start to divide. The length of the lag phase usually varies according to the procedure used in the preparation of the inoculum and culture conditions. The rate of growth and development also varies according to the prescribed culture conditions. These variable factors may shorten or lengthen the time before the *B. thuringiensis* culture matures, and elimination of the endotoxin-degrading proteases by washing is required to maintain the integrity of the

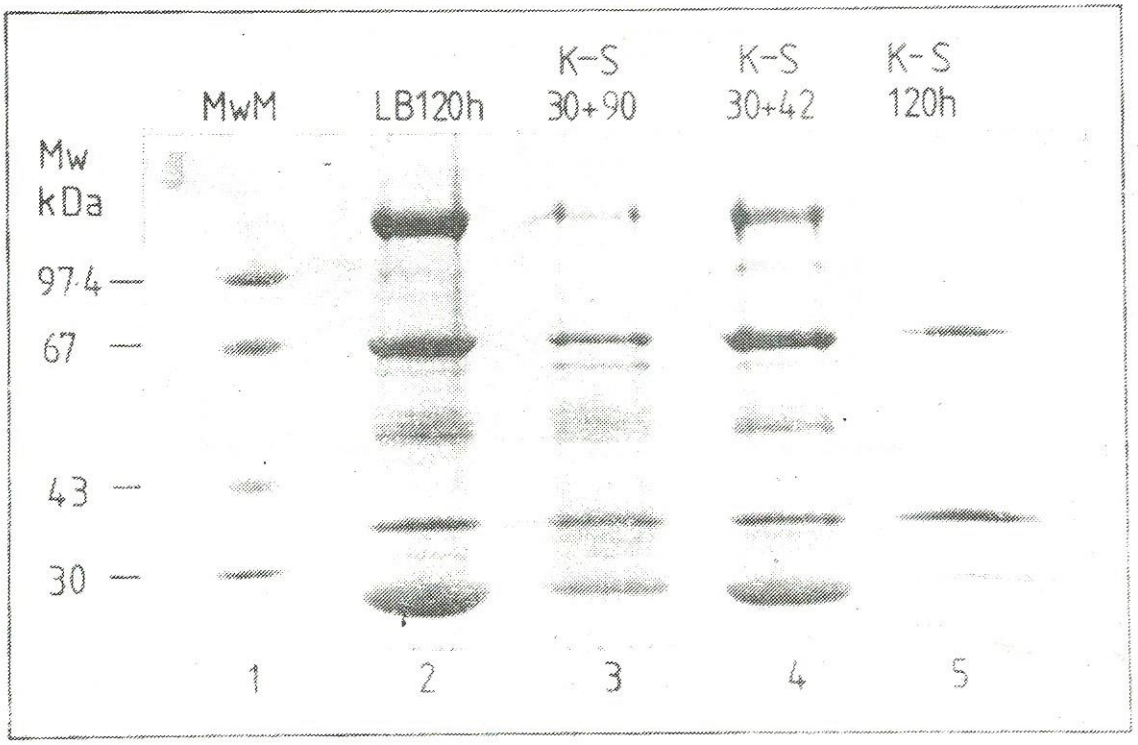

Fig. 1. SDS-PAGE analysis of *B. t. israelensis* crystal polypeptides produced in two different culture media under varied conditions, showing the effect of culture conditions on the integrity of the 130 kDa polypeptide.
Lane 1: Molecular weight markers; lane 2: crystals produced in Luria Broth under continuous culture for 120 hr, washed only at harvesting and purification; lane 3: crystals produced in K-S medium by substitution culture, by washing the sporangia 30 hr after inoculation and resuspending and incubating in deionized distilled water for an additional 90 hr; lane 4: crystals produced in K-S medium by substitution culture, washing the sporangia 30 hr after inoculation and resuspending and incubating the sporangia in deionized distilled water for 42 hr before purification of crystals; lane 5: crystals produced after 120 hr incubation in K-S medium by substitution culture method and only washing during harvesting. In all cases, crystal purification was done using a procedure modified from Pendleton and Morrison (1966) [see Fig. 2]. LB medium was the starting culture medium for the substitution cultures.

crystal toxin. Additionally, the vegetative cells will not sporulate simultaneously, neither will all the cells lyse at the same time. If the proteases are washed out of the culture before all the cells have finished sporulating, then cells that had not completed sporulation will continue to release proteases into cleaned system, leading to contamination. If washing is done until after the cells have began to lyse, some crystals will have already been released into the protease pool and will already have been attacked. A small amount of protease contamination leading to limited degradation, therefore, appears to be unavoidable. With all this in mind, we would recommend washing at 30 hr and further resuspension for about 42 hr when culturing in K-S medium.

Several non-radioactive gels of *B.t. israelensis* crystals processed by the washing procedure, demonstrated a clear presence of the 130 kDa polypeptide band (see Fig. 1, others not shown). Autoradiographs of gels of crystals labelled at very low specific radioactivity, faintly showed the presence of the polypeptide, which disappeared with increased specific radioactivity (Otieno-Ayayo et al., 1992). We therefore suggest that the 130 kDa plypeptide is susceptible to proteolysis, and, to some extent, radioactivity. It is also possible that the cleavage of the polypeptide is catalysed by metal ions (Bodanszky and Martinez 1983), which are present in K-S medium.

It is important to consider the protease-crystal interactions in the autolysate complex so that the native *B. thuringiensis* crystal and its constituent polypeptides are stabilized prior to any separation or analysis of the crystal proteins.

Successful removal of the contaminating proteases during crystal purification is essential to the maintenance of the complete endotoxin polypeptide profile. As production and activation of these proteases also varies according to the *B.*

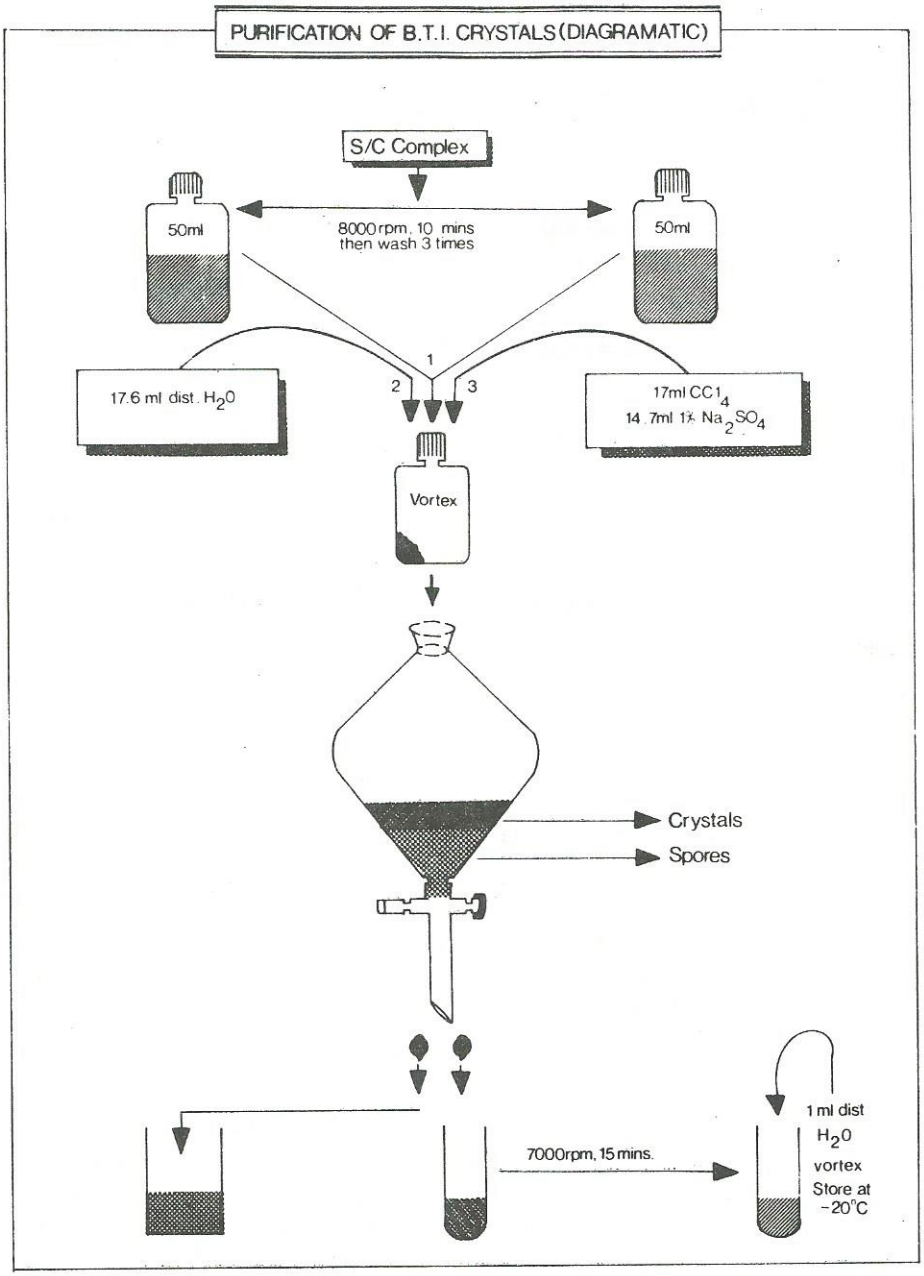

Fig. 2. Harvesting and purification of *B. t. israelensis* crystals from the spore/crystal complex: diagramatic representation constructed from the method described by Pendleton and Morrison (1966). Original drawing by Otieno-Ayayo (1989).

thuringiensis strain and culture medium used, these are additional factors which should be addressed in any studies on *B. thuringiensis* crystal proteins.

Acknowledgements—We wish to thank Dr. Maurice O. Odindo for the valuable suggestions during the preparation of the manuscript. Thanks to Prof. Thomas R. Odhiambo for encouragement during the period of fellowship. Miss Ngi-Song' Adéle Josee translated the abstract into French. This work was supported by a fellowship from the International Atomic Energy Agency (IAEA), No. C6/KEN/8806.

REFERENCES

Ang B. J. and Nickerson K. W. (1978) Purification of the protein crystal from *Bacillus thuringiensis* by zonal gradient centrifugation. *Appl. Environ.*

Microbiol. 36, 625–626.

Bodanszky M. and Martinez J. (1983) Side Reactions in Peptide Synthesis, in *the Peptides: Analysis, Synthesis, Biology* (Edited by Erhard Gross and Johannes Meinnofer), Vol.5 pp. 111–216. Academic Press Inc.

Bulla L. A. Jr., Bechtel D. B., Kramer K. J., Sethna Y. I., Aronson A. I. and Fitz-James P. C. (1980) Ultrastructure, physiology and biochemistry of *Bacillus thuringiensis*. *CRC. Crit. Rev. Microbiol.* 8, 147–204.

Chilcott C. N., Kalmakoff J. and Pillai J. S. (1983) Characterization of proteolytic activity associated with *Bacillus thuringiensis* var *israelensis* crystals. *FEMS Microbiol. Lett.* 18, 37–41.

Goodman N. S., Gottfried R. J. and Rogoff M. H. (1967) Biphasic system of separation of spores and crystals of *Bacillus thuringiensis*. *J. Bacteriol.* 94, 485.

Hanson R. S., Peterson J. A, and Yousten A. A. (1970) Unique biochemical events in bacterial sporulation. *Annu. Rev. Microbiol.* 24, 53–90.

Mahillon J. and Delcour J. (1984) A convenient procedure for the preparation of highly purified parasporal crystals of *Bacillus thuringiensis*. *J. Microbiol. Methods* 3, 69–75.

Nickerson K. W. and Swanson J. D. (1981) Removal of contaminating proteases from *Bacillus thuringiensis* parasporal crystals by density gradient centrifugation in NaBr. *European J. Appl. Microbiol. Biotechnol.* 13, 213–215.

Otieno-Ayayo Z. N., Khawaled K., Zaritsky A. and Chipman D. M. (1992) Degradation of *Bacillus thuringiensis* ssp. *israelensis* δ-endotoxin *in vitro* and in mosquito larval gut. *Biocontrol Sci. Technol.* (in press).

Pendleton I. R. and Morrison R. B. (1966) Separation of the spores and crystals of *Bacillus thuringiensis*. *Nature* 212, 728–729.

Sommerville H. J. (1978) Insect toxin in spores and protein crystal of *Bacillus thuringiensis*. *Trends Biochem. Sci.* 60, 181–197.

Thomas W. E. and Ellar D. J. (1983) *Bacillus thuringiensis* var *israelensis* crystal δ-endotoxin effects on insect and mammalian cells *in vitro* and *in vivo*. *J. Cell Sci.* 60, 181–197.