J. Indian Inst. Sci. 62 (C), Apr 1980, Pp. 1–11
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A simple technique for purification of the parasporal crystal (δ-endotoxin) of *Bacillus thuringiensis* var *thuringiensis*

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Received on March 27, 1980.

Abstract

A simple technique is described for the large scale purification of the *B. thuringiensis* var *thuringiensis* protein crystal. The method is based on the property of preferential aggregation of crystals and their subsequent separation from the spores in a column containing 0·001 M NaH_2PO_4, 0·4 M NaCl and 10 per cent glycerol at pH 5·5. The separation of crystals from spores is dependent on the conditions of pH, presence of sodium chloride in the culture medium as well as in the column buffer and presence and content of cellular debris in the spore crystal mixture. About 99 per cent pure crystal and spore preparations are obtained with high yields.

Key words: Parasporal crystal, δ-endotoxin, crystal aggregation, spore-crystal complex (scc), spore-crystal mixture (scm), crystal recovery, *Bacillus thuringiensis* var *thuringiensis* (*B.t.t.*).

1. Introduction

Bacillus thuringiensis is an economically important microbial insecticide and it shares the status of many chemical compounds that are used in controlling economically and biomedically important insects. The insecticidal principle resides in the parasporal protein crystal which is highly toxic when ingested by lepidopteran larvae[1]. However, efficient separation of spores from crystals is a prerequisite for studying the chemistry of the crystal itself. The spores and crystals can be efficiently separated from each other because of the differences in their density, surface properties and solubility. The earliest methods for obtaining pure crystals relied on spontaneous germination and autolysis of spores, followed by repeated differential centrifugation of crystals[2,3]. These techniques were followed by several diphasic systems in which organic solvent emulsions were formed. The crystals remained in the aqueous phase. Among the solvents employed are trifluorotrichloroethane[4], tetrabromomethane[3], carbontetrachloride[5], and chloroform[6]. Unfortunately, the extractions have to be repeated several times to achieve acceptable purity. Moreover, the yields are very low and the danger of crystal modi-

fication by the organic solvents is ever present. The last objection has been overcome by the use of diphasic systems containing dextran sulfate 500 and polyethylene glycol 6,000 ; the spores preferentially enter the phase richer in polyethylene glycol[7,8].

The above methods are very laborious and time consuming with very low crystal yields and loss of spores which cannot be recovered from the organic phase.

Probably the best method for the crystal preparation is isopycnic density gradient centrifugation in CsCl[9], or in certain X-ray contrasting agents such as Renografin[10-13]. Both these methods give crystal preparations of high purity. But the high cost and non-availability of CsCl and Renografin make these methods highly uneconomical. Also, the requirement for larger quantities of purified crystal for its biochemical characterization make these methods time consuming as the technique cannot be used for large scale purification. Nickerson *et al*[14] have described an isopycnic zonal centrifugation method for the large scale purification of the δ-endotoxin crystals employing NaBr gradients in a Beckman J–21B centrifuge equipped with a JCF–Z40–1900 titanium rotor which cannot be used in other available centrifuges. This places a restriction on the general use of this method.

We report here a simple, rapid and inexpensive technique for the efficient separation of the crystals and spores of *B.t.t.* based on the property of preferential aggregation of the protein crystals under specified conditions. Large quantities of relatively pure crystals as well as spores can be obtained within a short time without the disadvantages of any of the earlier methods. This method yields 99 per cent pure crystals with about 60 per cent crystal recovery.

2. Methods, results and discussion

2.1. *Procedure for separation of crystals and spores*

Growth of the organism : *B.t.t.* is grown in nutrient broth containing 0·3 to 0·4 per cent NaCl. Presence of NaCl in the culture broth is found to be a prerequisite for the phenomenon of preferential crystal aggregation to occur and their subsequent separation from the spores. No crystal aggregation is observed when NaCl is omitted from the nutrient broth. The culture is grown for 72 hr at 30° C on a rotory shaker (250 rpm) and the spore-crystal complex (scc) is harvested by centrifugation in a Sorvall GSA_8 rotor at 7,000 rpm for 10 min. The spore crystal complex is washed twice by suspending in distilled water followed by centrifugation at 7,000 rpm for 20 min.

Lysis of the vegetative cells : The scc has to be processed prior to the separation of crystals from spores. The vegetative cells, if any, in the scc are lysed by the following procedure. The scc is suspended in 1M NaCl containing 0·01 per cent Triton X-100 and magnetically stirred at 0–5° C or at room temperature for 2 hr at an OD_{600} = 10·0. The homogeneous suspension is centrifuged at 7,000 rpm for 10 min in a Sorvall

GSA_3 rotor. The pellet is suspended in appropriate amount of distilled water and sonicated 5 times, each of 30 sec duration. The suspension is diluted and then centrifuged at 7,000 rpm for 15 min. The pellet is resonicated and then centrifuged at 7,000 rpm for 20 min. The process is repeated once more but this time centrifuged for 30 min. It is observed that excessive or complete removal of cellular debris from the scc results in the loss of the phenomenon of preferential crystal aggregation. Care must be taken to avoid the excess loss of cellular debris during centrifugation either by increasing the length of the run or the centrifugal force, as the centrifugation of the spore-crystal suspension in distilled water results in slow pelleting. The spore-crystal mixture (scm) did not contain any vegetative cells at this stage. The lyophilized scm when solubilized in 0·0135 N NaOH, the OD_{280} of the supernatant is about 0·60 per mg dry weight per ml.

2.2. *Separation of crystals from spores*

Step I: Initial separation

The scm obtained from 6 litres of growth medium is suspended in appropriate amount of distilled water and sonicated 5 times each of 30 sec duration. The suspension is made 0·001 M in NaH_2PO_4, 0·4 M in NaCl and 0·01 per cent in Triton X–100 at an $OD_{600} = 5\cdot0$ and allowed to stand at 0 to 5° C in a 1 L beaker. The crystals preferentially aggregate under these conditions and the aggregates start settling at the bottom of the beaker. After 2 hr, the supernatant rich in spores is decanted and centrifuged to recover spores. The sediment rich in crystals is resonicated and again suspended in the above suspension buffer. The process is repeated 3 times and the crystal rich fraction is centrifuged. The pellet is washed 3 times with distilled water and each time centrifuged at 10,000 rpm for 10 min in an SS_{34} rotor and can be stored at − 20° C before further processing.

Step II: Final separation

The final step involves the suspension of the above crystal-rich fraction in the suspension buffer A, which is 0·001 M in NaH_2PO_4, 0·4 M in NaCl, 0·01 per cent in Triton X-100 and 2 per cent in glycerol and sonicated. The final suspension has an $OD_{600} \doteq 5\cdot0$. About 30 ml of the crystal-rich suspension is then layered on to the top of the supporting solution B, which is 0·4 M in NaCl and 10 per cent in glycerol in a glass column of 20 × 4 cm (Fig. 1). The crystal aggregates fall to the bottom of the column leaving the remaining spores at the top of the column. The crystals are collected through the outlet of the column. The process is repeated once or twice to achieve reasonable purity. Finally, the crystals are collected and centrifuged.

Step III: Removal of cellular debris

The final crystal preparation contains cellular debris and it must be removed to obtain pure crystals. The debris can be removed by sonication of the crystal fraction in distilled

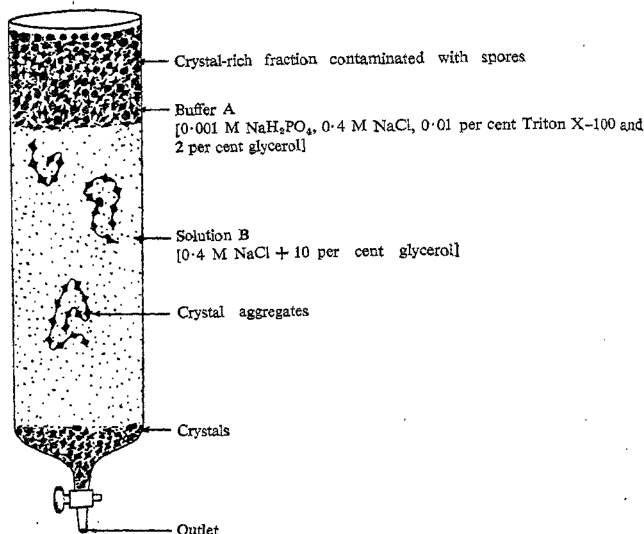


FIG. 1. Final purification of crystals in a glass column (20 × 4 cm).

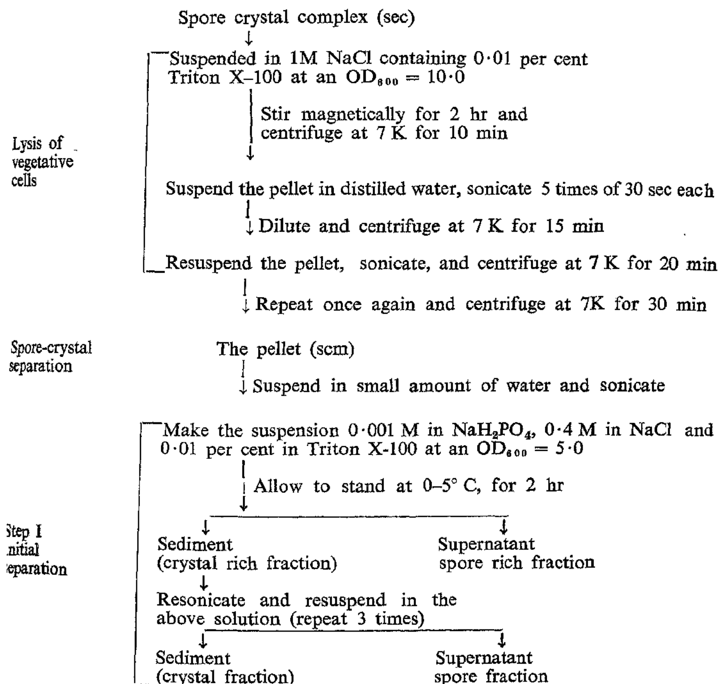
water followed by differential centrifugation. The crystals are suspended in distilled water and sonicated 5 times, each of 30 sec duration, and centrifuged at 4,000 rpm for 10 min in a Sorvall SS_{34} rotor. Crystals form a compact pellet and the cellular debris remains in the supernatant which is removed by decantation. The process is repeated 5 to 6 times when almost all the debris has been removed. The pure crystal preparation is then lyophilized to constant weight and stored at —20° C. The overall procedure for the separation of crystals from spores is represented in scheme 1.

The purity of the crystal preparation is determined by the viable spore count methods[11, 17], carbolfuchsin staining method[18], as well as by determining the protein content of the crystal preparation.

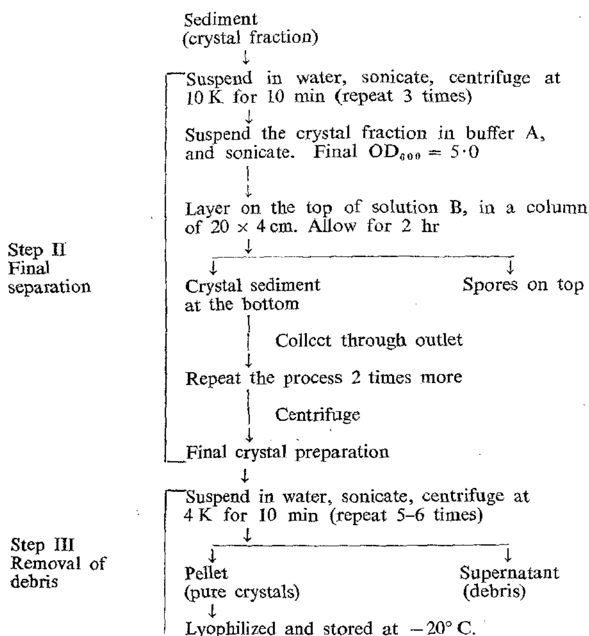
The crystal preparation is suspended in distilled water, sonicated and plated on nutrient agar in serial dilution for the spore count. The results of the average values obtained in three independent experiments are reported in Table I. It is observed that in all cases 99 per cent purification of crystals is obtained.

The crystal purity is also measured by the protein content of the crystal preparation. The protein content of the final crystal preparation is comparable to that of the crystal preparation obtained by Renografin density gradient centrifugation[10]. Protein values obtained on various steps during crystal purification are given in Table II.

In the spore fractions obtained during the initial separation of crystals (scheme 1) supernatant OD_{280} of 0·2 to 0·25 is observed on dissolving 1 mg of spore fraction in 0·0135 N NaOH for 5 hr after removal of debris. Microscopic examination of these spore fractions after staining with basic carbolfuchsin showed the presence of one to two crystals per 100 spores, representing 98–99 per cent pure spore preparations. As our



SCHEME 1 (*Contd.*)



SCHEME 1. Purification of parasporal crystals of *B. t.t.*

main objective was to purify the crystals, further purification of the spore fractions was not attempted.

The applicability of the technique, developed for the purification of crystals of *B. t.t.* to the purification of crystals of other varieties of *B. thuringiensis* has been investigated. The results are shown in Table III.

2.3. *Effect of pH and salt concentration on the phenomenon of crystal aggregation*

The effect of the concentration of NaH_2PO_4 and that of NaCl, effect of combination of both NaH_2PO_4 and NaCl and the effect of Triton X-100 on the crystal aggregation has been investigated. pH as well as the ionic strength of the suspension buffer affects the preferential crystal aggregation. Increase in pH beyond $5 \cdot 5$ results in the loss of the

Table I

Purity of crystal preparations by spore-count method

Sl. No.	Initial count [a]	Final count [b]	Per cent purity of crystals	Per cent crystal recovery [c]
(i)	* 30×10^6	$0{\cdot}29 \times 10^6$	99·03	58 (29)
(ii)	30×10^6	$0{\cdot}31 \times 10^6$	98·97	60 (30)
(iii)	30×10^6	$0{\cdot}30 \times 10^6$	99·00	60 (30)

100 mg by dry weight of *B. t.t.* scm is taken in each of the three experiments. The per cent purity of the crystals is estimated by the viable spore count method[11,17] and by carbolfuchsin staining method[18]. 1-2 spores per 100 crystals were observed with phase contrast optics in the latter method.

(*a*) *Initial count* is the spore count of scm per mg dry weight.

(*b*) *Final count* is the spore count of pure crystal preparation per mg dry weight.

(*c*) *Per cent crystal recovery* : Ignoring the content of debris it is assumed that 100 mg of scm contains 50 mg of spores and 50 mg of crystals.

$$\text{Per cent crystal recovery} = \frac{\text{Actual crystal recovery}}{\frac{1}{2}\,(\text{wt of scm started with})} \times 100$$

But, in practice, most of the debris comes into the crystal fraction. The actual final recovery of crystals, indicated in parenthesis, is estimated after removal of the cellular debris and lyophilization to constant weight.

preferential crystal aggregation, although one can find non-specific aggregation between crystals and spores above 0·005 M buffer solutions. Sodium chloride alone can cause crystal aggregation but the spore contamination is high. Below pH 5·0, the crystals got deformed with the resultant fusion of crystals on repeated separations.

The OD_{600} at which the scm is suspended affects the purity of the final crystal preparation. Separations at high concentrations of scm result in high contamination of crystal preparation with spores. The crystal purity and crystal recovery have been estimated under various conditions of pH, salt concentration and the concentration of scm, the detail results of which are summarized above but not presented here. In all the purification steps, maximal pure crystal preparation, without affecting the crystal structure, has been achieved when the scm is suspended at an $OD_{600} = 5{\cdot}0$ in a solution which is 0·001 M in NaH_2PO_4, 0·4 M in NaCl and 0·01 per cent in Triton X-100.

Table II

Purity of crystal preparations by protein estimation

Preparation	OD_{280} per mg dry wt per ml
scm	0·60
Cr^1	0·73
Cr^2	0·85
Cr^3	0·98
Cr^4	1·02
Cr^5	1·06
Crystals by Renografin method	1·10+

1 mg of the lyophilized sample is solubilized in 1 ml of 0·0135 N NaOH for 5 hr and centrifuged. The OD_{280} of the supernatant is measured to give the protein content of the preparation. Debris is removed from the crystal fractions but not from the scm.

Cr^1–Cr^5 = Crystal preparation after *i*th step of separation.

\+ = Value observed earlier by Y. I. Shethna while working at the Biological Research Unit, U.S. Grain Marketing Research Centre, Manhattan, Kansas 66502, U.S.A.

Table III

Applicability of the purification procedure to other varieties of *B. thuringiensis*

	Bacillus thuringiensis var	Initial count ×10⁶/mg	Final count ×10⁶/mg	Per cent crystal purity	Per cent crystal recovery
(i)	*thuringiensis*	30	0·30	99·00	59
(ii)	*sotto*	25	1·55	93·80	35
(iii)	*galleriae*	23	1·3	94·35	30
(iv)	*kenyae*	27	2·0	92·65	27
(v)	*tolworth*	22	3·15	85·68	11
(vi)	*kurstaki*	24	3·25	86·46	9
(vii)	*entomocidus*	21	3·55	83·17	5

Each experiment is started with 100 mg by dry weight of the respective scm. The figures in each case represent the average of three independent determinations. Crystals of *B. thuringiensis* var *alesti*, var *dendrolimus*, var *subtoxicus*, var *aizawai* and var *morrisoni* do not aggregate under these conditions. The very little sediment obtained could not be further purified.

The strains used have been obtained from Dr. E. Afrikain, Director, Institute of Microbiology, Abovian City, Armenian SSR, USSR.

Surface crystal antigens as well as the charges on the crystal surface may play a role in the phenomenon of crystal aggregation. Lowering the pH, at a suitable ionic strength provides the best conditions for the preferential aggregation of crystals which may be due to the interaction between the crystal antigens. We compared the occurrence of aggregation of crystals of different varieties of *B. thuringiensis* with their known crystal antigens[3, 15, 16] in order to elucidate whether surface crystal antigens influence the phenomenon of aggregation (Table IV). It appears that the crystal antigens are not the sole determinants of the property of aggregation.

Only *B. thuringiensis* var *thuringiensis* could give the best results under these conditions. While this method is partially successful with a few varieties, a few others tested did not show the phenomenon of crystal aggregation under the same conditions. Different varieties of *B. thuringiensis* may require modified conditions under which the phenomenon of crystal aggregation can be observed. The non-applicability of these conditions for varieties other than *B.t.t.* may be due to the differences in the structure, conformation and surface antigens of the crystals.

Table IV

Effect of crystal antigens on the phenomenon of aggregation

B. thuringiensis var	Crystal antigen (Ref. 3, 15, 16)	Aggregation
thuringiensis	*a d i*	++
sotto	*c*	+
galleriae	*f h*	+
kenyae	*e*	+
tolworth	*e*	±
kurstaki		±
entomocidus	*a c*	±
subtoxicus	*a*	—
morrisoni	*g*	—
dendrolimus	*d i*	—
alesti	*h i*	—

++ = 99 per cent purity and about 60 per cent crystal recovery.
\+ = 90–95 per cent purity and about 25–35 per cent crystal recovery.
± = 80–85 per cent purity and about 5–10 per cent crystal recovery.
— = No significant crystal aggregation.

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