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The mechanism of sunlight-mediated inactivation of *Bacillus thuringiensis* crystals

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Detailed photostability studies were carried out using purified δ-endotoxin crystals from *Bacillus thuringiensis* subspecies HD-1 and HD-73. The mechanism and time course of sunlight inactivation was investigated by: (*a*) monitoring the tryptophan damage in the intact crystals by Raman spectroscopy, (*b*) amino acid analysis and (*c*) biological assays using insects. The results demonstrate that, for purified HD-1 or HD-73 crystals, the 300–380 nm range of the solar spectrum is largely responsible for bringing about crystal damage and consequent loss of toxicity. Purified *Bacillus thuringiensis* crystals that were exposed to fermentation liquor after cell lysis were more quickly degraded by sunlight than were crystals from cells that were lysed in water. This effect is attributed to adsorption of chromophores by crystals exposed to the fermenter liquor and the subsequent ability of these chromophores to act as photosensitizers. The importance of a photosensitization mechanism in crystal degradation was further emphasized by irradiating *Bacillus thuringiensis* crystals *in vacuo*. The latter crystals were found to be less damaged (20% tryptophan loss after 24 h irradiation by the solar spectrum) compared with crystals from the same sample irradiated in air (60% tryptophan loss). Other methods of decreasing exposure of the crystals to oxygen, e.g. by using glycerol as a humectant, were also found to be successful in controlling photodamage. The results concerning photodegradation support a photosensitization mechanism involving the presence of exogenous (and possibly endogenous) chromophores which create singlet oxygen species upon irradiation by light.

INTRODUCTION

The proteinaceous δ-endotoxin produced by *Bacillus thuringiensis* (Bt) var. *kurstaki* accumulates in the cells as a crystalline inclusion body. Upon ingestion of Bt material by lepidopteran larvae, lysis of midgut epithelial cells occurs, followed eventually by larval death (Lüthy & Ebersold, 1981; Knowles & Ellar, 1987). Since the Bt proteins are highly specific for their insect targets, the Bt system is valued as an environmentally safe bioinsecticide.

The sunlight-mediated inactivation of Bt preparations in the field is a problem affecting the efficacy and economics of the bioinsecticide (Beegle *et al.*, 1981). Studies on this topic in the literature have considered the wavelength of light responsible for inactivation (Ishigura & Miyazono, 1982), the production of possible photosensitizers (Harms *et al.*, 1986) and the utility of photoprotectants (Morris, 1983). However, most studies have used crude Bt preparations containing spores, cell debris and fermentation and formulation material in addition to the parasporal Bt crystals. Since the latter is a key component of toxicity, we have set out to determine the details and mechanism of sunlight degradation in purified Bt crystals. In a previous publication (Pozsgay *et al.*, 1987) we have demonstrated that sunlight irradiation, using a solar simulator, gave rise to the destruction of up to 35% of tryptophan residues in Bt crystals, as detected by Raman spectroscopy. These studies have now been extended to include such factors as time-dependence, wavelength dependence and O_2-dependence of crystal damage monitored by amino acid analysis, Raman spectroscopy and bioassays. The results allow us to identify photosensitization as the major mechanism leading to photodegradation.

MATERIALS AND METHODS

Fermentation and crystal purification

Bt var. *kurstaki* strain HD-1 or HD-73 cells were grown in 1 litre of half-strength Tryptone/soya-broth medium for 24 h and used to inoculate a 25-litre fermenter containing the same medium. Purification of crystals after lysis was described previously (Carey *et al.*, 1986).

For studies involving the effect of exogenous chromophores on crystal photostability, the protocol was modified. After 44 h fermentation at 28 °C with 250 rev./min agitation and half-volume/volume aeration, half of the fermenter load was harvested. The pellet containing unlysed cells was washed with distilled water, 1 M-NaCl/0.01% Triton X-100 solution and then water to remove the medium. The washed cells were resuspended in water and lysed by stirring at 4 °C overnight. The second part of the fermenter load was allowed to lyse in the medium and the crystals subsequently harvested.

The 65 kDa toxin from strain HD-73 was purified as described previously (Bietlot *et al.*, 1989).

Amino acid analysis

Samples were hydrolysed in a Waters workstation by 6 M-HCl vapour and/or 4 M-methanesulphonic acid at 105 °C for 24 h and derivatized with phenyl isothiocyanate (PITC; Pierce) ac-

Abbreviations used: Bt, *Bacillus thuringiensis*; PITC, phenyl isothiocyanate; LD_{50}, dose of toxin required to kill 50% of insects tested; I_{1450}, intensity of Raman peak near 1450 cm^{-1}; I_{1555}, intensity of Raman peak near 1555 cm^{-1}.

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cording to the Waters protocol. The analyses were carried out by a Waters PICO-TAG system equipped with an automatic injector and photodiode-array detector.

Bioassay

LD_{50} (dose of toxin required to kill 50 % of insects tested) data were obtained for the HD-1 strain by force-feeding fourth-instar silkworm (*Bombyx mori*) larvae and for the HD-73 strain by force-feeding sixth-instar, 1-day-old Eastern-spruce-budworm (*Choristoneura fumiferana*) larvae. The dose volume was almost exclusively 2 μl. Mortality was scored after 24 h for silkworm and 3 days for Eastern spruce budworm. Each LD_{50} assay involved three replicates of ten insects.

Irradiation by solar simulator

Samples were irradiated by a solar simulator (Oriel Corporation, Stratford, CT, U.S.A.) delivering a solar spectrum equivalent to the light passing through 1.5 layers of the Earth's atmosphere. For bioassay after irradiation, 3–6 mg of purified crystals or toxin samples were spread on a 6 cm^2 area of a glass microscope slide. For analysis of the effects of irradiation by Raman spectroscopy or amino acid analysis, 0.5–1 mg of protein was applied over a 1 cm^2 area. The irradiating light power equalled 70 mW/cm^2 [measured by a Scientech (Boulder, CO, U.S.A.) calorimetric power meter]. The temperature increase at the sample surface was measured by using a thermocouple and found to be not greater than 4–5 °C.

For wavelength-dependence studies, 4 cm × 4 cm Corning glass filters were placed over the samples. The filters with 350, 370, 400, 500, 600 nm cut-off [Corning numbers: CS 0-52, 7380; CS 0-51, 3850; CS 3-74, 3391; CS 3-69, 3486; CS 2-60, 2408 (respectively)] exclude the light below the wavelength mentioned and pass wavelengths above (i.e. to the red of) the cut-off. Typically the effect of the irradiation time on purified crystal samples was analysed after exposing the samples to the solar simulator for 4, 8, 16, 24, and 52 h.

Photoprotection experiments were carried out by suspending HD-1 crystals in 5, 20 or 50 % (v/v) glycerol solutions in water, then exposing the crystal suspension, placed as a drop on a glass slide, to the solar spectrum for 24 h.

For solar irradiation in the absence of O_2, a 2 mg crystal suspension was dried on each of two narrow microscope slides. One of these was sealed in an evacuated square-section quartz tube. The slides (one in the presence, and the second in the absence, of O_2) were exposed to the solar spectrum for 24 h. The amount of tryptophan damage was determined by amino acid analysis.

RESULTS AND DISCUSSION

Between 90 and 95 % of cells contain parasporal crystals, which, after controlled lysis, can be purified as described elsewhere (Carey *et al.*, 1986). Typically 1–2 g of var. *kurstaki* HD-1 or HD-73 crystals could be prepared from a 24-litre fermentation volume. For the studies detailed here we used preparations with a crystal-to-spore ratio of 1000:1 or better. Such crystals were stable in water at 4 °C, pH 6.0, and no degradation could be detected (using SDS/PAGE) over a period of 4 years.

Photoinactivation of crystals

The possible loss of toxicity of Bt preparations due to exposure to sunlight has been pointed out by a number of authors (Yamvrias, 1962; Raun *et al.*, 1966; Sneh *et al.*, 1983). Experiments in this laboratory (Pozsgay *et al.*, 1987) have been performed on purified crystals from HD-1 and NRD-12 strains and have demonstrated major loss in toxicity after irradiation by sunlight. Recently very similar results have been obtained for HD-73 crystals. The irradiation was carried out under controlled conditions by a solar simulator whose spectrum is equivalent to that of the solar spectrum (Fig. 1). Raman-spectroscopic analysis demonstrated that about 35 % of tryptophan side chains in the crystal protein are destroyed after 24 h irradiation. The loss of indole side chains was subsequently confirmed by amino acid analysis, which also showed that ~ 30–40 % of imidazole side chains had been destroyed. Under these conditions almost total activity loss occurs. Very similar results have been obtained for crystals of HD-73. However, there are variations in the amount of damage per unit time of irradiation depending on the history of the sample (see below).

Wavelength-dependence

Irradiation studies were carried out by mounting Corning glass filters above each sample so that the sample was bathed only in the light transmitted by the filter. HD-1 crystals, control and irradiated under the filters for 24 h, were analysed by Raman spectroscopy (Carey *et al.*, 1986). Samples irradiated in the same batch were tested for toxicity towards silkworm larvae. Tryptophan degradation was measured using the intensity of the tryptophan Raman peak near 1555 cm^{-1} relative to the line near 1450 cm^{-1} (due predominantly to the CH_2 scissoring mode from methylene groups of amino acid side chains and arbitrarily assigned an intensity of 10.0). On this scale the 1555 cm^{-1} tryptophan mode had an intensity of 3 for the control sample. If the 600, 500 and 400 nm cut-off filters were used for the irradiated

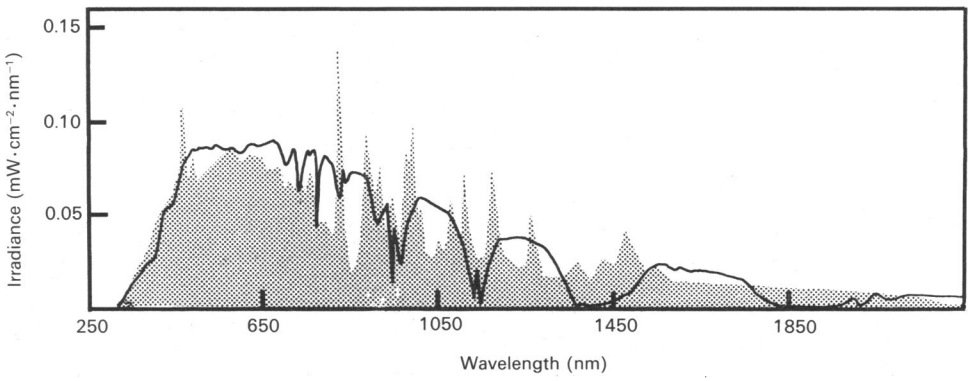

Fig. 1. Solar spectrum (continuous line) and Oriel-solar-simulator (stippled area) irradiance (sources: Solar Energy Research Institute, Golden, CO, U.S.A., and Oriel Solar Simulator literature)

Table 1. Locating the damaging wavelength range by 24 h irradiation of HD-1 crystals under glass filters

Wavelength range of irradiation	Method of detection... Bioassay [LD_{50} (μg of Bt/larva)]	Raman spectroscopy [$(I_{1450}/I_{1555}) \times 10$]
Control (non-irradiated)	0.15	3.0
Above 600 nm	0.19	3.1
Above 500 nm	0.26	3.2
Above 400 nm	0.40	3.0
Above 370 nm	0.55	–
Above 350 nm	2.55	–
Total spectrum	> 4	2.0

samples, the 1555 cm^{-1} intensity remained at 3 units (Table 1). Since these filters cut off light below 600, 500 and 400 nm respectively, the results indicate that little or no photodegradation occurs upon irradiating with the 400 nm to the i.r. portion of the solar spectrum. By contrast, samples irradiated with the full solar spectrum had a 1555 cm^{-1} peak intensity of ~ 2, indicating that about one-third of the tryptophan peak intensity had been lost. This result strongly suggests that the 300–400 nm spectral region is primarily responsible for degradation.

The bioassay results are in good accord with the Raman data. The former show that the LD_{50} values do not change greatly when 600, 500, 400 and 380 nm cut-off filters are used (Table 1). Again these data demonstrate that photoinactivation is minimal for the solar spectrum above 380 nm. However, slight and progressively increasing damage does appear to accompany the appearance of irradiation towards the blue end of the visible spectrum. The crystals underwent considerably more inactivation when the 360 nm filter was used. Since this blocks at shorter wavelengths than 360 nm, it is evident that the 360–380 nm region of the solar spectrum accounts for a substantial portion of photodegradation. Since filters with cut-offs between 360 and 300 nm were not available, we could not compare the effects of 'adding' the portion of the solar spectrum in this range. However, given the likely broad nature of the absorption bands of the chromophore responsible for photodegradation (see below) we believe that the entire 300–380 nm region causes photodegradation, although as 300 nm is approached the light from the solar spectrum reaches zero intensity after passing through the equivalent of $1\frac{1}{2}$ layers of the Earth's atmosphere (Fig. 1). The quantity of $1\frac{1}{2}$ layers of atmosphere is chosen as an average value because at most latitudes sunlight does not strike along the normal to the Earth's surface. Consequently, the light travels a greater distance than that defined by the direction along the normal.

Role of exogenous chromophore

Pure protein containing no prosthetic groups or modified amino acids is not degraded to any marked extent by sunlight (Pozsgay *et al.*, 1987), since the solar spectrum decreases to zero intensity at 300–305 nm (Fig. 1) and the nearest protein absorption band is due to indole side chains with an absorption maximum in the 280–285 nm region. Thus the facile degradation of Bt crystals demonstrates that a chromophore, absorbing in the 300–380 nm range, must be associated with the protein. This could be in the form of an exogenous non-covalently bound residue or covalently bound, e.g. as a modified amino acid, such as kynurenine, derived from a tryptophan side chain. In either case the chromophore would bring about degradation by the process of photosensitization, which involves the chromophore absorbing a photon and passing excited-electronic-state energy to a neighbouring oxygen molecule, converting it from a triplet to a singlet state. The resulting singlet oxygen is highly reactive, with indole and imidazole residues being favoured targets. In addition, a number of minor free-radical reactions such as attack on sulphur atoms and polypeptide-chain cross-linking could, and in all likelihood does, occur.

The detection and identification of very small amounts of modified amino acids in a large protein such as Bt protoxin or toxin is not an easy task and is rendered very difficult by the masking effect of exogenous chromophores that may be present. However, it has proved possible to generate data on the latter. Exogenous chromophores absorbed on to proteins within Bt crystals or adsorbed on the surface of the crystals themselves could come from two sources: either from the cytoplasm of the Bt cells during crystal deposition, or from the fermentation medium after cell lysis.

The possible role of chromophores associated with the fermentation medium was investigated by comparing the properties of HD-1 crystals that had been in contact with fermentation liquor with those that had been in contact only with water after lysis. Crystals were purified from the same fermentation batch after lysis in the fermentation medium or in water. The latter crystals do not have the opportunity to adsorb fermentation-medium-borne chromophores, since the water-lysed cells were washed repeatedly before lysis. In addition, the same culture was grown on agar plates prepared in the same medium. Purified crystals from fermenter-lysed, water-lysed and agar-grown sources were subject to 0–52 h of the full solar spectrum. Table 2 compares the results of silkworm-larvae bioassays for these samples. For no irradiation, all the samples have very similar toxicities. Interestingly, however, the fermenter-lysed crystals and also the agar-grown crystals are more sensitive to irradiation as compared with the water-lysed samples. At 8 h irradiation the LD_{50} of the fermenter-lysed crystals is 10 times higher than the water-lysed, and twice that of the agar-grown, material. The fermenter-lysed material is essentially atoxic after 16 h

Table 2. Photostability of different irradiated HD-1 samples

Irradiation time (h)	LD_{50} (μg of Bt/silkworm larva) Fermenter-lysed	Water-lysed	Agar-grown
0	0.11	0.09	0.04
4	0.37	0.21	0.3
8	3.15	0.37	1.48
16	> 19.1	0.91	2.35
24	> 14.7	1.23	4.23
52	> 15.5	> 6.86	> 14.6

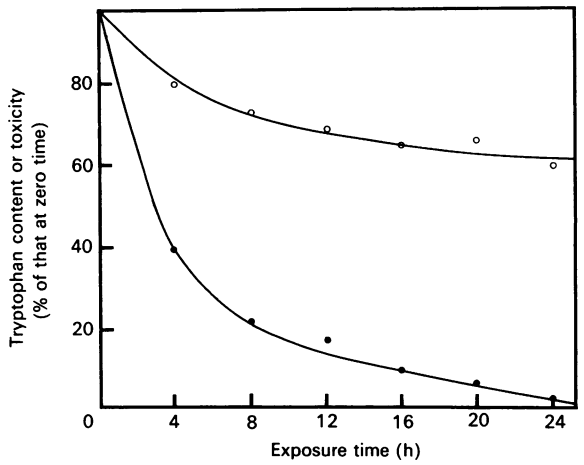

Fig. 2. Decrease in the tryptophan content of HD-1 crystals (○) and toxicity (●) as a function of time, monitored by amino acid analysis and bioassay respectively

irradiation, whereas water-lysed crystals require ~ 52 h and the agar-grown ~ 24 h exposure to render them atoxic. We have noted that the stability of samples to sunlight degradation changes from fermentation batch to fermentation batch, depending on the length of time the crystals are in contact with the medium (M. Pusztai & P. R. Carey, unpublished work).

For water-lysed HD-1 crystals, Fig. 2 compares the decrease in toxicity and the loss of tryptophan (by amino acid analysis) as a function of the time of irradiation. The curves show similar behaviour, with major initial losses being followed by slower degradation after 4–8 h. The reduction of tryptophan to about 65% of its original value after 24 h exposure is in accord with Raman data (Pozsgay *et al.*, 1987).

The identity of the chromophore(s) adhering to the Bt crystals has not been determined. In all likelihood there are a number of different chemical entities involved; a plethora of candidates exist in both the Bt-cell cytoplasm and in the fermentation medium. As might be expected, some chromophores can be removed by extensive washing (and detected by fluorescence; M. Pusztai & P. R. Carey unpublished work), but some are tightly bound.

Role of molecular oxygen

Further evidence for a photosensitization mechanism can be found by comparing the effect of sunlight on Bt crystals in the presence or absence of molecular oxygen. HD-1 crystals were irradiated in air for 24 h, and a second sample was irradiated for the same time, in an evacuated quartz tube. The tube passed more than 95% of the incident light at all wavelengths of the solar spectrum. Amino acid analysis showed that only 41% of tryptophan residues remained after irradiation in air, whereas 77% of the tryptophan residues remained for the sample irradiated under vacuum. The low tryptophan value for the air-irradiated sample (compared with the values quoted above) is often found for crystals exposed to the fermentation broth for a number of days, as was this HD-1 sample. The sample under vacuum is certainly more stable to sunlight, providing strong evidence in favour of a photosensitization-type mechanism. The approx. 20% tryptophan damage seen for the evacuated sample is probably due to presence of a small amount of remaining oxygen molecules that remains even after extensive 'pumping down'.

A further means of decreasing the effective oxygen content of the crystals is to keep them under water during irradiation. We found that HD-1 crystals under water retained 100% tryptophan and biological activity after 40 h irradiation. This theme was explored further by testing the stabilizing property of a humectant. Data involving the use of glycerol are shown in Table 3. Water-lysed HD-1 crystals were treated with glycerol and irradiated for 24 h. The use of 20% glycerol provided significant protection, and with 50% glycerol, most of the activity of Bt crystals was retained. In the latter case the crystals remained moist throughout the irradiation procedure.

Table 3. Photostability of water-lysed HD-1 crystals irradiated for 24 h in the presence of glycerol

[Glycerol]*	LD_{50} (μg of Bt/larva)
0 (Non-irradiated)	0.07
0 (Irradiated)	1.10
5	0.97
20	0.57
50	0.12

* Glycerol/crystal suspension (%, v/v) before exposure.

Table 4. Comparison of photostability of HD-73 crystals and the purified toxin (65 kDa) generated from the crystals

	Tryptophan remaining (%)	PF_{50}* (μg/larva)
Crystal HD-73		
Non-irradiated	100	26
24 h-irradiated	53	91
Toxin		
Non-irradiated	100	22
24 h-irradiated	72	55

* PF_{50} is a pupal-failure level of 50%.

Photostability of purified toxin

Although the activated toxin has not been investigated to the extent of intact crystals, the purified 65 kDa protein (Bietlot *et al.*, 1989) appears to remain photolabile. Table 4 compares the amino acid and bioassay results for irradiated and non-irradiated HD-73 crystals and toxin prepared from the same crystals. For both samples, irradiation for 24 h results in loss of a substantial portion of tryptophan and a concomitant diminution in toxicity. The source of the toxin's photolability has not been resolved. It may be due to the presence of very tightly, but non-covalently bound, exogenous chromophores travelling within the toxin core. Alternatively a covalently linked chromophore could be involved, either resulting from the breakdown of an indole ring to form a kynurenine, or by linkage of an unidentified chromophore to an unknown side chain.

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