

Characterization of melanin produced by a wild-type strain of *Bacillus cereus*

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Abstract *Bacillus cereus* 58 (Bc58) is a UV-resistant wild type strain that has an ability to produce a sorrel pigment induced by L-tyrosine. The Fourier-transform infrared (FT-IR) spectra and chemical tests of its pigment are similar to that of the standard melanin (Sigma). A bioassay shows that the LC₅₀ of a *Bacillus thuringiensis* (Bt) formulation added with the melanin of Bc58 and exposed to UV for 5 h is 16.1 µg/ml, which is similar to that of the Bt formulation without UV treatment, however, it is almost double that of the Bt formulation exposed to UV without the melanin of Bc58. The result of SDS-PAGE indicates that the melanin of Bc58 can protect the insecticidal crystal proteins from degradation. This suggests that it is an excellent UV protective agent for the insecticidal crystal proteins of the Bt formulation.

Keywords *Bacillus cereus*, melanin, UV protection

1 Introduction

Melanins, which widely exist in animals, plants and some microorganisms, are brown to black pigments. They are not essential for growth and development in biology but play an important role in improving survival and competitiveness (Alois and Michael, 1986). In general, melanins are macromolecules formed by oxidative polymerization of phenolic and/or indolic compounds. They are considered an effective protective screen of organisms, which gradually evolve and form for resistance to UV and ionizing radiation in the environment (Sarna, 1984).

Melanins have great application potential in agriculture and industry. There have been many reports about their resisting ultraviolet light and acting as a sink for free

radicals. Research has revealed that melanin produced by *Streptomyces* show photoprotection of the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis* (Liu et al., 1993). It was shown that melanin is an excellent photoprotective agent. Several research groups have obtained *Bacillus thuringiensis* (Bt) mutants producing melanin from *Bacillus thuringiensis* subsp. *kurstaki* and other strains by successive rounds of UV irradiation (Deepak et al., 2002; Hoti and Balaraman, 1993; Pater et al., 1996). Many reports have described melanin-producing microorganisms. Aronson reported that the *Bacillus cereus* (Bc) strain produced like-melanin but had no further research for pigment (Aronson and Wermus, 1965).

Some Bc can be used as bacteria fertilizer and widely applied in agriculture. Because Bc and Bt are genetically very closely related, some researches report that the *cry* gene of Bt was introduced into Bc and successfully expressed the insecticidal crystal proteins (Sun et al., 1994; Chen et al., 2004).

In this paper melanin produced by the wild strain Bc58 was firstly identified and its UV protection to insecticidal proteins was researched, which laid a foundation for the further construction of multifunctional genetically engineered Bc strain.

2 Materials and methods

2.1 Origins, media and insects

Origins of isolates used in this study included Bc46–49, 51–55, 58–59, 61, 63, 67, 69, 71–73, 77, 79, 89, 9509, 9633, 63303-3, 63304, and 63304-2. Bt15A3 was isolated from soil, and belongs to serotype H21, *Bacillus thuringiensis* subsp. *colmeri*, which exhibits high toxicity against lepidopteran pests (Chen et al., 2002). All strains were maintained on LB medium at 4°C before use and stored in this lab. Insects used for bioassay were the larva of *Helicoverpa armigera*. Casein

medium consists of 1 g glucose, 5 g NaCl, 0.1 g CaCl₂, 10 g bacto-tryptone, 4 g casein and 1 g L-tyrosine/l, pH 7.0. The medium was sterilized by autoclaving for 30 min at 8 lb/sq. inch in a liquid cycle, with 1.5% agar for the solid medium. LB medium was prepared with the routine method.

2.2 Selecting *Bacillus cereus* strains for producing pigment

Bacillus cereus (Bc) strains were inoculated by scribe onto casein medium and incubated at 30°C. Those strains which produced the pigment were recorded.

2.3 Preparation of crude melanin

The cultures of Bc58 in casein medium were collected into centrifuge bottles and centrifuged at 6000 rpm for 15 min at 4°C to remove bacteria and other debris. The supernatant was adjusted to pH 13 with 10 M NaOH to ensure complete polymerisation, then to pH 2 with 5 M HCl. The precipitated melanin was centrifuged at 12000 rpm for 30 min to remove the supernatant, and then 10 M NaOH was added to dissolve the precipitate. 0.2 V chloroform was mixed with the above solution to deproteinise the melanin pigment. The mixture was centrifuged at 12000 rpm for 10 min, and the supernatant was adjusted to pH 2 and then centrifuged again as above. The crude melanin was obtained after it was washed once with 100% methanol (0.1 vol) and 70% ethanol (0.1 vol) respectively and air-dried.

2.4 Pigment characteristic

The melanin sample was mixed with potassium bromide at a ratio of 1:3 and then infrared (IR) spectra between 4000/cm and 500/cm were taken on a Perkin-Elmer Fourier-transform infrared (FT-IR) spectrophotometer. Synthetic melanin from Sigma was used as a standard.

To observe the chemical test of the pigment, a 0.1 mg/ml pigment solution and standard melanin solution were respectively mixed with 30% H₂O₂ and NaClO at a ratio of 1:1. FeCl₃ solution was added to the pigment solution to observe the chemical reaction.

2.5 UV protection for Bt formulation by bioassay

Bt15A3 powder was diluted to 1 g/ml and mixed with Bc58 fermentation supernatant with melanin and bioassay buffer at a 1:9 ratio, which was irradiated at a distance of about 30 cm from the UV source at 254 nm for 5 h with gentle stirring. Another two samples 0.1 g/ml of Bt15A3 dilution and Bc58 fermentation supernatant without radiation were used as control. The bioassays of these four samples were carried out as described previously (Zhong et al., 1990).

2.6 Preparation of Bt crystal protein

Bt15A3 insecticidal crystal protein was prepared according to the routine method, and stored at -70°C for use.

2.7 SDS-PAGE analysis of Bc58 melanin UV protection for crystal protein

Two samples of crystal protein of Bt15A3 with melanin solution and bioassay buffer respectively were irradiated at a distance of about 30 cm from the UV source at 254 nm for 5 h with gentle stirring. The crystal protein without irradiation was used as control. SDS-PAGE analysis was performed by the routine method.

3 Results

3.1 *Bacillus cereus* strains producing pigment

In 27 Bc strains transplanted, there were 4 strains including Bc48, 55, 58 and 69 which had the ability to produce a red-brown diffusible pigment on the casein medium after incubating from 24 h to 72 h. Among them Bc58 produced more red-brown pigment than the other three strains. The pigment production of Bc58 and 48 started after culturing for 24 h, Bc55 started after 36 h and Bc69 started after 60 h.

3.2 Characteristic of the Bc58 pigment

When grown aerobically with tyrosine, Bc58 produced a red-brown, extracellular pigment. The extracted pigment was insoluble in hot or cold water, mineral acids such as HCl and HAc, and organic solvents such as aether, alcohol and chloroform. But chemical tests demonstrated that it was easily soluble in alkaline solvents such as NaOH and Na₂CO₃. Strong oxidants such as NaClO and H₂O₂ could bleach it. A red-brown precipitate was produced when FeCl₃ was added to the pigment solution. These properties were the same with the standard melanin from Sigma.

FT-IR spectroscopy is chosen to further characterize the Bc58 pigment because it is regarded as the most-informative method for well-resolved and detailed structural analysis of melanins (Bilińska, 1995; Pierce and Rast, 1996). The FT-IR bands from melanin show definite signature peaks at the wave numbers of 3000 nm (3333 cm⁻¹) ascribed to -OH or -NH stretching, and peak at the wave numbers of 6000 nm (1667 cm⁻¹) ascribed to -COOH stretching. From Fig. 1 these two peaks at 3405 cm⁻¹ and 1625 cm⁻¹ from Sigma melanin and at 3439 cm⁻¹ and 1634 cm⁻¹ from Bc58 pigment were observed respectively, which demonstrated that Bc58 pigment had a high degree of similarity to standard melanin from Sigma. But because of some impurities, there were some different absorbance peaks from standard melanin in the FT-IR spectra of the Bc58 pigment.

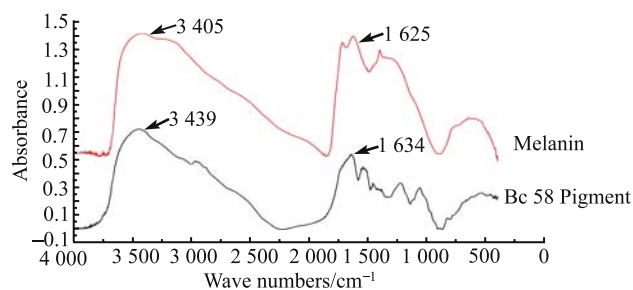


Fig. 1 FT-IR spectra of pigment produced by Bc58 and standard melanin

3.3 UV protection efficacy to insecticidal activity of Bt formulation.

The pigment of Bc58 was nontoxic against *Helicoverpa armigera* neonate larvae. Table 1 shows that the LC_{50} of a Bt formulation added with the pigment of Bc58 and exposed to UV for 5 h was $16.1 \mu\text{g/mL}$, which was similar to that of Bt formulation without UV treatment, however, it was almost double that of the Bt formulation exposed to UV without the pigment of Bc58. It was demonstrated that the pigment produced by Bc58 was an excellent UV protective agent to Bt formulation.

Table 1 LC_{50} ($\mu\text{L/ml}$)^{a)} against *Helicoverpa armigera* neonate larvae of Bt 15A3 formulations with and without pigment following UV irradiation

Sample	$LC_{50}/(\mu\text{L} \cdot \text{mL}^{-1})$	r
Bc58 pigment	Non-toxin	–
Bt formulation	15.2 ± 2.6	0.9939 ± 0.0031
Bt formulation following UV irradiation 5 h	32.7 ± 6.4	0.9851 ± 0.0080
Bt formulation with pigment of Bc58 following UV irradiation 5 h	16.1 ± 2.3	0.9787 ± 0.0141

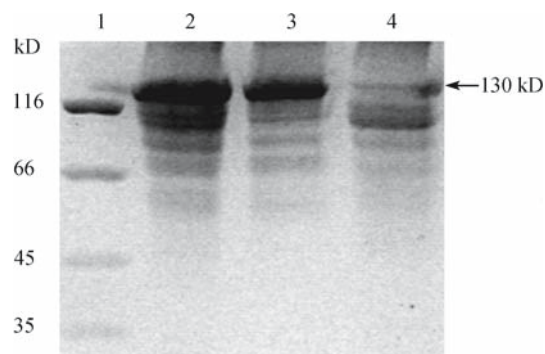
a): The LC_{50} was $\bar{x} \pm \text{SD}$ by different four bioassays

3.4 UV protection efficacy to insecticidal crystal proteins by SDS-PAGE method

Insecticidal crystal proteins with and without the pigment of Bc58 treated by UV irradiation for 5 h were used to test by the SDS-PAGE method. Results are shown in Fig. 2. Compared to crystal proteins without irradiation, after UV irradiation, only a few of crystal proteins, which were added to the Bc58 pigment, were degraded. Most of the protoxin with a molecular mass of 130 kD were retained. However, most of the crystal proteins that did not add to the Bc58 pigment were degraded. Among them 130 kD protoxin, whose molecular mass was the largest and played an important role in insecticidal activity, was degraded severely. The results demonstrated that the pigment of Bc58 could protect the insecticidal crystal proteins from degradation.

4 Discussion

When used in the field, ultraviolet and ionizing radiation can cause Bt insecticides to lose toxicity. These irradiations



1: Protein Marker; 2: Crystal proteins without UV irradiation; 3: Bt crystal protein with the pigment of Bc58 after UV irradiation; 4: Bt crystal protein without the pigment of Bc58 after UV irradiation
Fig. 2 SDS-PAGE profiles of crystal proteins after UV irradiation for 5 h

can cause water and other substances in the cell to ionize. Chemical free radicals are then formed, and the most important one is hydroxyl radical ($-\text{OH}$) (Wang et al., 1999). Free radicals react with macromolecules, such as DNA and protein in the cell to inactivate them. Grossweiner (1976) found that when exposed to sunlight, about 20% histidine residues in Bt crystal proteins was destroyed. Histidine and tryptophan residues are major elements of the core structure of crystal proteins. After the core structure is changed it will influence the combination with insect midgut cell receptors to reduce the insecticidal activity. The result of SDS-PAGE in this study found that the 130 kD protoxin band of crystal proteins that added to the pigment of Bc58 after UV irradiation was closely similar to that of crystal proteins without irradiation. However, the protoxin of crystal proteins without the pigment of Bc58 after UV irradiation was largely degraded. It is speculated that protoxin was easily attacked by UV because its magnitude was the largest and its amount was the most in Bt crystal protein. However, the proof of this cause needs further exploring.

To address the problem of the sensitivity to UV irradiation of the Bt formulation, some chemical screens have been found (Cohen et al., 2001; Dunkle and Shasha, 1989; Morris, 1983). Addition of cationic chromophores such as acriflavine and methyl green, as components of the formulation, can achieve UV-protection. However, these chemical screens have some negative impacts to the natural environment (Dunkle and Shasha, 1989; Margulies et al., 1988). Constructing an effectual engineering strain is another novel way to reduce the inactivity of Bt formulation in the field. Bc and Bt are genetically very closely related and some scholars think Bc is actually Bt except that it does not produce insecticidal crystal proteins, so Bc has been considered as a better receptor for expressing the Bt toxin gene (Chen et al., 2004). In this lab Bc9509, which could increase the yields of crops, had successfully been transferred by pHT-1C with Bt *Cry* gene and overexpressed Bt insecticidal crystal proteins. The Bc engineering strain showed insecticidal activity to *Spodoptera exigua*, and the former studies found that Bc58, which did not produce enterotoxins and hemolysins, was safe for mammals,

therefore, it had a potential to be a receptor for constructing an engineering strain.

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