University of Nebraska - Lincoln
DigitalCommons@University of Nebraska - Lincoln

Papers in Microbiology | Papers in the Biological Sciences

August 1993

Nonenzymatic Glycosylation of Lepidopteran-Active *Bacillus thuringiensis* Protein Crystals

Meenakshi Bhattacharyi
University of Nebraska-Lincoln

Bradley A. Plantz
University of Nebraska-Lincoln, bplantz2@unl.edu

Jane D. Swanson-Kobler
University of Nebraska-Lincoln

Kenneth W. Nickerson
University of Nebraska-Lincoln, knickerson1@unl.edu

Follow this and additional works at: https://digitalcommons.unl.edu/bioscimicro

 Part of the Microbiology Commons

Bhattacharyi, Meenakshi; Plantz, Bradley A.; Swanson-Kobler, Jane D.; and Nickerson, Kenneth W., "Nonenzymatic Glycosylation of Lepidopteran-Active *Bacillus thuringiensis* Protein Crystals" (1993). *Papers in Microbiology*. 30.
https://digitalcommons.unl.edu/bioscimicro/30

This Article is brought to you for free and open access by the Papers in the Biological Sciences at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Papers in Microbiology by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1993, p. 2666–2672 Vol. 59, No. 8
0099-2240/93/082666-07$02.00/0
Copyright © 1993, American Society for Microbiology

Nonenzymatic Glycosylation of Lepidopteran-Active *Bacillus thuringiensis* Protein Crystals

MEENAKSHI BHATTACHARYA, BRADLEY A. PLANTZ, JANE D. SWANSON-KOBLER,† AND KENNETH W. NICKERSON*

School of Biological Sciences, University of Nebraska, Lincoln, Nebraska 68588-0343

Received 22 January 1993/Accepted 29 May 1993

We used high-pH anion-exchange chromatography with pulsed amperometric detection to quantify the monosaccharides covalently attached to *Bacillus thuringiensis* HD-1 (Dipel) crystals. The crystals contained 0.54% sugars, including, in decreasing order of prevalence, glucose, fucose, arabinose/rhamnose, galactose, galactosamine, glucosamine, xylose, and mannose. Three lines of evidence indicated that these sugars arose from nonenzymatic glycosylation: (i) the sugars could not be removed by *N*- or *O*-glycanases; (ii) the sugars attached were influenced both by the medium in which the bacteria had been grown and by the time at which the crystals were harvested; and (iii) the chemical identity and stoichiometry of the sugars detected did not fit any known glycoprotein models. Thus, the sugars detected were the product of fermentation conditions rather than bacterial genetics. The implications of these findings are discussed in terms of crystal chemistry, fermentation technology, and the efficacy of *B. thuringiensis* as a microbial insecticide.

Bacillus thuringiensis produces protein crystals that are toxic to the larval stage of many insects and nematodes. These toxins have generated a tremendous amount of academic and industrial interest because of their potential as an alternative to chemical insecticides. Much of this research has focused on the molecular biology of toxin production. Höfte and Whiteley (20) compared the nucleotide sequences for 42 crystal toxin genes and assigned them to 14 toxin families. This approach will likely continue because of the huge number of strains yet to be examined. For instance, Martin and Travers (31) reported the isolation of fully 8,916 *B. thuringiensis* strains from soil samples obtained throughout the world. A second approach is to examine the toxin's mode of action and then characterize the toxin receptors located in the larval gut (18). In this regard, it is important to determine whether the toxin is a protein toxin or a glycoprotein toxin.

The presence of covalently attached sugars on purified *B. thuringiensis* crystals has been extremely controversial. Many studies (Table 1) have indicated that the toxin is indeed a glycoprotein toxin. Carbohydrate values ranging from 0.5 to 12% have been reported. However, Huber et al. (23) detected ≤0.1% neutral sugars, and they noted that the apparent sugar content decreased progressively as the crystals were washed repeatedly. Also, the functional significance of the attached sugars is called into question by the evident toxicity of the *B. thuringensis* crystal toxin when expressed in the bacteria *Escherichia coli* (43), *Pseudomonas* spp. (37), *Caulobacter crescentus* (46), and *Agmenellum quadruplicatum* (2), in the yeasts *Saccharomyces cerevisiae* and *Pichia* spp., and in plants such as tobacco (5) and tomato (15). The gram-negative bacterial hosts would be unable to carry out protein glycosylation in any fashion, while the glycosylation patterns in the eukaryotic hosts would likely be very different from those in the gram-positive bacilli.

What is the origin of the grossly divergent crystal glycosylation values reported in the literature (Table 1)? Possible problems include the use of assay methods which would detect neutral sugars but not aminosugars, extraction of spore and crystal mixtures rather than purified crystal preparations, and the use of Renografin gradients to accomplish crystal purification. Renografin is the *N*-methyl-D-glucamine salt of 3,5-diacetylamino-2,4,6-triiodobenzoate, and its presence could give artifactually high values in the anthrone test for neutral sugars (40). However, another possible explanation is nonenzymatic glycosylation (47).

Nonenzymatic glycosylation is a variation of the classical Maillard reaction between reducing sugars and amino acids. The sugars attach to free, nonionized amino groups such as the N terminus and lysine side chains. The initial step is the formation of a Schiff base between an amino group and the open-chain form of the sugar. At this stage, the reaction is still fully reversible. However, when the Schiff base (an aldimine) undergoes the Amadori rearrangement to a ketamine, the attachment becomes effectively irreversible. Because of the requirement for a nonionized amino group, the reaction is pH dependent. It proceeds rapidly at pHs of ≥8 and slowly at pHs of ≤6 (12). Much of the recent interest in nonenzymatic glycosylation of proteins derives from the discovery of elevated levels of glycosylated albumins and hemoglobins in the blood of diabetic patients (12, 24, 47). Indeed, the high frequency of cataracts and blindness in diabetic patients is probably due to nonenzymatic glycosylation of the lens protein in their eyes.

The present article reports that a majority of the sugars covalently attached to lepidopteran-active *B. thuringiensis* crystals are the products of nonenzymatic glycosylation. Thus, their presence is determined not by bacterial genetics but instead by the environmental parameters and fermentor conditions operative after the crystals are released.

MATERIALS AND METHODS

Bacillus thuringiensis subsp. *kurstaki* (HD-1) was obtained from a single-colony isolate from Dipel (Abbott Laboratories, North Chicago, Ill.). The cells were grown on

* Corresponding author.
† Present address: U.S. Army Corps of Engineers, Missouri River Division, Omaha, NE 68144.

TABLE 1. Values reported for the carbohydrate content of purified insecticidal proteins from *Bacillus* species

Bacterium	Carbohydrate (%)	Reference[a]
B. thuringiensis M	0.5	Holmes and Monro (21)
B. thuringiensis Berliner	12	Bateson and Stainsby (6)
B. thuringiensis HD-1	5.6	Bulla et al. (7)
B. thuringiensis (16 strains)	0.5–2.4	Swanson (45)
B. thuringiensis subsp. *israelensis*	≥11.2	Tyrell et al. (48)
B. thuringiensis subsp. *israelensis*	6	Insell and Fitz-James (25)
B. sphaericus	12	Narusu and Gopinathan (34)
B. thuringiensis HD-263	5	Aronson and Arvidson (4)
B. thuringiensis subsp. *israelensis*	2.7	Pfannenstiel et al. (40)

[a] Arranged chronologically.

GYS (36) medium with rotary agitation at 150 rpm on a New Brunswick Scientific G-52 shaker at room temperature. GYS medium (36) contained 0.2% $(NH_4)_2SO_4$, 0.2% yeast extract, and 0.05% K_2HPO_4 (pH 7.3), to which were aseptically added, after autoclaving, 0.1% glucose, 0.02% $MgSO_4$, 0.008% $CaCl_2 \cdot 2H_2O$, and 0.005% $MnSO_4 \cdot 4H_2O$. After sporulation (2 to 3 days), the crystals were purified on sodium bromide gradients (1) modified to include 7.5% ethanol throughout, washed eight times in either distilled water or 0.01 M K_2HPO_4 (pH 7.5), and lyophilized.

Other conditions for cell growth and harvesting. (i) **Commercial slurry.** Crystals were purified from a concentrated slurry taken directly from commercial fermentors (Abbott Laboratories). The slurry was diluted 10:1 in water, and the crystals were purified on NaBr gradients as described above.

(ii) **Variations of GYS.** Three variations of GYS were used (see Table 5). In the first, glucose was omitted. In the second, glucose was supplemented with 0.4% sodium glutamate. In the third, glucose was replaced by 0.4% sodium glutamate. The procedures for crystal purification were as described above.

(iii) **Solid medium.** HD-1 cultures were grown on Bacto-Nutrient Agar plates (Difco, Detroit, Mich.). They were grown as a lawn of cells for about 5 days. After sporulation, the cells were scraped off and washed with distilled water, and the crystals were purified.

(iv) **Harvest time.** HD-1 cultures were monitored by phase-contrast microscopy so that the cells were harvested as soon as the sporangia had lysed, thereby releasing the crystals, or as soon as the mature crystals and spores could be seen within the sporangia. The sporangia were washed three times and lysed in 0.1% Triton X-100, whereupon the released crystals were purified in the usual way.

Separation of the P_1 and P_2 proteins by preparative cell electrophoresis. The Bio-Rad model 491 Prep Cell System was used to separate the 130-kDa (P_1) and 65- to 70-kDa (P_2) proteins by continuous-elution electrophoresis. A 5-cm resolving gel (6% acrylamide) was polymerized in a 28-mm-diameter tube. A 2-cm stacking gel (4% acrylamide) was polymerized on top of the resolving gel. The discontinuous buffer system of Laemmli (27) was used. NaBr-purified crystals (3 to 6 mg) were dissolved at room temperature in sodium dodecyl sulfate (SDS) sample buffer containing 0.5 M dithiothreitol (8). The disulfides were blocked by incubation in 0.5 M iodacetamide for 30 min before loading on the gel. The gel was electrophoresed at 50 mA for 1 h, and then the current was reduced to 40 mA. The SDS running buffer was pumped at the rate of 1 ml/min. The elution chamber outlet was connected to a fraction collector through a UV monitor attached to a chart recorder. Portions (20 μl) of each 5-ml fraction were analyzed for their protein content by SDS-polyacrylamide gel electrophoresis (PAGE) on a Bio-Rad Mini-gel system.

The two protein peaks corresponded to pure bands of P_1 and P_2. Each peak was pooled separately and concentrated either by dialysis and lyophilization or by acetone precipitation followed by dialysis and lyophilization. Protein fractions (2.5 mg) were then hydrolyzed and analyzed by Dionex chromatography as described below.

Hydrolysis of the crystal protein. The crystal proteins were acid hydrolyzed in order to release the sugars as monosaccharides for analysis. Purified crystals (2.5 mg) were hydrolyzed in a screw-cap microcentrifuge tube in either 2 M trifluoroacetic acid (TFA; high-pressure liquid chromatography [HPLC] grade) or 4 N HCl (diluted from 6 N constant-boiling HCl) for 4 h at 100°C. The acid was evaporated on a Savant Speed-Vac concentrator, after which the pellet was dissolved in distilled water. The samples were then filtered through an ion-exchange cartridge (On-Guard A; Dionex Corp., Sunnyvale, Calif.) prior to sugar analysis.

Sugar analysis of the crystal glycoprotein. The high-pH anion-exchange chromatography with pulsed amperometric detection system used for monosaccharide analysis (16) was that marketed by Dionex Corp. All procedures followed their protocol. The Carbopac PA 1 anion-exchange column was washed for 15 min with 200 mM NaOH and then with 2.5 mM NaOH for 15 min. After sample injection (100 μl), the sugars were separated by elution with isocratic NaOH (2.5 mM) for 35 min. All eluants were prepared by dilution of 50% NaOH (Fisher Scientific). This system gave highly reproducible retention times. Detection of the separated monosaccharides was done by pulsed amperometric detection with a gold working electrode. To minimize baseline distortion, 300 mM NaOH was added to the postcolumn effluent via a mixing tee at a flow rate of 1 ml/min. The data were integrated and plotted with a Dionex 4400 integrator. 2-Deoxyglucose was used as an internal standard. Sugar contents (in micrograms per 100-μl injection volume, containing 2.5 mg of crystal) were converted to nanograms of sugar per milligram of crystal protein. The retention times and elution volumes of the samples were compared with those of a standard mixture containing fucose, galactosamine, galactose, glucosamine, glucose, xylose, and mannose.

Detection of glycoproteins after SDS-PAGE. Two procedures were used for detection. The first used biotin hydrazide and streptavidin-conjugated horseradish peroxidase (Pierce, Rockford, Ill.), and the second used digoxigenin-*X*-hydrazide and antidigoxigenin-conjugated alkaline phosphatase (Boehringer Mannheim Corp., Indianapolis, Ind.). In each case, the glycoprotein was treated with sodium periodate to cleave the bond between adjacent hydroxyl groups in the carbohydrate moiety, after which the newly formed aldehydes were reacted with the respective hydrazides. The derivatized proteins were separated by SDS-PAGE (8), transferred to nitrocellulose paper, and detected enzymatically. All protocols were as recommended by Pierce and Boehringer Mannheim. Peptide *N*-glycosidase F and *O*-glycosidase were purchased from Boehringer Mannheim.

Incubation of crystal protein with radioactive glucose. The procedures followed for labeling were those recommended

TABLE 2. Carbohydrate content of NaBr-purified crystals from *B. thuringiensis*

Strain no. (NRRL)	Serotype	% Glucose equivalents[a]
B-4039	Thuringiensis	1.9
B-4041	Alesti	1.3
	Kurstaki (HD-1)	1.2
B-4042	Sotto	1.5
B-4043	Dendrolimus	1.4
B-4044	Kenyae	2.4
B-4045	Galleriae	1.8
B-4056	Canadensis	0.5
B-4046	Entomocidus	1.9
B-4057	Subtoxicus	1.0
B-4048	Aizawai	1.7
B-4049	Morrisoni	1.3
B-4050	Tolworthi	0.7
B-4058	Darmstadtiensis	1.8
B-4059	Toumanoffi	0.7
B-4060	Thompsoni	1.6

[a] Each value is the average for triplicate samples agreeing within at least ±0.3%.

TABLE 3. HPLC analysis of the carbohydrate content of *B. thuringiensis* HD-1 (Dipel) crystals[a]

Sugar[b]	Content (ng/mg)		Stoichiometry[c] (residues/135 kDa)
	2 M TFA	4 N HCl	
Fucose	1,720	380	1.30
Arabinose/rhamnose	480	130	0.36
Galactosamine	180	100	0.13
Galactose	380	400	0.29
Glucosamine	150	100	0.11
Glucose	2,310	520	1.75
Xylose	140	80	0.11
Mannose	80	80	0.06
Total	5,440	1,790	4.11
%Sugar	0.54%	0.18%	

[a] Crystals obtained from a concentrated slurry from production fermentors (Abbott Laboratories) were purified on NaBr gradients and washed 15 times in distilled water prior to analysis.

[b] Sugars arranged in order of elution. Values are the averages of ≥6 separate determinations agreeing within ± 20%.

[c] Sugar content detected via hydrolysis in 2 M TFA (0.54%) normalized to number of residues expected per 135-kDa protein subunit.

by Dolhoffer and Wieland (12). All incubations were carried out in sterile 1.5-ml microcentrifuge tubes with filter-sterilized (0.2 μm) buffers and glucose solutions. Purified crystal protein (2.5 mg) was incubated in 1 ml of 50 mM glucose (30 μCi of [6-^{3}H]glucose; New England Nuclear)–10 mM sodium phosphate with 0.02% sodium azide for 72 h at 25°C with constant shaking (50 rpm on a New Brunswick Scientific G2 rotary shaker). Final pHs ranged from 4.9 to 9.0. Unbound radioactivity was removed by precipitating the crystal proteins with 7% trichloroacetic acid and centrifugation for 10 min at 10,000 rpm at room temperature. The pellets were washed three times by repeated suspension in distilled water, precipitation with trichloroacetic acid, and centrifugation. The final pellets were dissolved in Beckman Ready Value scintillation fluid, and radioactivity was counted in an LKB model 1219 Rackbeta liquid scintillation counter.

For some incubations, crystals (2.5 mg) were solubilized for 2 h in 50 mM NaOH at 37°C, after which the pH was adjusted to ca. 8.0 and the proteins were incubated with [6-^{3}H]glucose as described above. Bovine serum albumin (2.5 mg) in radioactive glucose–10 mM sodium phosphate (pH 8.1)–0.02% sodium azide served as the positive control.

RESULTS

Covalent attachment. The presence of low levels of sugars on NaBr-purified *B. thuringiensis* insecticidal crystals is a common phenomenon. Table 2, taken from reference 45, shows that crystals from 16 different serotypes of *B. thuringiensis* possessed from 0.5 to 2.4% glucose equivalents, as determined by the phenol-sulfuric acid assay (17). These sugars were not removed by repeated washing with 0.01 M K_2HPO_4 (pH 7.5). In view of the known pH dependence of nonenzymatic glycosylation (12), the pH of the washing buffer might explain otherwise contradictory results in different laboratories. Additionally, to eliminate the possibility of trapped sugar molecules occurring fortuitously within the crystal interior, *B. thuringiensis* subsp. *kurstaki* crystals were dialyzed for 5 days against 8 M urea at pH 8 and then for 2 days against four changes of distilled water. This procedure, introduced by Lecadet (28), causes the crystals to swell and lose their refractility. The interchain disulfide bonds remain intact, and morphologically indistinguishable crystals are reformed during the dialysis against distilled water (28). However, the sugar contents of native and urea-dialyzed crystals were identical (45).

Confirmation that the sugars detected (Table 2) were indeed covalently attached came from the use of two procedures for the detection of glycoproteins after their separation by SDS-PAGE. In one procedure, the sugars were labeled with biotin hydrazide and detected with streptavidin-horseradish peroxidase. In the second, the sugars were labeled with digoxigenin hydrazide and detected with antidigoxigenin-alkaline phosphatase. In both cases, the high-molecular-weight P_1 proteins from HD-1 crystals were stained as if they were glycoproteins. Significantly, in both cases, the hydrazide-reactive material could not be removed by enzymatic treatment with either peptide *N*-glycosidase F or *O*-glycosidase. Thus, the hydrazide-reactive material was not attached to the crystal protein via either of the common eukaryotic carbohydrate attachment mechanisms, i.e., N-linkage to Asn or O-linkage to Ser/Thr.

Sugar identity and stoichiometry. Purified *B. thuringiensis* crystals were hydrolyzed and then analyzed for their sugar contents by the Dionex HPLC carbohydrate analysis system (Table 3). Acid hydrolysis conditions can influence the identity of the sugars released, and consequently, hydrolyses in both 4 N HCl and 2 M TFA were used (Table 3). Typical eukaryotic glycoproteins contain GlcNAc (deacetylated to glucosamine) and mannose, in defined stoichiometric ratios. However, the sugars detected in our *B. thuringiensis* crystals (Table 3) did not fit this pattern. Unusual sugars such as fucose were prevalent, and when calculated on a per-protein-chain basis, the sugar ratios were also unusual (Table 3). Most of the sugars detected were present at substantially less than one residue per protein chain. Clearly the eukaryotic model did not apply. Note, however, that the attachment patterns may be heterogeneous. The sugar residues may be preferentially attached to proteins on the surface of the crystals.

Preparative SDS-PAGE. As further evidence that the carbohydrates detected are covalently attached, the P_1 (130 to 135 kDa) and P_2 (65 to 70 kDa) proteins were separated by preparative SDS-PAGE and then analyzed for their carbo-

TABLE 4. HPLC analysis of the P_1 and P_2 proteins purified by preparative SDS-PAGE

Sugar	Content (ng/mg)	
	P_1 protein	P_2 protein
Fucose	440	144
Arabinose/rhamnose	4	6
Galactosamine	0	3
Galactose	6	0
Glucosamine	56	72
Glucose	468	848
Xylose	58	106
Mannose	21	23
Total	1,053	1,202
% Sugar	0.105%	0.12%

hydrate contents. Both protein fractions contained attached sugars (Table 4).

Alternative growth conditions. Further evidence that crystal glycosylation (Tables 3 and 4) had a nongenetic origin came from analysis of crystals produced following a variety of growth and harvesting conditions (Table 5). Each column in the table represents crystals from the same organism, *B. thuringiensis* HD-1 (Dipel). Medium variations included supplementation of yeast extract with combinations of glucose and glutamate (Table 5). In each case, the overall levels of glycosylation and the percentages of the individual sugars attached were distinctly different. Harvest time also influenced the levels of sugars attached. The carbohydrate content of the crystals increased as the time between sporulation and harvesting became longer (Table 5). The comparatively high sugar contents of the crystals analyzed in Table 2 may be the result of harvesting 3 to 5 days after sporulation (45). The crystals were also glycosylated when formed on nutrient agar plates (Table 5). Note that in three cases (nutrient agar, glutamate-yeast extract-salts, and yeast extract-salts only), crystal glycosylation occurred even when no sugars were added to the bacterial growth medium.

Attachment of [6-^{3}H]glucose. Another method for demonstrating nonenzymatic glycosylation involves mixing a protein with [6-^{3}H]glucose and then monitoring the incorporation of that radioactivity into trichloroacetic acid-precipitable material (11, 12). Experiments with intact and solubilized *B. thuringiensis* crystals showed the incorporation of 4.0×10^3 and 1.3×10^4 cpm, respectively, equivalent to 0.044 and 0.09% glucose attachment, respectively. A bovine serum albumin control gave 0.28% glucose attachment. Each of these protein-glucose incubations was conducted at pH 8.1. A similar experiment for intact crystals at pH 4.9 gave only 1.9×10^3 cpm, equivalent to 0.021% glucose attachment.

DISCUSSION

We have shown that insecticidal crystals from *B. thuringiensis* HD-1 (Dipel) contain low levels (≤0.54%) of covalently bound sugars. Four observations support the conclusion that these sugars are the product of nonenzymatic glycosylation: (i) the sugars could not be removed by *N*- or *O*-glycanases; (ii) the levels of attached sugars were influenced by the medium in which the bacteria were grown and the time at which the crystals were harvested; (iii) unusual sugars such as fucose were present; and (iv) the sugars were present in non-stoichiometric ratios, usually far less than one residue per protein chain. The implications of nonenzymatic glycosylation as the likely cause of the otherwise disparate values published for crystal glycosylation (Table 1) fall into three general categories—the chemistry of the crystal itself, fermentation technology during crystal production, and the specificity and toxicity of the crystals produced.

With regard to crystal chemistry, the sugars identified from *B. thuringiensis* HD-1 crystals (Table 3) agree nicely with those reported by Tyrell et al. (48) for *B. thuringiensis* subsp. *israelensis* crystals, i.e., glucose, mannose, fucose, rhamnose, xylose, and galactosamine. Because we used acid hydrolysis to release the sugars, any *N*-acetyl groups present on glucosamine or galactosamine would have been cleaved prior to analysis. Similarly, any sulfated or phosphorylated sugars would not have been detected because those esters would also have been hydrolyzed by the acid treatment. However, the possible presence of phosphorylated sugars on the *B. thuringiensis* crystals is attractive because it could explain the findings of Watson and Mann (50). Using autoradiography of acid-hydrolyzed, ^{32}P-labeled *B. thuringiensis* HD-1 crystals, they detected [^{32}P]phosphothreonine and a large spot (see Fig. 4 in reference 50) which was later identified as $^{32}P_i$ (30). This $^{32}P_i$ could have been released

TABLE 5. Effect of growth and harvesting conditions on the carbohydrate content of *B. thuringiensis* HD-1 crystals

Sugar[a]	Content[b] (ng/mg)						
	GYS			Glutamate, 2 days	GGYS, 2 days	YS, 2 days	Nutrient agar, 2 days
	2 days	At lysis	Artificial lysis				
Fucose	164	220	96	668	112	204	72
Arabinose/rhamnose	54	46	24	398	76	104	120
Galactosamine	104	72	40	148	52	368	80
Galactose	32	0	4	128	32	1,172	360
Glucosamine	356	176	80	144	96	60	360
Glucose	448	168	204	280	428	900	428
Xylose	60	56	24	44	28	80	46
Mannose	104	52	72	76	24	52	36
Total	1,322	790	544	1,886	848	2,940	1,502
% Sugar	0.13%	0.08%	0.05%	0.19%	0.08%	0.29%	0.15%

[a] Sugars arranged in order of elution. Values are the averages of ≥2 separate determinations agreeing within ±20%.

[b] Medium variations: GYS, glucose-yeast extract (YE)-salts; YS, YE-salts; GGYS, glucose-glutamate-YE-salts; glutamate, glutamate-YE-salts. Harvest variations: 2 days, ca. 2 days after sporulation; at lysis, ca. 18 h after sporulation; artificial lysis, after first visible spore and crystal formation.

from the crystals by acid hydrolysis of attached phosphorylated sugars.

Nonenzymatic glycosylation provides an alternative explanation for the very large (≥500-kDa) crystal proteins often detected by reducing SDS-PAGE (7, 8, 35). Many of these crystal proteins are too large to cross the stacking gel-separating gel interface, while others are too large to leave the sample well. This phenomenon becomes more prevalent as the crystals age (35); in crystals which have been stored for a year or more, these giant proteins can constitute 40 to 50% of the total protein (35). This protein polymerization could result from the gradual formation of covalent cross-links, possibly in the form of lysinoalanine linkages (35). An alternative explanation (47) would be that the nonenzymatically glycosylated proteins underwent cross-linking and polymerization by a mechanism similar to the Maillard-type browning reactions. This process is thought to be associated with the accelerated aging of tissues in diabetics (47). It may also be associated with the formation of giant proteins during the storage of *B. thuringiensis* crystals (8, 35).

Nonenzymatic glycosylation is a chemical reaction in which the precise nature of the protein is of little significance. Thus, the phenomenon should be applicable for all extracellular microbial proteins, particularly those present during prolonged incubations. This caveat should be even more relevant for proteins whose enzymatic activity creates additional reducing sugars. Four possible examples are the chloride-stimulated cellobiosidase from the gram-negative bacterium *Fibrobacter succinogenes* (formerly *Bacteroides succinogenes*), reported (22) to contain 8 to 16% carbohydrate; the pectin methylesterase from *Clostridium thermosaccharolyticum* (49); and the cellulase and xylanase from a *Bacillus* sp., which were reported to contain 11.5 and 20% carbohydrate, respectively (38).

There are three reasons why the insecticidal *B. thuringiensis* crystals are more likely than other proteins to be glycosylated. (i) For most bacilli, the culture pH rises to ≥8 during sporulation. (ii) After sporulation, most researchers allow the autolytic process to go to completion. Thus, the crystals are harvested 2 to 5 days after sporulation. (iii) As a consequence, the spores and crystals are released into a milieu containing a multitude of carbohydrate degradation products which had been part of the sporangia and vegetative cells. Residual sugars from the growth medium may also be present. Thus, the sugars found on the crystals may originally have been part of the teichoic or teichuronic acids (3), the glycoprotein crystalline cell surface layer, also known as the S-layer (32, 33), or some other part of the *Bacillus* cell envelope (3). As examples, teichuronic acid from *Bacillus megaterium* contains rhamnose (51), and the cell surface S-layer from *Bacillus stearothermophilus* contains rhamnose, *N*-acetyl mannuronic acid, glucose, and *N*-acetylglucosamine (32, 33).

Nonenzymatic glycosylation is likely responsible for the sugars detected on lepidopteran-active *B. thuringiensis* crystals. Consequently, our view of the *B. thuringiensis* product as a glycoprotein toxin shifts from the realm of bacterial genetics to that of fermentation conditions. This shift makes comparisons from one laboratory to another even more difficult. Factors which must be considered now include the time between sporulation and harvesting; the pH, temperature, and residual nutrient levels present during this interval; strain differences, which might be accompanied by differences in the polymers present in the cell envelope; and differences in the growth medium, which might influence the polymers present in the bacterial cell envelope. For instance, the percent teichoic and teichuronic acid composition is often determined by the availability of Mg^{2+}, K^+, and phosphate ions in the growth medium (14).

With regard to fermentation technology, nonenzymatic glycosylation now provides a rational explanation for three long-standing observations in the fermentation industry: (i) the existence of batch-to-batch variability, (ii) the realization that merely starting with the same microorganism does not guarantee the same product, and (iii) the empirical finding for *B. thuringiensis* that maintaining a pH of ≤6 during and after sporulation is desirable for maximum toxicity. In this light, different pHs during the period following sporulation could even shift the host specificity of *B. thuringiensis* from one insect to another.

How does nonenzymatic glycosylation alter the toxicity of *B. thuringiensis* crystals? Data which address this point were provided by Scherrer et al. (42). They grew *B. thuringiensis* subsp. *thuringiensis* in a yeast extract-salts medium containing six levels of glucose varying from 0 to 1.5%. The crystals produced at the different glucose concentrations were bioassayed against larvae of the cabbage butterfly *Pieris brassicae*. Significantly, the toxic activities were not linearly related to the protein content of the crystals. On a per-milligram-of-protein basis, the crystals produced at the different glucose concentrations varied up to fourfold in their toxicity (42).

How could these changes in toxicity occur? Three possible mechanisms are suggested by the fact that nonenzymatic glycosylation occurs preferentially on lysine side chains at alkaline pHs. (i) Glycosylation could occur in the highly alkaline midguts of lepidopteran (13) and dipteran (10) larvae. The extent of glycosylation would depend on the free sugars available and thus would also depend on larval diet. Many examples are known in which the effectiveness of the *B. thuringiensis* crystal toxin is dependent on the composition of the larval diet (26, 41, 44). In most cases, these effects have been attributed to the presence of allelochemicals in the plant food ingested by the insect larvae. (ii) The *B. thuringiensis* protoxin is activated in the larval gut by trypsin-like enzymes. However, trypsin only cleaves peptide bonds on the carboxyl side of lysine or arginine residues (19). Thus, glycosylation of the lysine amino groups could alter the proteolytic cleavage pattern of these toxins. The resulting toxicity shift could be in either direction. Glycosylation could block a necessary step in toxin activation or could create a protease-resistant domain by protecting a critical lysine-containing cleavage site. The latter possibility becomes more likely when one considers the heterogeneity of which lysine side chains are glycosylated. As an example, for human albumin (24), only 10 of the 59 lysines were glycosylated, and fully 33% of that glycosylation occurred at Lys-525. Favored glycosylation sites included Lys-Lys, Lys-His, and Lys-His-Lys sequences as well as those Lys residues located near disulfide bonds (24). (iii) Lysine side chains could themselves be necessary for toxicity. For the mosquito-active *B. thuringiensis* subsp. *israelensis* toxin (39), lysine modification led to a dramatic drop in toxicity. However, the situation appears to be somewhat different for lepidopteran-active *B. thuringiensis* toxins. Choma and Kaplan (9) found that lysine-derivatized HD-73 toxin retained full activity towards spruce budworm larvae, while Yan and McCarthy (52) found that lysine-derivatized HD-524 toxin retained full cytolytic activity towards a cell line from the cabbage looper, *Trichoplusia ni*. Our current research involves a comparison of the 50% lethal concentrations of

differentially glycosylated *B. thuringiensis* crystals towards several lepidopteran larvae.

ACKNOWLEDGMENTS

This research was supported by grants from Abbott Laboratories, North Chicago, Illinois; Entotech, Davis, California; the U.S. Department of Agriculture (86-CRCR-1-1973); the National Science Foundation (DCB-8919166); and the Midwest Plant Biotechnology Consortium (593-0009-03).

REFERENCES

1. **Ang, B. J., and K. W. Nickerson.** 1978. Purification of the protein crystal from *Bacillus thuringiensis* by zonal gradient centrifugation. Appl. Environ. Microbiol. **36**:625–626.
2. **Angsuthanasombat, C., and S. Panyim.** 1989. Biosynthesis of 130-kilodalton mosquito larvicide in the cyanobacterium *Agmenellum quadruplicatum* PR-6. Appl. Environ. Microbiol. **55**:2428–2430.
3. **Archibald, A. R.** 1989. The *Bacillus* cell envelope, p. 217–254. *In* C. R. Harwood (ed.), Bacillus. Plenum Press, New York.
4. **Aronson, J. N., and H. C. Arvidson.** 1987. Toxic trypsin digest fragment from the *Bacillus thuringiensis* parasporal protein. Appl. Environ. Microbiol. **53**:416–421.
5. **Barton, K. A., H. R. Whiteley, and N.-S. Yang.** 1987. *Bacillus thuringiensis* δ-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. Plant Physiol. **85**:1103–1109.
6. **Bateson, J. B., and G. Stainsby.** 1970. Analysis of the active principle in the biological insecticide *Bacillus thuringiensis* Berliner. J. Food Technol. **5**:403–415.
7. **Bulla, L. A. Jr., K. J. Kramer, and L. I. Davidson.** 1977. Characterization of the entomocidal parasporal crystal of *Bacillus thuringiensis*. J. Bacteriol. **130**:375–383.
8. **Calabrese, D. M., K. W. Nickerson, and L. C. Lane.** 1980. A comparison of protein crystal subunit sizes in *Bacillus thuringiensis*. Can. J. Microbiol. **26**:1006–1010.
9. **Choma, C. T., and H. Kaplan.** 1992. *Bacillus thuringiensis* crystal protein: effect of chemical modification of the cysteine and lysine residues. J. Invertebr. Pathol. **59**:75–80.
10. **Dadd, R. H.** 1975. Alkalinity within the midgut of mosquito larvae with alkaline-active digestive enzymes. J. Insect Physiol. **21**:1847–1853.
11. **Day, J. F., R. W. Thornburg, S. R. Thorpe, and J. W. Baynes.** 1979. Nonenzymatic glucosylation of rat albumin: studies in vitro and in vivo. J. Biol. Chem. **254**:9394–9400.
12. **Dolhoffer, R., and O. H. Wieland.** 1979. Glycosylation of serum albumin: elevated glycosyl-albumin in diabetic patients. FEBS Lett. **103**:282–286.
13. **Dow, J. A. T.** 1984. Extremely high pH in biological systems: a model for carbonate transport. Am. J. Physiol. **246**:R633–636.
14. **Ellwood, D. C., and D. W. Tempest.** 1969. Control of teichoic acid and teichuronic acid biosyntheses in chemostat cultures of *Bacillus subtilis* var. *niger*. Biochem. J. **111**:1–5.
15. **Fischoff, D. A., K. S. Bowdish, F. J. Perlak, P. G. Marrone, S. M. McCormick, J. G. Niedermeyer, D. A. Dean, K. Kusano-Kretzmer, E. J. Mayer, D. E. Rochester, S. G. Rogers, and R. T. Fraley.** 1987. Insect tolerant transgenic tomato plants. Bio/Technology **5**:807–813.
16. **Hardy, M. R., R. R. Townsend, and Y. C. Lee.** 1988. Monosaccharide analysis of glycoconjugates by anion exchange chromatography with pulsed amperometric detection. Anal. Biochem. **170**:54–62.
17. **Hirs, C. H. W.** 1967. Glycopeptides. Methods Enzymol. **11**:411–413.
18. **Hofmann, C., H. Vanderbruggen, H. Höfte, J. Van Rie, S. Jansens, and H. Van Mellaert.** 1988. Specificity of *Bacillus thuringiensis* δ-endotoxins is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. Proc. Natl. Acad. Sci. USA **85**:7844–7848.
19. **Hofmann, K., and M. Bergmann.** 1939. The specificity of trypsin II. J. Biol. Chem. **130**:81–86.
20. **Höfte, H., and H. R. Whiteley.** 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. **53**:242–255.
21. **Holmes, K. C., and R. E. Monro.** 1965. Studies on the structure of parasporal inclusions from *Bacillus thuringiensis*. J. Mol. Biol. **14**:572–581.
22. **Huang, L., C. W. Forsberg, and D. Y. Thomas.** 1988. Purification and characterization of a chloride-stimulated cellobiosidase from *Bacteroides succinogenes* S85. J. Bacteriol. **170**:2923–2932.
23. **Huber, H. E., P. Lüthy, H.-R. Ebersold, and J.-L. Cordier.** 1981. The subunits of the parasporal crystal of *Bacillus thuringiensis*: size, linkage, and toxicity. Arch. Microbiol. **129**:14–18.
24. **Iberg, N., and R. Flückiger.** 1986. Nonenzymatic glycosylation of albumin in vivo: identification of multiple glycosylated sites. J. Biol. Chem. **261**:13542–13545.
25. **Insell, J. P., and P. C. Fitz-James.** 1985. Composition and toxicity of the inclusion of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. **50**:56–62.
26. **Krischik, V. A., P. Barbosa, and C. F. Reichelderfer.** 1988. Three trophic level interactions: allelochemicals, *Manduca sexta* (L.,), and *Bacillus thuringiensis* var. *kurstaki* Berliner. Environ. Entomol. **17**:476–482.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
28. **Lecadet, M. M.** 1967. Action comparée de l'urée et du thioglycolate sur la toxine figurée de *Bacillus thuringiensis*. C. R. Acad. Sci. **264**:2847–2850.
29. **Lechner, J., and F. Wieland.** 1989. Structure and biosynthesis of prokaryotic glycoproteins. Annu. Rev. Biochem. **58**:173–194.
30. **Mann, Nicholas.** Personal communication.
31. **Martin, P. A. W., and R. S. Travers.** 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl. Environ. Microbiol. **55**:2437–2442.
32. **Messner, P., and U. B. Sleytr.** 1991. Bacterial surface layer glycoproteins. Glycobiology **1**:545–551.
33. **Messner, P., and U. B. Sleytr.** 1992. Crystalline bacterial cell-surface layers. Adv. Microb. Physiol. **33**:213–275.
34. **Narusu, M. L., and K. P. Gopinathan.** 1986. Purification of larvicidal protein from *Bacillus sphaericus* 1593. Biochem. Biophys. Res. Commun. **141**:756–761.
35. **Nickerson, K. W.** 1980. Structure and function of the *Bacillus thuringiensis* protein crystal. Biotechnol. Bioeng. **22**:1305–1333.
36. **Nickerson, K. W., G. St. Julian, and L. A. Bulla, Jr.** 1974. Physiology of spore-forming bacteria associated with insects: radiorespirometric survey of carbohydrate metabolism in the 12 serotypes of *Bacillus thuringiensis*. Appl. Microbiol. **28**:129–132.
37. **Obukowicz, M. G., F. J. Perlak, K. Kusano-Kretzmer, E. J. Meyer, and L. S. Watrud.** 1986. Integration of the delta endotoxin gene of *Bacillus thuringiensis* into the chromosome of root colonizing strains of pseudomonads using Tn*5*. Gene **45**:327–331.
38. **Paul, J., and A. K. Varma.** 1992. Glycoprotein components of cellulase and xylanase enzymes of a *Bacillus* sp. Biotechnol. Lett. **14**:207–212.
39. **Pfannenstiel, M. A., G. A. Couche, G. Muthukumar, and K. W. Nickerson.** 1985. Stability of the larvicidal activity of *Bacillus thuringiensis* subsp. *israelensis*: amino acid modification and denaturants. Appl. Environ. Microbiol. **50**:1196–1199.
40. **Pfannenstiel, M. A., G. Muthukumar, G. A. Couche, and K. W. Nickerson.** 1987. Aminosugars in the glycoprotein toxin from *Bacillus thuringiensis* subsp. *israelensis*. J. Bacteriol. **169**:796–801.
41. **Salama, H. S., M. S. Foda, and A. Sharaby.** 1984. Novel biochemical avenues for enhancing *Bacillus thuringinesis* potency against *Spodoptera littoralis* [Lep: Noctuidae]. Entomophaga **29**:171–178.
42. **Scherrer, P., P. Lüthy, and B. Trumpi.** 1973. Production of δ-endotoxin by *Bacillus thuringiensis* as a function of glucose concentrations. Appl. Microbiol. **25**:644–646.
43. **Schnepf, H. E., H. C. Wong, and H. R. Whiteley.** 1987. Expression of a cloned *Bacillus thuringiensis* crystal protein in *Esche-*

richia coli. J. Bacteriol. **169**:4110–4118.

44. **Sivamani, E., N. Rajendran, R. Senrayan, T. N. Ananthakrishnan, and K. Jayaraman.** 1992. Influence of some plant phenolics on the activity of δ-endotoxin of *Bacillus thuringiensis* var. *galleriae* on *Heliothis armigera*. Entomol. Exp. Appl. **63**:243–248.
45. **Swanson, J. D.** 1980. A biochemical investigation of the entomocidal crystal produced by *Bacillus thuringiensis*. M. S. thesis, University of Nebraska, Lincoln.
46. **Thanabalu, T., J. Hindley, S. Brenner, C. Oei, and C. Berry.** 1992. Expression of the mosquitocidal toxins of *Bacillus sphaericus* and *Bacillus thuringiensis* subsp. *israelensis* by recombinant *Caulobacter crescentus*, a vehicle for biological control of aquatic insect larvae. Appl. Environ. Microbiol. **58**:905–910.
47. **Thorpe, S. R., and J. W. Baynes.** 1982. Nonenzymatic glycosylation of proteins in vitro and in vivo, p. 113–132. *In* The glycoconjugates, vol. III. Academic Press, New York.
48. **Tyrell, D. J., L. A. Bulla, R. E. Andrews, K. J. Kramer, L. I. Davidson, and P. Nordin.** 1981. Comparative biochemistry of entomocidal parasporal crystals of selected *Bacillus thuringiensis* strains. J. Bacteriol. **145**:1052–1062.
49. **Van Rijssel, M., G. J. Gerwig, and T. A. Hansen.** 1993. Isolation and characterization of an extracellular glycosylated protein complex from *Clostridium thermosaccharolyticum* with pectin methylesterase and polygalacturonate hydrolase activity. Appl. Environ. Microbiol. **59**:828–836.
50. **Watson, G. M. F., and N. H. Mann.** 1988. Protein phosphorylation in *Bacillus thuringiensis* during growth and δ-endotoxin production. J. Gen. Microbiol. **134**:2559–2565.
51. **White, P. J., and C. Gilvarg.** 1977. A teichuronic acid containing rhamnose from cell walls of *Bacillus megaterium*. Biochemistry **16**:2428–2435.
52. **Yan, X., and W. J. McCarthy.** 1991. Chemical modification of *Bacillus thuringiensis* subsp. *thuringiensis* (HD-524) trypsin-activated endotoxin: implication of tyrosine residues in lepidopteran cell lysis. J. Invertebr. Pathol. **57**:101–108.