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Amino Sugars in the Glycoprotein Toxin from *Bacillus thuringiensis* subsp. *israelensis*

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The carbohydrate content of purified *Bacillus thuringiensis* subsp. *israelensis* crystal toxin was determined by six biochemical tests, column chromatography on an amino acid analyzer, and the binding of 11 fluorescent lectins. The crystals contained approximately 1.0% neutral sugars and 1.7% amino sugars. The amino sugars consisted of 70% glucosamine and 30% galactosamine. No *N*-acetylneuraminic acid (sialic acid) was detected. The presence of amino sugars was confirmed by the strong binding of fluorescent wheat germ agglutinin and the weak binding of fluorescent soybean agglutinin. These lectins recognize *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine, respectively. The lectin-binding sites appeared evenly distributed among the protein subunits of the crystal. The sugars were covalently attached to the crystal toxin because wheat germ agglutinin still bound alkali-solubilized toxin which had been boiled in sodium dodecyl sulfate, separated by polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. This study demonstrates the covalent attachment of amino sugars and indicates that the *B. thuringiensis* subsp. *israelensis* protein toxins should be viewed as glycoprotein toxins. The crystals used in the present study were purified on sodium bromide density gradients. Studies employing crystals purified on Renografin density gradients can give artificially high values for the anthrone test for neutral sugars.

The bacterium *Bacillus thuringiensis* subsp. *israelensis* produces a protein crystal that is toxic to the larval stage of many mosquito and blackfly species. Consequently it is an important component of many mosquito abatement programs. An understanding of the mode of action of these mosquito toxins on the molecular level is desirable. Such an understanding requires a structure-function analysis of both the protein toxin and its target(s) in the larval gut. The size and amino acid composition of several *B. thuringiensis* toxins have already been determined (6, 21, 29, 30). Moreover, the amino acid sequences for the 28-kilodalton (kDa) subunit of the *B. thuringiensis* subsp. *israelensis* crystal (31) and the 134-kDa lepidoptera-active toxin from *B. thuringiensis* subsp. *kurstaki* have been deduced (25) from the cloned DNA sequences of their respective genes.

However, the possible presence of carbohydrates attached to these protein toxins has not yet been resolved. In particular, reports on the carbohydrate content of purified *B. thuringiensis* subsp. *kurstaki* crystals range from 0.1 to 12% (4, 5, 12, 13). These differences in reported values may be due to different methods of crystal purification and the limitations of the analytical procedures selected. Additionally, it is necessary to prove the covalent attachment of sugars, not merely their presence. Huber et al. (13) detected ≤0.1% neutral sugars in extensively (more than 10 times) washed *B. thuringiensis* subsp. *kurstaki* crystals. Moreover, they noted that the apparent sugar content decreased with each washing. This observation led them to suggest that the sugars detected were not, in fact, covalently attached to the crystal proteins but instead were products of sporulation and cell lysis, still adhering to the crystals owing to insufficient washing (13).

Similar disagreements exist with regard to the carbohydrate content of *B. thuringiensis* subsp. *israelensis* crystals. Insell and Fitz-James (14) reported a hexose content of 6%, while Tyrell et al. (29) reported a carbohydrate content "severalfold greater than for the other subspecies." Significantly, the same group had previously reported (5) that *B. thuringiensis* subsp. *kurstaki* crystals contained 5.6% carbohydrate, consisting of 3.8% glucose and 1.8% mannose. In the present report, we reexamined the carbohydrate content of *B. thuringiensis* subsp. *israelensis* crystals. This analysis included six colorimetric tests and the chromatographic separation of amino sugars on an amino acid analyzer as well as the ability of purified *Bacillus* crystals to bind 11 different fluorescent lectins. Our data demonstrate that the larvicidal toxins in the *Bacillus* crystal should be viewed as glycoprotein toxins since at least the lectin-specific carbohydrates are covalently attached to specific sites on the protein subunits.

MATERIALS AND METHODS

Toxin preparation. A single-colony isolate of *B. thuringiensis* subsp. *israelensis* taken from a Bactimos powder (courtesy of Brian Federici, University of California, Riverside) was grown on GGYS medium (20). After sporulation, the protein crystals were purified on NaBr gradients as described previously (3, 21). The crystals were solubilized for 2 h at 37°C in 50 mM NaOH with 10 mM EDTA at pH 11.7 (21) followed by centrifugation at 15,000 × *g* for 10 min. Protein concentrations in the supernatants were determined from the A_{280} ($E^{1\%}_{1\,cm}$ = 11.0 [29]).

Hydrolysis was performed on *B. thuringiensis* subsp. *israelensis* crystal protein by heating for 6 h at 95°C in 4 N HCl under nitrogen. After hydrolysis, the HCl was removed by vacuum desiccation. Neutral carbohydrates in the presence of amino acids interfere with the determination of

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TABLE 1. Detection of carbohydrates in *B. thuringiensis* subsp. *israelensis* toxin by colorimetric assays[a]

Assay	Carbohydrates measured	Intact crystals	Alkali-solubilized toxin[b]	Acid-hydrolyzed toxin[c]
Phenol-sulfuric acid	Neutral sugars	0.7 ± 0.07	0.5 ± 0.04	ND[d]
Anthrone	Neutral sugars	1.0 ± 0.02	0.1 ± 0.01	ND
Orcinol	*N*-Acetylneuraminic acid	0	0	ND
Lee and Montgomery	Neutral sugars and hexosamines	3.5 ± 0.05	ND	3.8 ± 0.15
Elson-Morgan	Hexosamines	0	ND	1.8 ± 0.15[e]
Morgan-Elson	Hexosamines and N-acetylated hexosamines	0	ND	1.7 ± 0.12[e]

[a] A 1- to 2-mg sample of purified toxin was used per assay. Assays were performed as described in Materials and Methods. Results are the percentage of carbohydrate ± standard error. Each number represents a minimum of four replicates.
[b] Crystal toxin was solubilized at pH 11.75 for 2 h at 37°C. After centrifugation at 15,000 × *g*, the supernatant was analyzed for carbohydrate content.
[c] Crystal toxin was hydrolyzed in 4 N HCl at 95°C for 6 h. After vacuum desiccation, the neutral sugars were removed by cation-exchange chromatography.
[d] ND, Not determined.
[e] Using glucosamine as the standard.

amino sugars, and these were removed by cation-exchange chromatography on AG 50W-X2 (Bio-Rad Laboratories, Richmond, Calif.) (2, 11).

Determination of carbohydrate content of crystal protein by colorimetric assays. A number of colorimetric assays were performed to determine the content of neutral sugars, amino sugars, and *N*-acetylneuraminic acid (sialic acid). The phenol-sulfuric acid (2, 11), anthrone (2, 11), orcinol (24), Lee and Montgomery (17), and Elson-Morgan (2, 28) and Morgan-Elson (2, 28) determinations were performed as described previously. A 1-to 2-mg sample of purified toxin was used per assay. Glucose was used as a standard for the phenol-sulfuric acid and anthrone assays, and *N*-acetylneuraminic acid was used as a standard for the orcinol assay. Because acid hydrolysis of the glycoprotein removes the acetyl moiety from N-acetylated carbohydrates (1, 9), glucosamine and galactosamine were used as standards for the Lee and Montgomery and Elson-Morgan reactions.

Chromatographic separation of amino sugars. Amino sugars were separated by chromatography on a Beckman amino acid analyzer (9). Hydrolyzed crystal protein (2 mg) was lyophilized and suspended in 1 ml of 0.2 M sodium citrate, pH 2.2. Samples were applied to a column of PA-35 resin (0.9 by 10.5 cm) maintained at 60°C. Flow rates of 100 and 35 ml/h for buffer and ninhydrin solutions, respectively, were used. The 0.158 M sodium citrate buffer of Plummer (23) was used for chromatography. Glucosamine and galactosamine (10 μg/ml) were used as standards.

Lectin binding to intact crystals. Purified *Bacillus* crystals (5 mg) were mixed with 10 μl of fluorescein isothiocyanate-labeled lectin (1 mg/ml) (EY Laboratories, San Mateo, Calif.). The mixture was incubated for 30 min at room temperature with occasional agitation. The crystals were pelleted by centrifugation for 5 min at 15,000 × *g*. The pellet was washed three times with 1 ml of buffer and then resuspended in 3 ml of buffer and read on a Perkin-Elmer 44A fluorescence spectrophotometer with 490 and 525 nm as excitation and emission wavelengths, respectively.

Lectins utilized were purified from *Concanavalia ensiformis* (concanavalin A), *Griffonia simplicifolia* (GS I and GS II), *Dolichos biflorus* (DBA), *Maclura pomifera* (MPA), *Ulex europaeus* (UEA-1), *Glycine max* (soybean agglutinin [SBA]), *Arachis hypogaea* (PNA), *Triticum vulgaris* (wheat germ agglutinin [WGA]), *Bauhinia purpurea* (BPA), and *Limulus polyphymus* (LPA). Incubation conditions were as recommended by EY Laboratories. The buffer for concanavalin A was 50 mM Tris hydrochloride (pH 7.0)–150 mM NaCl–1 mM $CaCl_2$–1 mM $MnCl_2$. The buffer for GS I and GS II binding was 10 mM sodium phosphate (pH 7.45)–150 mM NaCl (PBS) plus 5 mM $CaCl_2$. The buffer for LPA was 50 mM Tris hydrochloride–150 mM NaCl–10 mM $CaCl_2$. The PBS buffer was used for all other lectins.

Lectin binding to solubilized toxin. Solubilized toxin was applied to nitrocellulose membranes by vacuum filtration with a dot-blot apparatus. The membrane was incubated in 3% bovine serum albumin in PBS to block nonspecific binding sites. The nitrocellulose was washed two times with PBS. The WGA-horseradish peroxidase conjugate (200 μg) (Sigma Chemical Co., St. Louis, Mo.) was then added in 20 ml of PBS plus 1.0% bovine serum albumin. It was incubated for 2 h at room temperature and then washed four times in PBS and one time in 20 mM Tris hydrochloride–500 mM NaCl (pH 7.5). Horseradish Peroxidase Color Development Reagent (containing 4-chloro-1-naphthol) (Bio-Rad Laboratories) was used to detect lectin binding. Protein controls were bovine serum albumin which is not a glycoprotein and ovalbumin which contains mannose and *N*-acetylglucosamine.

Lectin binding to individual crystal proteins. Solubilized crystal proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12.5% gels, using the discontinuous system of Laemmli (16). The proteins were then transferred to nitrocellulose membranes with a Hoeffer transblot apparatus operating at maximum voltage for 1 h. Transfer buffer consisted of 192 mM glycine, 25 mM Tris, and 20% methanol. One portion of the nitrocellulose was stained with naphthol blue black and scanned with a Hoeffer densitometer to determine the relative amount of protein transferred to nitrocellulose in each band. The remaining nitrocellulose was used for WGA-horseradish peroxidase conjugate binding as described above. The nitrocellulose was then scanned to quantitate the relative amount of WGA binding to each protein band.

RESULTS

Chemical determinations. *B. thuringiensis* subsp. *israelensis* crystals purified on sodium bromide gradients were analyzed by six different colorimetric methods (Table 1). The anthrone and phenol-sulfuric acid methods are specific for neutral sugars. In intact crystals, these assays detected 1.0 and 0.7% carbohydrate, respectively, whereas lower levels were detected in the pH 11.75 solubilized crystal protein (Table 1). This decrease in the neutral sugar content of the alkali-solubilized proteins may be due either to alkali-

labile protein-carbohydrate linkages (beta-elimination) or to incomplete solubilization of the *Bacillus* crystals (21), with the alkali-insoluble proteins being highly enriched in carbohydrates. The alkali-insoluble proteins typically make up 20% of the total crystal protein, and based on the phenol-sulfuric acid assay, the insoluble protein pellet contained 3.8% neutral sugars. A qualitatively similar enrichment for carbohydrates had previously been observed by Insell and Fitz-James (14).

However, neither the anthrone nor the phenol-sulfuric acid method detects amino sugars. To alleviate this difficulty, Lee and Montgomery (17) introduced a modification of the phenol-sulfuric acid method in which the hexosamines are first deaminated with nitrous acid. This procedure detected 3.5% total sugars (neutral and amino) in intact *B. thuringiensis* subsp. *israelensis* crystals and 3.8% total sugars in acid-hydrolyzed crystal protein (Table 1).

The Elson-Morgan reaction (2, 28) with acetylacetone detects free amino sugars, while the Morgan-Elson reaction (28) with acetic anhydride measures both free and N-acetylated amino sugars. These reactions require that the hexosamines be liberated before colorimetric determination, and as expected, hexosamines were only detected after their release by acid hydrolysis (Table 1). The Elson-Morgan reaction detected 1.8% amino sugars, while the Morgan-Elson reaction detected 1.7% amino sugars (Table 1). No attempts were made to optimize hydrolysis conditions. Standard hydrolysis conditions (4 N HCl at 95°C for 6 h) were used for both reactions (9). Since the conditions necessary for the hydrolysis of amino sugar-containing polymers vary tremendously (1), the data obtained represent minimum amounts of carbohydrate present. Last, no *N*-acetylneuraminic acid was detected in the *B. thuringiensis* subsp. *israelensis* protein by the orcinol method (Table 1).

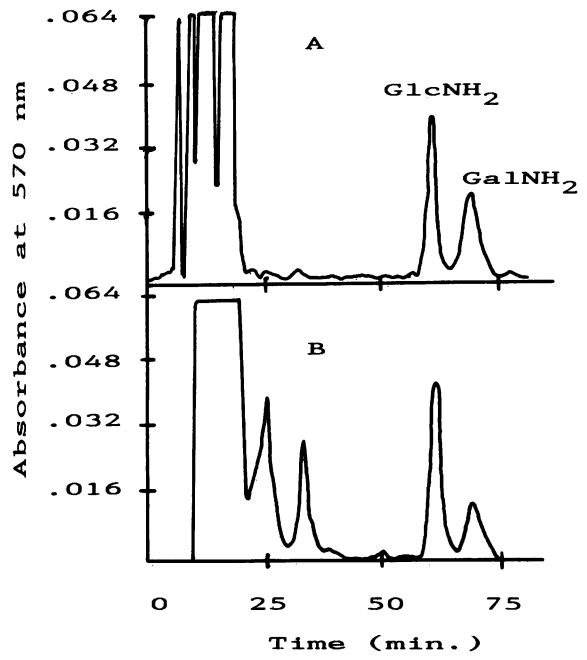

FIG. 1. Chromatographic separation of amino sugars on an amino acid analyzer. (A) Amino acid calibration mixture (500 nmol) plus 10 μg of both glucosamine and galactosamine per ml; (B) analysis of 0.5 mg of *B. thuringiensis* subsp. *israelensis* crystal protein after hydrolysis in 4 N HCl for 6 h. Abbreviations: $GlcNH_2$, glucosamine; $GalNH_2$, galactosamine.

TABLE 2. Binding of lectins by *B. thuringiensis* subsp. *israelensis* crystal toxin[a]

Lectin[b]	Major sugar specificity	Fluorescence
Concanavalin A	D-Mannose	0.06
GS I	D-Galactose	0.00
GS II	*N*-Acetyl-D-glucosamine	0.00
LPA	*N*-Acetylneuraminic acid	0.04
DBA	*N*-Acetyl-D-galactosamine	0.00
MPA	D-Galactose	0.00
UEA-1	L-Fucose	0.09
SBA	*n*-Acetyl-D-galactosamine	0.44
PNA	D-Galactose	0.00
WGA	*N*-Acetyl-D-glucosamine	3.76
BPA	*N*-Acetyl-D-galactosamine	0.00

[a] Crystals (5 mg) were incubated with 10 μg of fluoresein isothiocyanate-labeled lectins. The crystals were washed by centrifugation three times with 1 ml of buffer. Units given are arbitrary fluorescent units when the excitation wavelength is 490 nm and the emission wavelength is 525 nm. Experiments were repeated three times with similar results.

[b] Lectins utilized were purified from *Concanavalia ensiformis* (concanavalin A), *Griffonia simplicifolia* (GS I and GS II), *Dolichos biflorus* (DBA), *Maclura pomifera* (MPA), *Ulex europaeus* (UEA-1), *Glycine max* (soybean agglutinin [SBA]), *Arachis hypogaea* (PNA), *Triticum vulgaris* (wheat germ agglutinin [WGA]), *Bauhinia purpurea* (BPA), and *Limulus polyphymus* (LPA).

Chromatographic separation of amino sugars. D-Glucosamine and D-galactosamine are the only amino sugars known to be components of glycoproteins (9), and chromatographic analysis indicated that both of them were present in hydrolyzed *Bacillus* crystal protein (Fig. 1B). Only two amino sugar peaks were detected, and their retention times were identical to those for the glucosamine and galactosamine standards (Fig. 1A). Of the total hexosamines detected (8.7 ± 0.55 μg/mg of crystal protein), integration of the peak areas indicated 70 ± 2.7% glucosamine and 30 ± 2.7% galactosamine. This glucosamine/galactosamine ratio agrees very well with that calculated from the colorimetric tests for amino sugars. In the Elson-Morgan assay, glucosamine and galactosamine give identical color yields on a molar basis, whereas in the Morgan-Elson reaction galactosamine gives only 35% of the color of glucosamine (28). When both reactions are used, they provide two stimultaneous equations whose solution determines the amount of glucosamine and galactosamine present in an unknown mixture. For acid-hydrolyzed crystal protein (Table 1), these equations indicated approximately 80% glucosamine and 20% galactosamine.

Lectin binding. Lectins are proteins which contain highly specific binding sites for a wide range of carbohydrate structures. We tested 11 differenct fluorescent lectins for their ability to attach to intact *B. thuringiensis* subsp. *israelensis* crystals on the assumption that lectin binding would indicate the presence of the corresponding sugars. Of these 11 lectins, only WGA and SBA gave fluorescence intensities significantly above background (Table 2), with the fluorescence owing to WGA attachment being far more intense. The specifity of this binding was shown by competition with *N*-acetyl-D-glucosamine. In the presence of excess *N*-acetyl-D-glucosamine, the fluorescent WGA no longer bound *Bacillus* crystals.

Covalent attachment. We next sought to determine whether WGA was binding to the protein toxin itself or to some contaminant, possibly introduced during crystal prep-

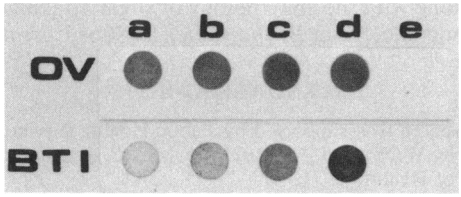

FIG. 2. Binding of WGA to ovalbumin (OV) and *B. thuringiensis* subsp. *israelensis* (BTI) protein. The crystal toxin was solubilized as described in Materials and Methods. The solubilized toxin and ovalbumin were applied to nitrocellulose membranes and incubated with WGA-horseradish peroxidase conjugate, and the color was developed as described in Materials and Methods. Amounts of proteins applied per dot for ovalbumin are 4 (a), 8 (b), 16 (c), 24 (d), and 0 (e) μg. Amounts of toxin protein applied are 18 (a), 36 (b), 72 (c), 108 (d), and 0 (e) μg.

aration. This point was clarified by demonstrating the continued ability of WGA to bind alkali (pH 11.75)-solubilized crystal protein. Two methods were used. In the first method, solubilized toxin was applied directly to nitrocellulose membranes and incubated with WGA-horseradish peroxidase conjugate (Fig. 2). In the second method, solubilized toxin was boiled in sodium dodecyl sulfate sample buffer (16) and resolved by polyacrylamide gel electrophoresis. The separated proteins were then transferred to nitrocellulose membranes and incubated with the WGA-horseradish peroxidase conjugate. In both experiments, *B. thuringiensis* subsp. *israelensis* crystal protein and the glycoprotein ovalbumin bound WGA, while the sugar-free bovine serum albumin control did not.

Distribution of WGA-binding residues. *B. thuringiensis* subsp. *israelensis* crystals are composed of multiple protein subunits ranging in size from 28 to 140 Kda (21, 29). The distribution of WGA-binding sites among these multiple protein subunits was quantified by transferring them from sodium dodecyl sulfate gels to nitrocellulose membranes and comparing their reactivity with WGA-horseradish peroxidase (Table 3). All the major protein bands bound WGA, and the ratio of bound WGA to protein was roughly equivalent for each band (Table 3). The lectin-binding sites appeared evenly distributed among the major classes of the protein subunits. Unfortunately, the precision of the method did not permit resolution within the major classes, i.e., 135 versus 140 kDa.

Possible artifacts. Most procedures for the purification of *B. thuringiensis* crystals involve either NaBr (3) or Renografin (26) gradients. Significantly, Renografin is the *N*-methyl-D-glucamine salt of 3,5-diacetylamino-2,4,6-triiodobenzoate, and *N*-methyl-D-glucamine is prepared from D-glucose and methylamine. If these gradient components were not completely removed after crystal purification, they could interfere with subsequent analysis of the carbohydrate content of the crystal. Accordingly, the anthrone, phenol-sulfuric acid, and Lee and Montgomery (17) assays were performed with increasing concentrations of either NaBr or Renografin. Sodium bromide did not interfere with any of these assays; it would be physically impossible to have an interfering level of NaBr present in crystal samples. Similarly, Renografin did not interfere with the phenol-sulfuric acid and Lee and Montgomery assays. However, Renografin did interfere with the anthrone reaction. If 0.13 mg of Renografin was present in a 1-mg sample of carbohydrate-free protein, the anthrone reaction would give an erroneous indication of 5% carbohydrate.

DISCUSSION

We used six colorimetric tests to analyze the carbohydrate content of the *B. thuringiensis* subsp. *israelensis* mosquito larvicidal protein crystals. Purified crystals contained roughly 2.7% total carbohydrate, consisting of 1.0% neutral sugars and 1.7% amino sugars (70% glucosamine and 30% galactosamine). The presence of both glucosamine and galactosamine is in qualitative and quantitative agreement with the binding of the fluorescent lectins WGA and SBA by intact crystals. Column chromatography on an amino acid analyzer confirmed the presence of amino sugars in purified crystals. It is important that the column chromatography detecting both glucosamine and galactosamine is conducted on acid hydrolysates of intact crystals rather than of alkali-solubilized crystal protein. This precaution avoids the danger of alkali-catalyzed epimerization of the *N*-acetylhexosamines (19) by which glucosamine and galactosamine could possibly interconvert. However, quantitative estimation of sugars in glycoproteins is considered to be a problem of extreme complexity (7), and the absolute levels of sugars present are less certain. Complete release of the sugars present inevitably leads to the destruction of some of those sugars (7). Additionally, the colorimetric tests for sugars are qualitative and nonstoichiometric (2). The amounts of amino sugars detected by column chromatography were substantially less than those detected by the colorimetric tests.

The crystals analyzed in this study were purified from sodium bromide density gradients. Two other groups have studied the carbohydrate content of crystals purified from Renografin gradients (14, 29). In both cases, the levels of sugar detected were much higher. Tyrell et al. (29) reported the presence of glucose, mannose, fucose, rhamnose, xylose, and galactosamine at a level severalfold greater than in crystals from other subspecies of *B. thuringiensis*. Since this group had previously reported 5.6% carbohydrate in crystals from *B. thuringiensis* subsp. *kurstaki* (5), the indicated carbohydrate level in *B. thuringiensis* subsp. *israelensis* crystals should be ≥11.2%. Similarly, Insell and Fitz-James (14) reported that *B. thuringiensis* subsp. *israelensis* crystals contained 6% hexose by dry weight using the anthrone test for neutral sugars.

Glycoproteins of bacterial origin are rather rare. They have been found in halobacterial flagellin (32), a surface protein from *Myxococcus xanthus* (18), and the paracrystalline S-layer proteins on the outer surface of many eubacteria and archaebacteria (27). Because of this rarity and because the presence of crystal glycoproteins from other *B. thuringiensis* subspecies has been controversial (5, 13), it was important to demonstrate that the amino sugars detected in *B. thuringiensis* subsp. *israelensis* crystals were, in fact, covalently bound to the protein toxin. Covalent attachment of at least the amino sugars is indicated because the crystal glycoprotein subunits could still be detected by WGA bind-

TABLE 3. Relative amount of WGA bound by *B. thuringiensis* subsp. *israelensis* protein subunits separated by sodium dodecyl sulfate-gel electrophoresis

Mol wt of *Bacillus* protein (10^3)	% Protein per band	% WGA bound per band
135–140	11	13
68–70	27	23
38–40	22	28
28	40	36

ing after (i) alkali solubilization, (ii) sodium dodecyl sulfate-gel electrophoresis, and (iii) transfer from polyacrylamide gels to nitrocellulose membranes. These observations indicate that the amino sugars are intrinsic crystal components and that the mosquito larvicidal toxin must be viewed as a glycoprotein.

B. thuringiensis subsp. *israelensis* crystals bound WGA strongly and SBA weakly, but they did not bind nine other fluorescent lectins. Binding by WGA indicates the presence of either *N*-acetyl-D-glucosamine or *N*-acetylneuraminic acid. However, *N*-acetylneuraminic acid was not detected chemically, and the lectin LPA, which is specific for *N*-acetylneuraminic acid alone, did not bind. Both WGA and SBA bind amino sugars preferentially, and their attachment to *B. thuringiensis* subsp. *israelensis* crystals confirms the presence of amino sugars. However, the absence of attachment by the other nine lectins cannot be used to eliminate other possible carbohydrates. Some lectins may be able to penetrate to the critical carbohydrate residues, while other lectins of the same saccharide-binding specificity may not (10). Finally, both GS II and WGA recognize beta-linked *N*-acetyl-D-glucosamine, but only the WGA bound the *Bacillus* crystals. In addition to the steric access problem already mentioned, this difference could reflect a more subtle aspect of lectin specificity. GS II binds only to terminal *N*-acetyl-D-glucosamine residues, while WGA has the ability to bind internal residues as well (10).

Our study used lectins to characterize purified *B. thurigiensis* subsp. *israelensis* crystals and as such it is quite different from that of DeLucca (8), which used lectins to distinguish the vegetative surface layers of 28 serovars of *B. thuringiensis*. However, there was no correlation between the binding specificity of the crystals and the vegetative cells. The lectins WGA and SBA bound *B. thuringiensis* subsp. *israelensis* crystals but did not bind *B.thurigiensis* subsp. *israelensis* vegetative cells (8), whereas the lectin from *G. simplicifolia* recognized *B. thuringiensis* subsp. *israelensis* vegetative cells (8) but not *B. thuringiensis* subsp. *israelensis* crystals.

N-Acetylglucosamine is most commonly attached to proteins at Asn-X-Ser and Asn-X-Thr sequences via *N*-glycosidic linkage to the amide N of asparagine (22). The amino acid sequence of the 28-kDa subunit from *B. thuringiensis* subsp. *israelensis* crystals has recently been deduced from the DNA sequence of its cloned gene (31). This sequence indicates a 27,340-dalton protein consisting of 259 amino acids. Significantly, the sequence contains four Asn-X-Thr tripeptide sites, at positions 104 to 106, 167 to 169, 170 to 172, and 246 to 248 (31). Four potential attachment sites are sufficient to accommodate the carbohydrate levels detected. If each attachment site contained a single *N*-acetylglucosamine residue, the resulting protein would be 3.14% carbohydrate.

The significance of glycoproteins in the *Bacillus* crystal extends beyond their structural chemistry. The amino sugars also serve an important function in larval pathogenicity (G. Muthukumar and K. W. Nickerson, manuscript in preparation) consistent with toxin binding to a lectinlike receptor in the larval gut. Knowles et al. (15) have demonstrated that toxicity of *B. thuringiensis* subsp. *kurstaki* crystals toward a lepidopteran cell line (*Choristoneura fumiferana* CF1 cells) is inhibited by preincubation of the toxin with both *N*-acetylgalactosamine and *N*-acetylneuraminic acid as well as with the lectins which bind these amino sugars. The occurrence of lectinlike receptors in the mosquito larval gut and their involvement in pathogenicity is currently being investigated along with the enzymology of sugar attachment in the procaryotic bacterium *B. thuringiensis* subsp. *israelensis*.

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