THE EFFECTS OF VARYING GLUCOSE MEDIA CONCENTRATION ON SPORES OF BACILLUS THURINGIENSIS VAR KURSTAKI HD-1

by

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ABSTRACT

The Effects of Varying Glucose Media Concentration on Spores of Bacillus thuringiensis var. kurstaki HD-1

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Bacillus thuringiensis spores were grown on nonsupplemented, 0.1, 0.5, or 1.0 percent glucose supplemented nutrient agar. Spores were isolated using a density gradient of Angiovist. The spore coats were removed through solubilization with a Tris buffer containing sodium dodecyl sulfate. Soluble versus total protein content for spores from each concentration was then analyzed by a dye-binding technique and the respective ratios were calculated. Sodium dodecyl sulfate polyacrylamide gel electrophoresis further analyzed the soluble and the total spore proteins.

CHAPTER 1

INTRODUCTION

Bacillus thuringiensis is a gram positive, rod shaped, spore-forming bacterium. At the time of sporulation, it forms a unique bipyramidal crystal which is used in commercial insecticides for mosquitoes, caterpillars, and blackflies. When the crystal is ingested by susceptible larvae, the alkaline pH and other midgut conditions hydrolyze the crystal into toxic subunits. This toxin causes midgut paralysis and the insect dies within twenty four hours.

Bacillus thuringiensis has two qualities which have led to its widespread popularity - low cost and target specificity. Studies have shown that even high levels of *B. thuringiensis* are harmless to non-target animals as well as to the environment (5). This non-target safety has allowed *Bacillus thuringiensis* products to be classified in the lowest toxicity category by the United States Environmental Protection Agency (1). An estimated four million kilograms of *B. thuringiensis* products are used annually in the Western world (5). Its frequent use is illustrated by the fact that over eighty percent of the lettuce grown in the United States is sprayed at least once during the last two months before harvest, a time when many harsher products are banned (6).

There are some problems with *Bacillus thuringiensis*, however. Although spores are normally protective structures, *Bacillus thuringiensis* spores have poor viability in the environment, necessitating frequent spraying (11). Ideally, the

spores would germinate, grow, and resporulate, thus creating a self-generating, perpetual larvicide.

Previous experiments have linked the changes in the glucose content of a growth medium to changes in crystal size (10). Since the spore coat is made of the same crystal proteins, the purpose of this study is to determine the effect of growth medium glucose content on the spore coat proteins. Several properties of the spore are reliant upon spore coat content (2). Therefore, it is possible that some undesirable spore properties (such as poor viability in the environment) could be attributed to differences in coat proteins. If the causes of such properties could be regulated through growth medium content, then a better larvicide product could be marketed.

CHAPTER 2

MATERIALS AND METHODS

Cultures

The strain used in this study was Bacillus thuringiensis var. kurstaki HD-1, which was isolated from the commercial product Dipel (Abbott Laboratories). Dipel is a dried powder mixture containing spores and crystals of B. thuringiensis. A small quantity of Dipel was placed in autoclaved distilled water and suspended with a vortex mixer. The solution was streaked onto nutrient agar (Difco) plates, incubated at room temperature over night, and several resulting colonies were checked microscopically for purity and ability to sporulate and form crystals. These colonies were collected in sterile distilled water using a sterile loop and used as the inoculum for preparing large volumes of spores and crystals.

Growth Media and Conditions

Nutrient agar (Difco) plates containing 0.0, 0.1, 0.5, or 1.0 percent glucose were inoculated with Bacillus thuringiensis using sterile cotton tipped swabs to obtain a lawn of bacteria. The plates were left at room temperature for five days, and then checked for complete sporulation and purity by microscopic inspection.

Collection and Purification of Spores and Crystals

The cultures were harvested from the surfaces of the plates with a sterile metal spatula and transferring the bacteria to sterile distilled water in 50 ml centrifuge tubes. The spores and crystals were suspended in a Vortex mixer and then collected by centrifugation at 9000 rpm for fifteen minutes. The supernatant was poured off and the pellets were suspended by the addition of 30 ml of 0.1 M NaCl with 0.1% Triton X-100. Spores and crystals were then collected by centrifugation for fifteen minutes at 9000 rpm. The spore and crystal mixture was washed three more times in sterile distilled water and stored at room temperature (see Figure 1).

Separation of Spores and Crystals

The spore and crystal suspension was combined with Angiovist 292 (Berlex Imaging) to make a final concentration of 50% (v/v). This solution was sonically treated for forty seconds in a Bransonic 221 sonic cleaner at 185 Watts to eliminate clumping of the spores and crystals, and then layered onto a discontinuous gradient of 60% and 70% Angiovist 292, prepared as noted by Milne, et. al. Separation was accomplished by ultra-centrifugation at 22,000 rpm for one hour. The pure spores formed a pellet at the base of the centrifuge tube. The supernatant was decanted and the spores were collected using

sterile pipettes. Purity of greater than 99% phase bright spores was verified by phase contrast microscopy.

Solubilization of Spore Coats

Spore coat protein was solubilized by the technique of Laemmli (8). One ml aliquots of each sample were dispensed into two ml Eppendorf tubes, and placed in a Brinkmann centrifuge for one minute. The supernatant was removed from each tube and 0.5 ml of Tris(hydroxymethyl)aminomethane (Tris) buffer at pH 6.8 containing sodium dodecyl sulfate and 2-mercaptoethanol (SDS-buffer) was then added. The spores were resuspended by use of a Vortex mixer and placed in a boiling water bath for one minute. The sample was then mixed by hand vortex and collected using an Eppendorf centrifuge, leaving the spore coat protein in the supernatant (see Figure 2.)

Disruption of Spores

Disruption of spores through sonication was necessary to deterimine both total spore protein and the protein content of spores which have had their coat proteins removed (stripped spores). The spore pellets (in 2 ml Eppendorf tubes) were suspended in 200 ul of SDS-buffer, and then sonically disrupted for six minutes (see Figure 2).

Protein Assay

Protein assays were performed in duplicate on spore coat protein, stripped spore protein, and total spore protein by the dye-binding method of Bradford (4). The sample was dispensed by 5 ul aliquots into 13 x 100 mm test tubes, and 5 ml of the reagent were added. A standard curve was prepared using absorbance readings for known concentrations of bovine serum albumin (fraction V). Readings for samples were compared to determine their protein content.

Polyacrylamide Gel Electrophoresis (PAGE)

Spore proteins were analyzed by SDS-PAGE by the method of Laemmli (8). After using the results from the protein assays to determine relative amounts of protein, 30 ug of each sample were loaded onto the 7.5% polyacrylamide gel at 40 mA at constant current for approximately 2.5 hours. The gel was stained using Coomassie Blue, and destained in a solution containing 10% acetic acid and 15% methanol.

CHAPTER 3

RESULTS

Assays of total spore protein and solubilized spore coat protein from the unsupplemented, 0.1, 0.5, and 1.0 percent glucose-supplemented media yielded the results presented in Figure 3. Results from the four groups of spores cannot be compared directly because the number of spores in each sample was not determined. Instead, the proportion of spore protein to total protein was of interest. As this proportion should not vary from spore-to-spore within a sample, it was unnecessary to make counts of spores.

The comparison of coat protein per total spore protein among the samples grown on unsupplemented, 0.1, 0.5, and 1.0% glucose-supplemented nutrient agar reveals similarities between the two samples with low glucose (unsupplemented and 0.1% glucose supplemented) and between the two with relatively high amounts of glucose (0.5 and 1.0%). The ratios of the 0.0 and the 0.1% samples are 97.5 and 96.6%, respectively. The ratios of the 0.5 and 1.0% samples, however, are 80.4 and 80.1%, respectively.

The 7.5% SDS-polyacrylamide gel reflects both the qualitative and quantitative differences between the samples. Since each well received 30 ug protein, the relative densities of the bands reflect the changes in the amount of specific proteins synthesized under each growth condition.

The main protein bands visible in the gel are located at approximately 127,500 Da and at approximately 63,500 Da. The relative amount of the larger protein decreases as the amount of

glucose in the media increases. The amount of the smaller major peptide is also inversely related to the amount of glucose, although the difference is not as obvious. Several less prominant bands are also visible.

CHAPTER 4

DISCUSSION

A previous study has shown that Bacillus thuringiensis spores grown under varying glucose concentrations display a wide range of characteristics vital to spore viability in the environment (12). Spores grown under low glucose conditions, such as the ones grown on the media containing 0.0 and 0.1 % glucose in this study, have relatively poor viability. They are fast germinating, low in dormancy (ie. require no activation), have poor resistence and are small in size. However, spores grown under high glucose conditions (such as 0.5 and 1.0 % in this study) are more stable. These spores are slow germinating, have adequate dormancy, better resistence, and larger size. Since the ideal spore would be able to germinate, grow, and resporulate in order to create a perpetual larvicide, the spores grown under high glucose conditions would be preferable.

Earlier studies have linked the change in the glucose content of the growth medium to the change in crystal size (10, 12). These same crystal proteins are found in the spore coat (12). Therefore, the purpose of this study was to determine if similar changes occurred to the proteins of the spore coat. It was expected that the proteins would be greater in number, amount, and complexity as the amount of glucose in the media increased. The influence of growth medium content on spore properties has been well documented (12). Several spore coat proteins are the same as the crystal proteins, and these crystal proteins have been shown to vary with different growth medium

content. These spore coat proteins could therefore be good candidates as the causitive agents in the variation of spore viability traits.

The dye-binding method of the Bradford protein assay was used to determine changes in the amounts of soluble versus total spore protein (4). The ratios of the percent of solubilized coat protein from the spores grown under 0.0 and 0.1% glucose were both approximately 97%, indicating that the vast majority of the protein in the spore was dedicated to the spore coat. The spores grown under high glucose conditions, however, had 15% less protein dedicated to the coat. This information did not support the hypothesis that changes in spore viability were due to differences in content of the spore coat. On the contrary, it implied that as the media glucose content increases, the percent coat protein from total protein diminishes. This would imply that increases in glucose content lead to increases in the complexity of the core proteins of the spore.

Equal quantites of protein were loaded into each lane of the SDS-polyacrylamide gel, so both qualitative and quantitative studies could be performed. The two main bands that were visible appear to be crystal proteins. Previous work has shown crystal proteins to migrate at 134 kDa and 66 kDa (3). The largest major band observed in this study is approximately 127.5 kDa and correlates with previous studies as the delta-endotoxin, which is also known as the P-1 crystal toxin (3). The other major band is approximately 63.5 kDa and correlates with the active crystal toxin (3). Both the P-1 and the active crystal toxin amounts are

inversely related to the amount of glucose in the media. Likewise, the general complexity of the spore coat proteins which normally are found in the region between 35 - 90 kDa tends to decrease as the media glucose increases.

Although the core proteins were not recovered in quantities sufficient to allow performance of gel electrophoresis, preliminary data from an assay of the small quantities recovered tended to support the rest of the study. As the media glucose increased, so did the amount of protein dedicated to the core. This change would not be visible on the gel as performed because many of the core proteins have been categorized as small proteins in the range of 5 - 7 kDa (3). Since the gel was capable of distinguishing proteins from from 29 - 205 kDa, the smaller core proteins would not have been detected. Evidence of undetected proteins in the electrophoresis lies in the fact that although equal amounts of protein were loaded in each well, less protein is visible in the lanes of the spores grown on 0.5 and 1.0% glucose-supplemented media than in the lanes of the spores grown on nonsupplemented or 0.1% glucose-supplemented media. This protein must be accounted for, and the only probable solution is that the missing protein ran off the gel in the form of proteins smaller than 29 kDa, such as the small core proteins of 5 - 7 kDa.

Ultraviolet resistence is associated with the small, acid-soluble proteins associated with the core (9). They are thought to be bound to the DNA so as to protect vulnerable regions from ultaviolet damage. Sensitivity to ultraviolet light is a major factor in the disappearance of Bacillus thuringiensis spores from

the soil (7). Changes in core protein content due to medium content, therefore, may be the origin of the ultraviolet sensitivity noted and may possibly be manipulated to improve the spore.

In conclusion, the results of this study suggest that changes in the viability of Bacillus thuringiensis spores grown under varying glucose conditions are due to changes in small proteins associated with the core of the spore. The spore coat proteins, however, are negatively related to the media glucose content. As the amount of glucose in the media increases, the amount of protein dedicated to the spore coat decreases.

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LIST OF FIGURES

FIGURE 1

PROTOCOL FOR THE HARVEST AND PURIFICATION
OF BACILLUS THURINGIENSIS SPORES

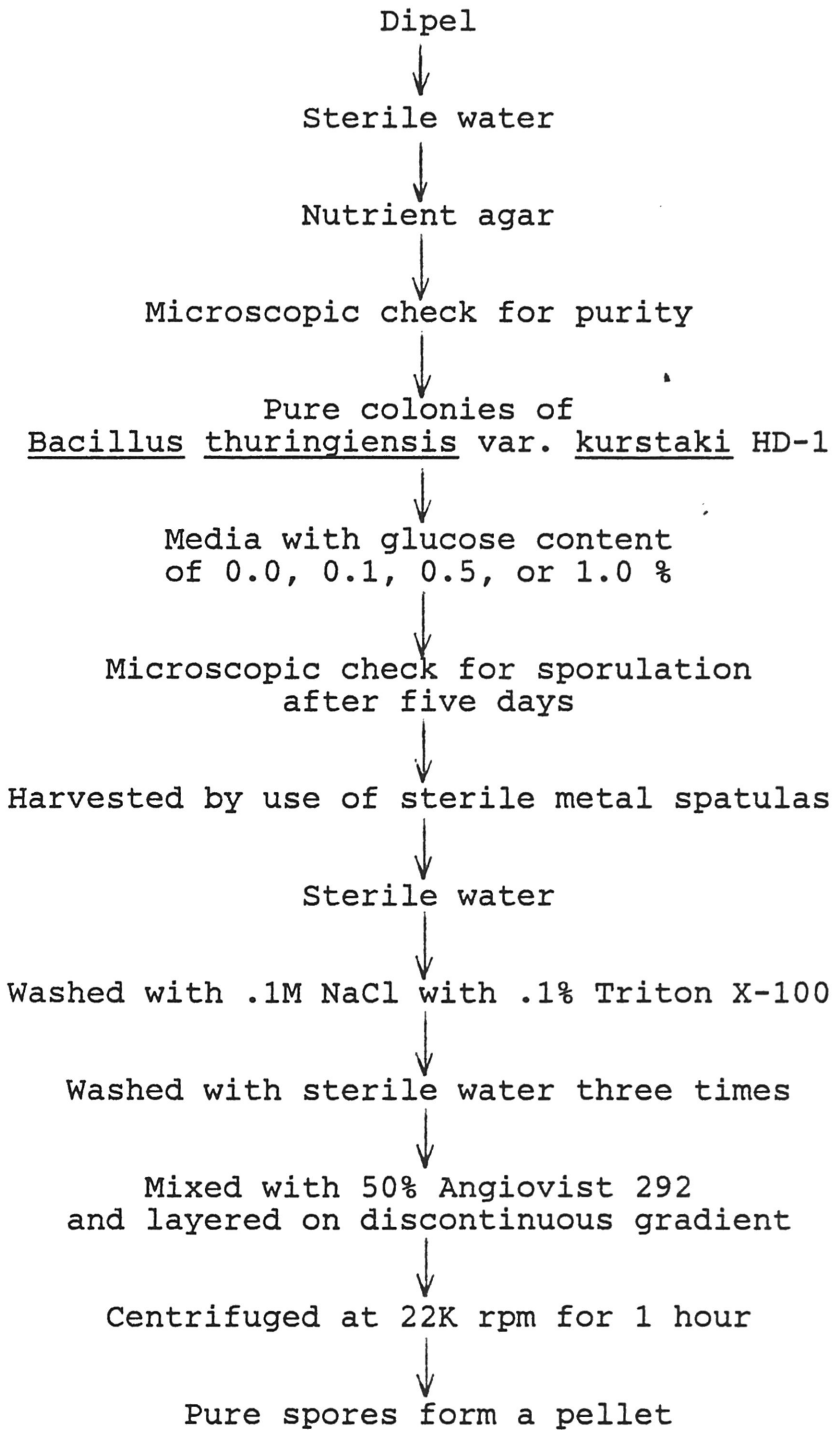

FIGURE 2

PROTOCOL FOR THE ANALYSIS OF PURE SPORES

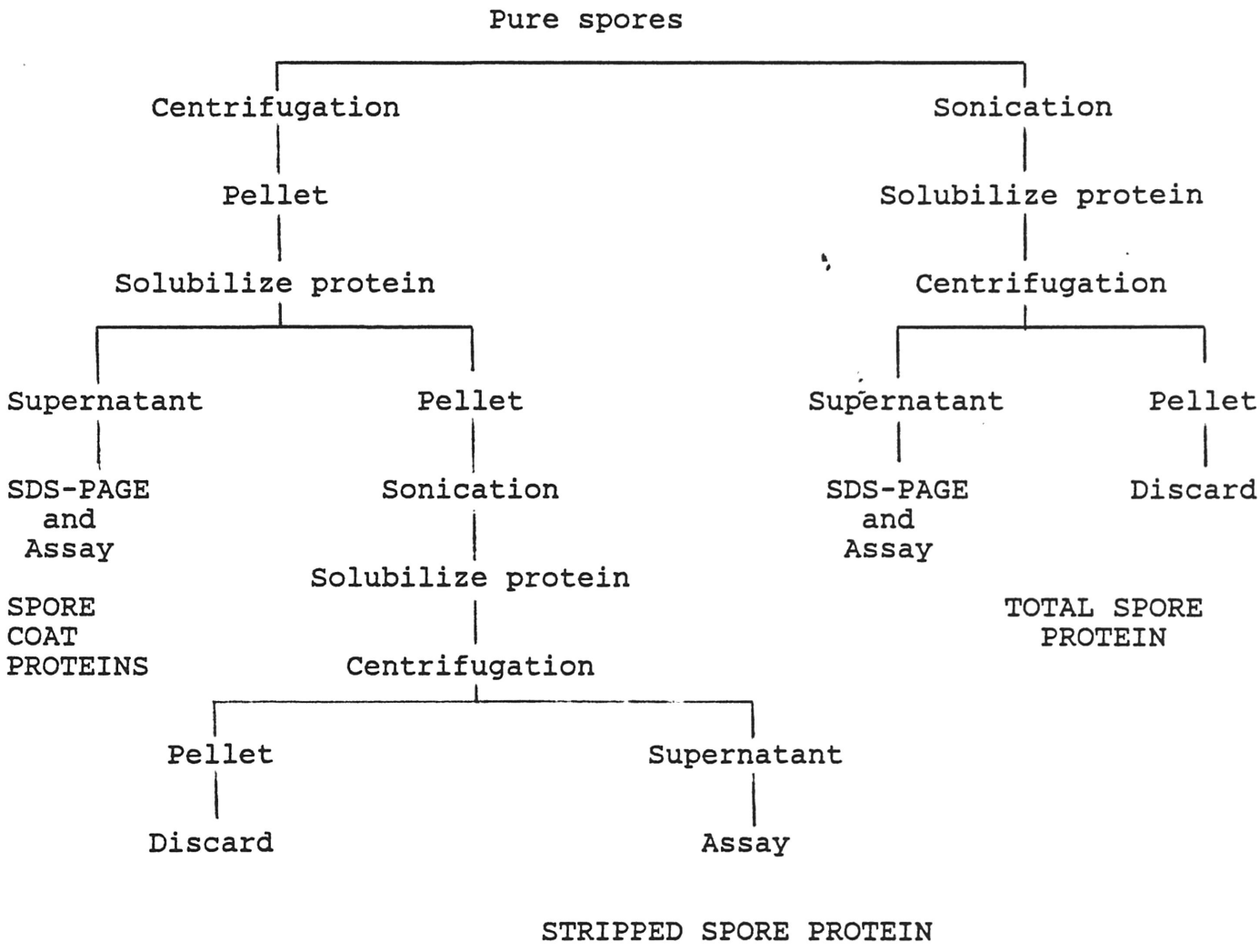

FIGURE 3. PROTEIN CONTENTS OF SOLUBILIZED SPORE COATS AND DISRUPTED SPORES.

Data were obtained by the dye-binding method of Bradford et. al. (3). Spores were produced on nutrient agar containing 0.1, 0.5, or 1.0% glucose. Unsupplemented medium served as the controls.

SPORE COAT PROTEIN	
% Glucose	ug/ul
none	13.1277 +/- .1339
0.1	13.2287 +/- .7468
0.5	7.1132 +/- 1.2662
1.0	1.2171 +/- .4536

TOTAL SPORE PROTEIN	
% Glucose	ug/ul
none	13.4703 +/- .9644
0.1	13.6928 +/- .2040
0.5	8.8504 +/- 1.2100
1.0	1.5199 +/- .6316

PERCENT SPORE COAT PROTEIN / TOTAL SPORE PROTEIN

% Glucose in Media	% Protein
none	97.46
0.1	96.58
0.5	80.37
1.0	80.07

FIGURE 4. PERCENT OF TOTAL PROTEIN IN SPORE COAT.

Percent protein was determined by comparing protein contents of solubilized coat proteins with solubilized proteins from disrupted spores.

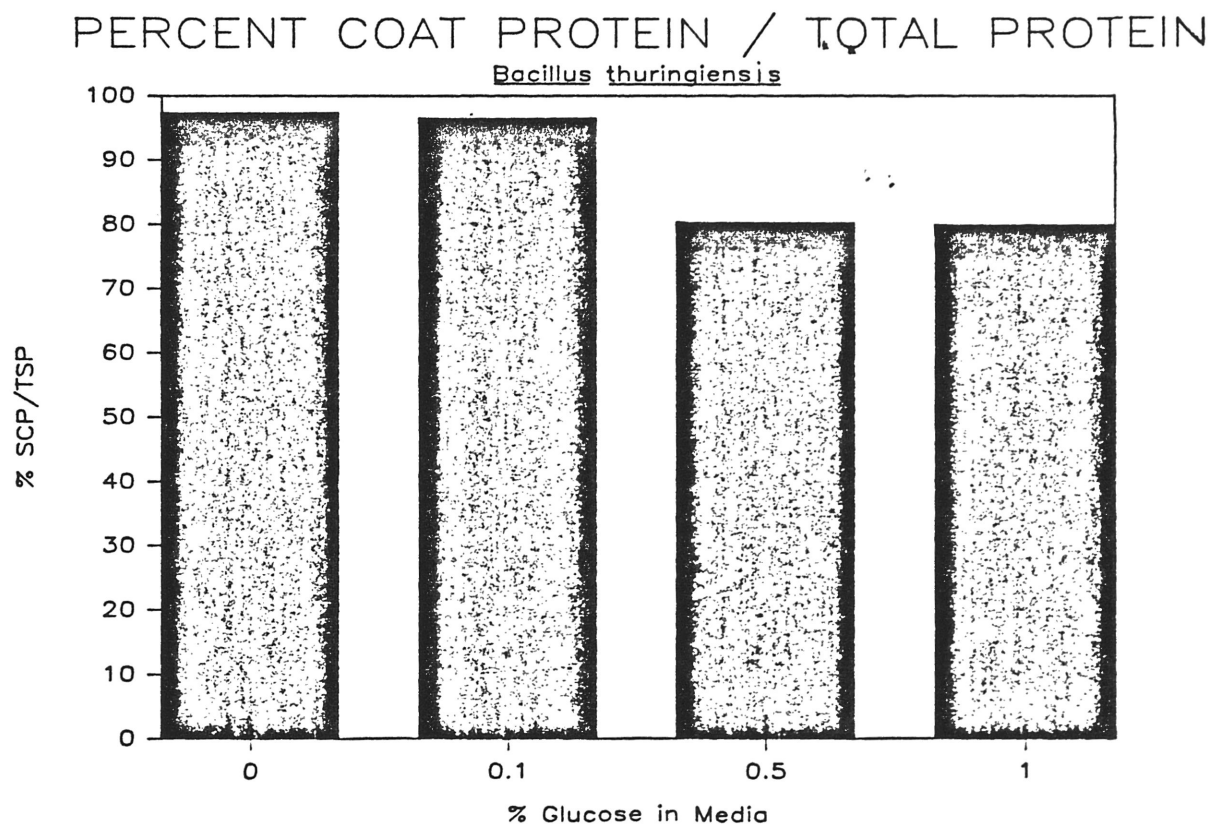

FIGURE 5. GEL ELECTROPHORESIS OF PROTEINS FROM SPORES.

Electrophoresis was done by the method of Laemmli (5). Samples from the unsupplemented, 0.1, 0.5, and 1.0% glucose-supplemented media were analyzed to compare solubilized coat protein with solubilized protein from disrupted spores.

Rf Values for the Molecular Weight Markers

Rf values	1.35	3.45	4.15	5.50	8.00	9.80
MW (kDa)	205	116	93	66	45	29

Correlation coefficient : r = 0.9946