Purification and Properties of an Extracellular Polysaccharide Containing Amino Sugars Formed by *Bacillus cereus*

By D. MIRELMAN, R. LOTAN, Yael BERNSTEIN,
H. M. FLOWERS AND N. SHARON

Department of Biophysics, Weizmann Institute of Science, Rehovoth, Israel

(Received 28 November 1972; revised 13 February 1973)

SUMMARY

A water-soluble polysaccharide containing amino sugars was isolated from the culture fluid of *Bacillus cereus* by alcohol precipitation and purified by chromatography on DEAE-cellulose followed by gel filtration. It was composed of *N*-acetylglucosamine, *N*-acetylgalactosamine and D-glucose, in the molar ratio 5:3:1. *N*-Acetylgalactosamine was found at the reducing end. The polymer gave a single peak in the analytical ultracentrifuge, corresponding to a mol. wt of 35 000.

INTRODUCTION

Glucosamine and galactosamine are the two amino sugars most commonly found in micro-organisms (Salton, 1965; Sharon, 1965). Although glucosamine is of very wide distribution, the occurrence of galactosamine in micro-organisms is rather limited. In some bacteria, galactosamine is a constituent of wall polysaccharides, lipopolysaccharides, or teichoic acids. A few microbial extracellular polysaccharides contain galactosamine. *Aspergillus parasiticus* secreted a viscous polysaccharide composed solely of galactosamine residues of which only one-third were *N*-acetylated (Distler & Roseman, 1960). Gorin & Eveleigh (1970) characterized an extracellular heteropolysaccharide produced by *Aspergillus nidulans* composed of *N*-acetylgalactosamine and D-galactose in a molar ratio of 1:1.8. Culture media of *Chondrococcus columnaris* contained a galactosaminoglycan in which half of the amino groups were not substituted (Johnson & Chilton, 1966).

We report here the isolation, purification and characterization of a water soluble heteropolymer excreted into the growth medium by *Bacillus cereus*.

METHODS

Organism. The strain used was isolated from soil in Nes-Ziona (Israel), and identified as *Bacillus cereus* (Breed, Murray & Smith, 1957).

Medium and culture. The medium contained Casamino acids, 3.0 g (Baltimore Biological Laboratories); Na₂HPO₄, 2.5 g; KH₂PO₄, 0.35 g; NaCl, 1.5 g; MgSO₄, 0.15 g; FeSO₄, 1 mg; MnSO₄, 1 mg; thiamine, 4 mg; nicotinic acid, 4 mg; and a carbon source, usually D-glucose, 5 g in 1 l of water. To study the production of polysaccharide under different growth conditions, 50 ml medium in 250 ml flasks was shaken on a rotary shaker at 200/min. For large-scale production, 1/l cultures in 6/l flasks were shaken at 80 strokes/min. Growth was monitored as absorbance of culture samples in a Klett-Summerson photoelectric colorimeter using filter no. 66 (640 to 700 nm).

Isolation of crude extracellular polysaccharide. After removal of cells by centrifugation at

7000g for 20 min, ethanol was added with mixing to the clear supernatant fluid to 70 % (v/v) and the mixture kept overnight at 5 °C. Insoluble material equal to about 10 % of the total amino sugar present in the polysaccharide was removed from the dialysed solution by centrifugation (13 000g, 10 min) and discarded. The clear supernatant fluid was lyophilized to afford the crude polysaccharide.

Ion-exchange chromatography. Crude polysaccharide (800 mg in 10 ml of water) was applied to a column (110 cm $\times$ 2.2 cm) of DEAE-cellulose (Whatman DE-52 microgranular, preswollen) which had been equilibrated with water. Elution was started with water (600 ml), then a mixing chamber containing water (1000 ml) linked to a reservoir of LiCl (0.4 M, 1000 ml) was attached to the column and a linear gradient applied. Fractions (6 ml) were collected at 18 ml/h and the elution pattern was followed by measuring the absorbance at 220 nm. Portions were analysed for neutral sugar and amino sugars. The concentration of LiCl was determined by measurement of conductivity.

Gel filtration. A portion of the major polysaccharide fraction (350 mg in 10 ml of water) from the DEAE-cellulose column was applied to a column of Sephadex G-100 (110 cm $\times$ 2.2 cm) equilibrated with water. Elution was carried out with water, and fractions (6 ml), collected at 30 ml/h, were analysed as described above.

Paper chromatography. Descending paper chromatography on Whatman no. 1 paper was carried out with the following solvent systems: A, butan-1-ol + methanol + water (3:1:1, by vol.); B, butan-1-ol + pyridine + water (6:4:3, by vol.); C, butan-1-ol + acetic acid + water (3:1:1, by vol.); D, ethyl acetate + acetic acid + formic acid + water (18:3:1:4, by vol.). Chromatographic standards were: D-glucose, D-galactose, D-mannose, D-glucosamine hydrochloride, D-galactosamine hydrochloride, D-mannosamine hydrochloride and ribitol.

The polysaccharide was hydrolysed in 2M-HCl for 4 h at 110 °C and the acid evaporated prior to application to the chromatogram. Reducing sugars and amino sugars were detected by silver nitrate (Trevelyan, Procter & Harrison, 1950) and by ninhydrin (0.5 %, w/v, in acetone) respectively. Ribitol and anhydrosorbitol were revealed by periodate-benzidine (Gordon, Thornburg & Werum, 1956).

Analytical methods. Neutral sugars were determined by the phenol-sulphuric acid method of Dubois, Gilles, Hamilton, Rebers & Smith (1956) using D-glucose as standard. The configuration of the glucose was determined in acid hydrolysates of the polysaccharide by D-glucose oxidase (Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.). Amino sugars were determined in hydrolysates (2 M-HCl under nitrogen for 4 h at 110 °C) by a modification of the Elson-Morgan method (Gardell, 1953), after removal of the acid by evaporation. Glucosamine and galactosamine in the acid hydrolysates were separated and estimated on the amino acid analyser (Beckman 120C). Uronic acids were tested for by the modified carbazole procedure (Davidson, 1966).

Infrared spectra were recorded on a Perkin-Elmer 237 spectrophotometer (pellets of 1.5 mg purified polysaccharide in 100 mg KBr). Optical rotation at 589 nm was measured on a Cary 29 instrument using the sodium D line. Molecular weight was determined using the Beckman Model E analytical ultracentrifuge at 59780 rev./min assuming a partial specific volume $\bar{V} = 0.67$.

For identification of the sugar moiety at the reducing end, the purified polysaccharide (10 mg) was dissolved in water (1.0 ml) and a solution of tritiated sodium borohydride (25 mCi, 87 mCi/mmol, Radiochemical Centre, Amersham, Buckinghamshire) in water was added. After 16 h at 4 °C, acetic acid was added to destroy excess borohydride and the solution was dialysed extensively against water. A sample of the polysaccharide containing 10^6 c.p.m. was hydrolysed (6 M-HCl, 4 h, 110 °C) and analysed according to the method of Bella

Fig. 1. Growth of *Bacillus cereus* and production of extracellular polysaccharide in 50 ml medium containing 0.5 % glucose in 250 ml flasks at 37 °C. ○ —○, Optical density (Klett units); □ —□, extracellular polysaccharide (mg/ml).

& Kim (1970) on an amino acid analyser with a radioactive flow-counter. Standards of D-galactosaminitol and D-glucosaminitol were analysed in parallel. Using these conditions D-galactosaminitol was eluted at 135 min and D-glucosaminitol at 145 min.

Total nitrogen (Steyermarck, 1961), organic and inorganic phosphorus (Chen, Toribara & Warner, 1956) and *N*-acetyl groups (Ingram, 1960) were estimated as described in the literature.

RESULTS AND DISCUSSION

Effects of growth conditions on polysaccharide production. No correlation between the growth curve of the bacterium and the production of polysaccharide was found (Fig. 1). The culture reached the stationary phase after 16 h, whereas the extracellular polysaccharide only attained a constant maximal value after 48 h. This behaviour resembles *Physarum polycephalum* (McCormick, Blomquist & Rusch, 1970); polysaccharide is released into the medium very slowly during logarithmic growth but extracellular polysaccharide increases 20-fold after the culture becomes stationary. This phenomenon may arise for one of the following reasons: (i) the polymer may be produced during the logarithmic phase, but not excreted until the stationary phase, perhaps because the permeability of the bacterial walls and membranes changes. Changes in the permeability of *Bacillus subtilis* membrane permitting competence occur only after a certain period of culture growth (Spizizen, 1958); (ii) the extracellular polysaccharide may be produced only when sporulation begins. Sporulation in *B. subtilis* begins at the end of the exponential phase of growth (14 to 16 h) and reaches a maximum after 36 to 40 h of the stationary phase (Boylan & Ensign, 1968). In our culture sporulation was first observed after 12 h of growth, at which time some polysaccharide could be detected; (iii) synthesis may be delayed until the culture reaches the stationary phase; (iv) the extracellular polysaccharide may originate by autolysis. This view can be excluded since no soluble polysaccharide could be detected in the solution when bacteria were allowed to autolyse for 24 h in buffer after reaching the stationary phase.

Polysaccharide production was maximal after 48 h at 37 °C. Using a standard glucose medium, approximately 300 mg crude polysaccharide/l was formed. No significant change in

Fig. 2. Elution pattern of amino sugar-containing polysaccharide from DEAE-cellulose column (110 cm $\times$ 2.2 cm). Elution was started with water (600 ml), then continued with a linear gradient of 0.4 M-LiCl (a mixing chamber containing 1000 ml water linked to a reservoir of 0.4 M-LiCl (1000 ml). $\bigcirc$ — $\bigcirc$, Absorbance; $\blacksquare$ — $\blacksquare$, amino sugars; $\triangle$ — $\triangle$, neutral sugar; $\bullet$ — $\bullet$, LiCl.

Table 1. Analysis of materials eluted from DEAE-cellulose and Sephadex columns

Material	Yield (mg)	Neutral sugar ($\mu\text{g/mg}$)	Amino sugar ($\mu\text{g/mg}$)	Ribitol*	Organic P ($\mu\text{g/mg}$)
Crude polysaccharide	800	95	520	+	11
Peak I DEAE-cellulose	110	82	315	—	3
Peak II DEAE-cellulose	510	70	730	—	4
Peak III DEAE-cellulose	77	240	190	+	76
Peak II after Sephadex	315	104	803	—	.

* Identified on a paper chromatogram in butan-1-ol+acetic acid+water (3:1:1, by vol.)

either the amount of polysaccharide or the amino sugar to neutral sugar ratio (5:1) was observed when glucose was replaced by sucrose, maltose, D-glucosamine, N-acetyl-D-glucosamine or beet sugar molasses. At 28 °C polysaccharide production was only 10 % of that at 37 °C, although the micro-organism concentration was 400 Klett units at both temperatures. At 45 °C, the absorbance of the medium after 48 h was 70 Klett units, and little polysaccharide could be isolated. Growth and polysaccharide production were unchanged over the range pH 6 to 9; no growth was observed below pH 5 or above pH 10.

Increase of the culture volume from 50 ml to 1 l resulted in a decrease of 50 % in polysaccharide yield, a phenomenon which we cannot explain. One-l cultures were nevertheless used for large-scale preparation of the polysaccharide.

Purification of crude polysaccharide. Crude polysaccharide (800 mg) was purified on a DEAE-cellulose column (see methods and Fig. 2). Peak I (10 mg material in an elution volume 200 to 270 ml water) was discarded. Peak II (510 mg) was eluted between 720 and 780 ml 0.02 M-LiCl, and 77 mg of more acidic material was eluted with 815 to 880 ml 0.15 M-LiCl (Peak III). The composition of these fractions is given in Table 1. The material in the

Fig. 3. Elution pattern of Peak II (obtained from DEAE-cellulose) from Sephadex G-100 column (110 cm $\times$ 2.2 cm). $\circ$ — $\circ$, Absorbance; $\blacksquare$ — $\blacksquare$, amino sugars; $\triangle$ — $\triangle$, neutral sugar.

third peak, which contained glucosamine, ribitol and phosphate, was apparently a teichoic acid. Peak II was further purified by gel filtration on Sephadex G-100 (Fig. 3). Ninety per cent of the material applied to the Sephadex column emerged in the first peak which was composed of amino sugars (80 %) and neutral sugars (10 %) (Table 1); the remaining 10 % emerged later and contained material absorbing at 280 nm (probably protein) together with small amounts of neutral sugar. Paper chromatography of hydrolysates of the purified polysaccharide revealed that glucose was the only neutral sugar and the amino sugars were glucosamine and galactosamine. All the glucose was of the D-configuration as determined by D-glucose oxidase.

On the amino acid analyser, acid hydrolysates of the crude polysaccharide and of the purified polymer gave a shoulder of material emerging after galactosamine. Treatment with acid (2 M-HCl, 4 h, 110 °C) of a control mixture of *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine and D-glucose (in the same ratio as in the hydrolysates) did not give the same shoulder and it may represent a small amount of an unidentified amino sugar. The ratio of glucosamine to galactosamine calculated from autoanalysis was 1.65:1. The amino sugars were completely *N*-acetylated since the polysaccharide did not react with ninhydrin or fluorodinitrobenzene. Total nitrogen (4.3 %) and *N*-acetyl (15.3 %) were in good agreement with the value expected for a fully acetylated polymer. Thus the purified polysaccharide apparently has the following composition: *N*-acetylglucosamine + *N*-acetylgalactosamine + D-glucose, 5:3:1. The fact that the polysaccharide is slightly retarded on DEAE-cellulose despite its apparent lack of negatively charged groups may perhaps result from hydrophobic interactions between the polysaccharide backbone and the cellulose.

Infrared spectroscopy indicated the presence of *N*-acetamido (amide bands at 1640 cm^{-1} and 1550 cm^{-1}) and no *O*-acetyl absorption. Bands characteristic for α - and β -glycosidic linkages (840 cm^{-1} and 890 cm^{-1} respectively) were also present. The purified polysaccharide

gave a single peak in the analytical ultracentrifuge and the calculated mol. wt was 35000 ($S = 2.54$; $D = 5.43 \times 10^7$). The optical rotation of the polysaccharide was $[\alpha]_D^{25} + 42.0^\circ$ (about 0.3 %, in water).

Reduction of the purified polysaccharide with sodium borohydride (3H) followed by acid hydrolysis yielded several radioactive compounds. Paper chromatography with solvent D revealed a number of slow migrating radioactive oligosaccharides and one compound which migrated as hexoaminitol. On the amino acid analyser, the radioactive hexosaminitol migrated at the same rate as a standard of galactosaminitol which clearly separated from glucosaminitol. Thus galactosamine is apparently the terminal reducing amino sugar.

The polysaccharide described in this paper is the only bacterial extracellular polysaccharide known to contain both *N*-acetylglucosamine and *N*-acetylgalactosamine in such high proportion. A similar capsular polysaccharide isolated from *Bacillus licheniformis* (Sharon, 1957) contained *N*-acetylglucosamine, *N*-acetylgalactosamine and D-galactose in a molar ratio of 1:1:2.

This work was supported by a grant from the United States Department of Agriculture, no. FG-IS-294.

REFERENCES

- BELLA, A. M., JUN. & KIM, Y. S. (1970). An improved method of separating glucosaminitol from galactosaminitol and their amino sugars on an amino acid analyser. *Journal of Chromatography* **51**, 314-315.
- BREED, R. S., MURRAY, E. G. D. & SMITH, N. R. (1957). *Bergey's Manual of Determinative Bacteriology*, 7th edn, pp. 617-618. Baltimore: Williams and Wilkins Co.
- BOYLEN, C. W. & ENSIGN, J. C. (1968). Ratio of teichoic acid and peptidoglycan in cell walls of *Bacillus subtilis* following spore germination and during vegetative growth. *Journal of Bacteriology* **96**, 421-427.
- CHEN, P. S., TORIBARA, T. Y. & WARNER, H. (1956). Microdetermination of phosphorus. *Analytical Chemistry* **28**, 1756-1758.
- DAVIDSON, E. A. (1966). Analysis of sugars found in mucopolysaccharides. In *Methods in Enzymology*, vol. VIII, pp. 52-55. Edited by E. F. Neufeld and V. Ginsburg. New York: Academic Press.
- DISTLER, J. J. & ROSEMAN, S. (1960). Galactosamine polymers produced by *Aspergillus parasiticus*. *Journal of Biological Chemistry* **235**, 2538-2541.
- DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**, 350-356.
- GARDELL, S. (1953). Separation on Dowex 50 ion exchange resin of glucosamine and galactosamine and their quantitative determination. *Acta chemica scandinavica* **7**, 207-215.
- GORDON, H. T., THORNBURG, W. & WERUM, L. N. (1956). Rapid paper chromatography of carbohydrates and related compounds. *Analytical Chemistry* **28**, 849-855.
- GORIN, P. A. J. & EVELEIGH, D. E. (1970). Extracellular 2-acetamido-2-deoxy-D-galacto-D-galactan from *Aspergillus nidulans*. *Biochemistry* **9**, 5023-5027.
- INGRAM, G. (1960). Determination of acetyl and benzoyl groups. In *Comprehensive Analytical Chemistry 1B*, pp. 634-651. Edited by C. L. Wilson and D. W. Wilson.
- JOHNSON, J. L. & CHILTON, W. S. (1966). Galactosamine glycan of *Chondrococcus columnaris*. *Science, New York* **152**, 1247-1248.
- MCCORMICK, J. J., BLUMQUIST, J. C. & RUSCH, H. P. (1970). Isolation and characterization of an extracellular polysaccharide from *Physarum polycephalum*. *Journal of Bacteriology* **104**, 1110-1118.
- SALTON, M. R. J. (1965). Chemistry and function of amino sugars and derivatives. *Annual Review of Biochemistry* **34**, 143-174.
- SHARON, N. (1957). Polysaccharide containing amino sugar from *Bacillus subtilis*. *Nature, London* **179**, 919.
- SHARON, N. (1965). Distribution of amino sugars in microorganisms, plants and invertebrates, pp. 1-45. In *The Amino Sugars 11A*. Edited by R. W. Jeanloz and E. A. Balazs. New York: Academic Press.
- SPIZIZEN, J. (1958). Transformation of a biochemically deficient strain of *Bacillus subtilis* by deoxyribonucleate. *Proceedings of the National Academy of Sciences of the United States of America* **44**, 1072-1078.
- STEYERMARK, A. L. (1961). In *Quantitative Organic Microanalysis*, pp. 151-187. (Edited by A. L. Steyermark). New York: Academic Press.
- TREVELYAN, W. E., PROCTER, D. P. & HARRISON, J. S. (1950). Detection of sugars on paper chromatograms. *Nature, London* **166**, 444-445.