# ISOLATION OF GLUCOSAMINYL-β(1-4)-MURAMIC ACID AND PHOSPHORIC ACID ESTER OF THIS DISACCHARIDE FROM ACID HYDROLYSATES OF PEPTIDOGLYCAN OF BACILLUS CEREUS AHU 1356 CELL WALLS

Yoshio Araki, Tokuji Nakatani, Ryu Makino,
Hideyuki Hayashi, and Eiji Ito

Department of Chemistry, Faculty of Science,
University of Hokkaido, Sapporo, Japan

Received January 4, 1971

Summary. Glucosaminyl-β(1-4)-muramic acid and glucosaminyl-β(1-4)-muramic acid-6-phosphate were isolated from acid hydrolysates of Bacillus cereus AHU 1356 cell wall peptidoglycan. The yield of the disaccharide suggests that the majority of the amino groups of the glucosamine residues in this peptidoglycan are nonsubstituted.

Analysis in an autoanalyzer after direct acid hydrolysis showed that the cell walls from Bacillus cereus strain AHU 1356 and the peptidoglycan preparation from these cell walls contain extremely small amounts of muramic acid, and that only a minor proportion of the phosphorus in this peptidoglycan is accounted for by muramic acid-6-phosphate (1). The structure of this unusual peptidoglycan, which is highly resistant to lysozyme, has been studied in terms of analysis of the fragments obtained by acid hydrolysis. The present communication describes the isolation and characterization of the disaccharide glucosaminyl-β(1-4)-muramic acid and a phosphoric acid ester of this disaccharide from the peptidoglycan hydrolysates.

Methods. Bacillus cereus strain AHU 1356, furnished by Dr. Y. Sasaki, University of Hokkaido, were grown in a medium containing 0.5 % yeast extract, 0.5 % peptone, 0.5 % meat extract, 0.2 % glucose, and 0.2 % $K_2HPO_4$ at pH 7.2, and harvested at 60 % maximal growth. The procedure for preparing cell walls, which is a modification of the method of Cummins and Harris (2), involved

disruption of cells in a sonic oscillator with glass beads for 7 min, heating of crude cell walls at 100° for 20 min, and successive treatments with ribonuclease, trypsin, and 0.5 % sodium dodecyl sulfate. Peptidoglycan was prepared by collecting the insoluble fraction after heating the cell walls in 0.1 N HCl at 60° for 12 hours (3).

Results. Analysis in an autoanalyzer of the peptidoglycan after hydrolysis in 4 N HCl for 12 hours at 95° indicated that it contains alanine, glutamic acid, α,ε-diaminopimelic acid, muramic acid, and glucosamine in a molar ratio of 1.8:1:1:0.2:2. Large-scale chromatography of the acid hydrolysate from peptidoglycan (426 mg) on a column of Dowex 50W gave two main peaks of reducing material (approximately 220 μmoles in Peak 2 and 250 μmoles in Peak 5 as glucosamine) as shown in Fig. 1.

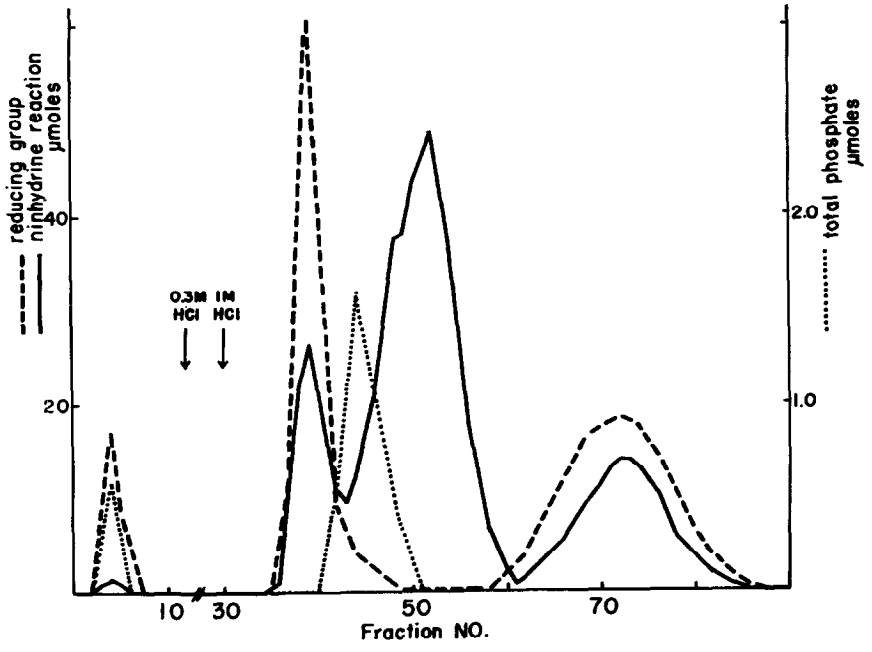


Fig. 1. Ion exchange chromatography of the acid hydrolysate from peptidoglycan. 426 mg of peptidoglycan was hydrolyzed in 60 ml of 4 N HCl at 100° for 4 hours. The hydrolysate after removal of HCl by lyophilization was applied to a column (3 X 26 cm) of Dowex 50W-X8, $H^+$ form. The column was eluted with water, 0.3 N, 1 N, and 2 N HCl. Fractions of 15 ml were collected. Ninhydrin-positive material, ——; reducing material (4), – – –; and total phosphate (5), ·····.

Peak 2 comprised glucosamine and muramic acid, while Peak 5 contained a single component which had a reducing group and was ninhydrin-positive. The reducing material in Peak 5 (S-1) was precipitated from a concentrated solution by adding acetone (yield 35.6 mg. Anal. Calc. for $C_{15}H_{30}N_2O_{11}Cl_2$: C, 37.12; H, 6.23; N, 5.77. Found: C, 36.03; H, 6.57; N, 5.59 %). A part of S-1 (20 mg) was treated in 1.5 ml of a solution containing 150 mg of $NaHCO_3$ and 250 µl of acetic anhydride at 0° overnight (6). After desaltation on columns of Dowex 50W ($H^+$ form) and Dowex 1 (acetate form), the N-acetyl derivative was precipitated with acetone and ethanol from a concentrated solution (yield 17 mg).

N-Acetylated S-1, which on hydrolysis, gave glucosamine and muramic acid in a molar ratio of 1:1, was in agreement with authentic samples of N-acetylglucosaminyl-β(1-4)-N-acetylmuramic acid, kindly given by Dr. Jeanloz and by Dr. Sharon, in its behavior on paper chromatography (Table 1), the Morgan-Elson reaction (8), and on digestion with β-N-acetylglucosaminidase (Table 2). Reduction of N-acetylated S-1 with $NaBH_4$ followed by acid hydrolysis and estimation in the autoanalyzer gave glucosamine and muramicitol. Treatment of original S-1 with fluorodinitrobenzene followed by hydrolysis and paper chromatography yielded DNP-glucosamine and DNP-muramic acid. These results are consistent with the structure glucosaminyl-β(1-4)-muramic acid for S-1.

On the treatment in 0.1 N HCl at 60°, more than 80 % of the organic phosphate in the walls was retained in the insoluble peptidoglycan preparation. After hydrolysis in 4 N HCl, most of the phosphate recovered was found in Peak 3 as shown in Fig. 1. The components of Peak 3 were again applied to a column of Dowex 50W, and eluted in a gradient of HCl giving a peak of phosphate which coincided with a peak of ninhydrin-positive

TABLE 1

Relative Rate of Migration on Paper Chromatography

| Samples | Solvent 1 | Solvent 2 | Solvent 3 |
|---|---|---|---|
| | $R_{alanine}$* | $R_{alanine}$* | $R_{alanine}$* |
| S-1 | 0.55 | 0.25 | 0.90 |
| S-2 | 0.15 | 0.00 | 0.56 |
| S-2, after phosphatase | 0.55 | 0.25 | 0.90 |
| | $R_{GlcNAc}$** | $R_{GlcNAc}$** | $R_{GlcNAc}$** |
| N-Acetylated S-1 | 0.66 | 0.40 | 0.80 |
| N-Acetylated S-2 | - | 0.05 | - |
| N-Acetylated S-2, after phosphatase | 0.66 | 0.40 | - |
| GlcNAc-$\beta$(1-4)-MurNAc (Jeanloz) | 0.66 | 0.40 | 0.80 |

Descending paper chromatography was carried out on Toyo No. 50 filter paper. Solvent 1, n-butanol-acetic acid-water (3:1:1); Solvent 2, n-butanol-pyridine-acetic acid-water (6:4:0.3:3); Solvent 3, isobutyric acid-0.5 N $NH_3$ (1.5:1).
* Compounds were detected by ninhydrin.
** Compounds were detected by fluorescence (7).

material and with that of reducing material. The ninhydrin-positive material in this peak was further purified by repeating paper chromatography in n-butanol-acetic acid-water (2:1:1) until a single spot of ninhydrin-positive material was obtained. The yields of the isolated compound (S-2) were 1.63, 2.60, and 1.40 μmoles as assayed for total phosphate (5), reducing groups (calculated as N-acetylglucosamine) (4), and ninhydrin-positive material (calculated as leucine), respectively.

Three quarters of S-2 was acetylated with acetic anhydride in a $NaHCO_3$ solution, and the product was purified by chromatography on columns of Dowex 50 (X8, $H^+$ form) and Dowex 2 (X8, acetate form) yielding N-acetylated S-2 (reducing groups, 1.28 μmoles; total phosphate, 1.02 μmoles).

TABLE 2

Relative Molar Color Yields in Modified Morgan-Elson Reaction

| Sample | 3 min | 35 min |
|---|---|---|
| N-Acetylated S-1 | 0.032 | 0.387 |
| N-Acetylated S-1, after β-N-acetylglucosaminidase | 1.90 | |
| N-Acetylated S-2 | 0.093 | 0.508 |
| GlcNAc-β(1-4)-MurNAc (Jeanloz) | 0.030 | 0.359 |
| N-Acetylglucosamine | 1.00 | 1.00 |

The modified Morgan-Elson reaction was carried out with 3 or 35 min of a heating time in borate (8). The color yields are given relative to an equimolar amount of N-acetylglucosamine tested under the identical conditions. The value for N-acetylated S-1 was calculated assuming it as GlcNAc-MurNAc. The value for N-acetylated S-2 was calculated from its phosphorus content. β-N-Acetylglucosaminidase was prepared from pig epididymis (10).

N-Acetylated S-2 was identified as N-acetylglucosaminyl-β(1-4)-N-acetylmuramic acid-6-phosphate from the following evidence: On analysis in the autoanalyzer after hydrolysis in 4 N HCl at 95° for 6 hours, it gave peaks corresponding to those of muramic acid-6-phosphate (1) and glucosamine, indicating the phosphate linkage on the muramic acid residue. It resembles N-acetylglucosaminyl-β(1-4)-N-acetylmuramic acid in the Morgan-Elson reaction (Table 2). Treatment with alkaline phosphatase from E. coli yielded an oligosaccharide, which was coincident with N-acetylglucosaminyl-β(1-4)-N-acetylmuramic acid in the behavior on paper chromatography (Table 1), and in other properties. Since 4-O of the muramic acid residue in N-acetylated S-2 is involved in the linkage with the glucosamine residue, the phosphate group should be linked to 6-O of the muramic acid residue. This compound gave a very weak fluorescence on paper under the conditions described by Sharon *et al.* (7), requiring

more than 0.2 μmole for its detection. While, for the detection of the product from the phosphatase treatment, 0.02 μmole was sufficient.

On treatment with alkaline phosphatase, original S-2 yielded a ninhydrin-positive reducing material, which was in agreement with S-1, glucosaminyl-β(1-4)-muramic acid, on paper chromatograms (Table 1). The above data are consistent with the structure glucosaminyl-β(1-4)-muramic acid-6-phosphate for S-2.

Discussion. N-Acetylglucosaminyl-β(1-4)-N-acetylmuramic acid is thought as a repeating unit of peptidoglycans from various bacterial walls. In the present experiments, a significant part of these repeating units were recovered as the disaccharide after hydrolysis under the conditions which ordinarily give rise to complete degradation into monosaccharides. The unusually high yield of the disaccharide strongly suggests that the glycosidic linkages in the corresponding proportion of the disaccharide units are acid-resistant probably because of the absence of the N-acetyl groups from their glucosamine residues. In accordance with this suggestion, analysis of the peptidoglycan which had been N-acetylated prior to acid hydrolysis, gave a molar ratio of 1:1:1.2, the composition of a typical peptidoglycan, for glutamic acid, muramic acid, and glucosamine. The yield of the phosphoric acid ester of this disaccharide appears to be explained similarly, and provides evidence for the phosphate linkages with 6-O of some muramic acid residues in the peptidoglycan.

In succeeding papers will be reported further evidence for the occurrence of the non-N-substituted glucosamine residues in peptidoglycans and the relationship between the content of these residues and the resistance of peptidoglycans to lysozyme (9).

Acknowledgements. The authors are grateful to Dr. Y. Sasaki for strain of B. cereus and Dr. R. W. Jeanloz and Dr. R. N. Sharon

for samples of N-acetylglucosaminyl-$\beta$(1-4)-N-acetylmuramic acid.

## REFERENCES


1. Liu, T-Y., and Gotschlich, E. C., J. Biol. Chem., 242, 471 (1967).
2. Cummins, C. S., and Harris, H., J. Gen. Microbiol., 14, 583 (1956).
3. Knox, K. W., and Hall, E. A., Biochem. J., 96, 302 (1965).
4. Park, J. T., and Johnson, M., J. Biol. Chem., 181, 149 (1949).
5. Lowry, O. H., and Roberts, N. R., J. Biol. Chem., 207, 1 (1954).
6. Heymann, H., Manniello, J. M., and Barkulis, S. S., J. Biol. Chem., 239, 2981 (1964).
7. Sharon, N., and Seifter, S., J. Biol. Chem., 239, PC2398 (1964).
8. Sharon, N., Osawa, T., Flowers, H. W., and Jeanloz, R. W., J. Biol. Chem., 241, 223 (1966).
9. Araki, Y., Nakatani, T., Hayashi, H., and Ito, E., Biochem. Biophys. Res. Commun., submitted for publication.
10. Findlay, J., and Levvy, G. A., Biochem. J., 77, 170 (1960).