OCCURRENCE OF NON-N-SUBSTITUTED GLUCOSAMINE RESIDUES IN LYSOZYME-RESISTANT PEPTIDOGLYCAN FROM BACILLUS CEREUS CELL WALLS

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

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

Received January 4, 1971

Summary. Three strains of Bacillus cereus have cell walls completely insensitive to lysozyme. The peptidoglycans from these cell walls are unique in their large contents of glucosamine residues with a free amino group. These lysozyme-resistant cell walls were converted to a sensitive form in terms of N-acetylation with acetic anhydride. This result indicates that the resistance of these cell walls to lysozyme is ascribable to the occurrence of the non-N-substituted glucosamine residues in the peptidoglycans.

The cell walls from several strains of Bacillus cereus are highly resistant to the treatment with lysozyme. Resistance of cell wall peptidoglycan to lysozyme has been accounted for by the presence of O-acetyl groups (1), the attachment of accessory polymers such as teichoic acid and polysaccharides (2), and the occurrence of free amino groups (3) and a high degree of cross linking in the peptide portion (4). After elimination of these factors, however, the peptidoglycan preparations from B. cereus still remain insensitive to lysozyme. Therefore, structure of these peptidoglycans has been studied in order to determine the factor responsible for their resistance to lysozyme. The present communication describes that in the peptidoglycan from B. cereus cell walls, a considerable proportion of the glucosamine residues has a free, non-N-acetylated amino group, and that the resistance of the cell walls from B. cereus to lysozyme should be attributed to the occurrence of these free amino groups.

Materials and Methods. The cells of B. cereus strains AHU 1355,

1356, and 1030 furnished by Dr. Y. Sasaki, University of Hokkaido, and another strain (strain T) furnished by Dr. J. L. Strominger, Harvard University, were grown in a medium containing 0.5 % peptone, 0.5 % yeast extract, 0.5 % meat extract, 0.2 % glucose, and 0.2 % K_2HPO_4 at pH 7.2. The cells of Micrococcus lysodeikticus ATCC 4698 were grown in a medium containing 2 % peptone, 0.5 % yeast extract, and 0.5 % NaCl. The cells harvested at 60 % maximal growth were disrupted by treating in a sonic oscillator with glass beads and cell walls were separated according to the method of Cummins and Harris (5) with a small modification. De-O-acetylation of cell walls was carried out by treating samples in 0.01 N NaOH for 1 hour at 37°. Peptidoglycan was prepared by collecting the insoluble fraction after heating cell walls in 0.1 N HCl at 60° for 12 hours (6). Components of the polysaccharide portion were removed by this treatment. N-Acetylation of cell walls and peptidoglycan was performed by treating several milligrams of samples in 0.6 ml of a solution containing 45 mg of $NaHCO_3$ and 20 µl of acetic anhydride at 0° overnight (7). Then, the insoluble material was collected, washed twice in water, and lyophilized. Reducing groups were assayed by the method of Park and Johnson (8) with glucose as a reference standard. Flavobacterium L-11 enzyme was kindly given by Dr. S. Kotani, Osaka University (9) and used as muramyl L-alanine amidase after purification by DEAE-cellulose column chromatography.

Results. The cell walls, de-O-acylated cell walls, and peptidoglycan prepared from B. cereus strains AHU 1355, 1356, and T were all insensitive to the treatment with lysozyme, while the cell walls from AHU 1030 were digested at a modest rate as shown in Fig. 1, A. Digestion with muramyl L-alanine amidase brought about solubilization of the cell walls and peptidoglycan from the

lysozyme-resistant strains. The solubilized glycan portion, which was separated from the peptide fragments by dialysis, was found to be still resistant to lysozyme as shown in Fig. 1, B, Curve 1.

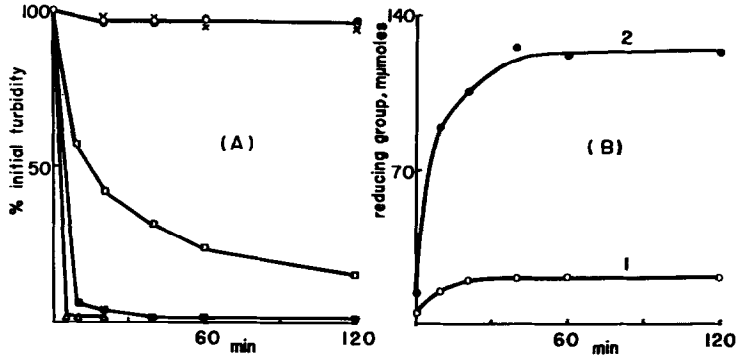

Fig. 1. A. Digestion of cell wall preparations with lysozyme as estimated by turbidity. 300 μg of cell walls, de-O-acetylated cell walls, or peptidoglycan were incubated at 37° in a suspension containing 40 mM Tris-Cl, pH 7.2, 15 μg of lysozyme (Sigma), and 0.5 % sodium azide in a final volume of 370 μl. The absorbance at 500 mμ is plotted as per cent of the initial value. O, cell walls; ×, de-O-acetylated cell walls; ●, peptidoglycan from B. cereus AHU 1356; □, cell walls from B. cereus AHU 1030; and Δ, cell walls from M. lysodeikticus ATCC 4698. The results obtained with preparations from B. cereus AHU 1355 and T were essentially the same as those with AHU 1356.

B. Digestion of muramyl L-alanine amidase-treated cell walls with lysozyme as estimated by reducing group liberation. 20 mg of B. cereus AHU 1356 cell walls were digested with excess muramyl L-alanine amidase at 37° in 20 ml of 10 mM Tris-Cl, pH 8.2 until the absorbance at 500 mμ of the reaction mixture decreased to 7 % of the initial value. The reaction mixture was centrifuged at 13,000 rpm for 45 min and the supernatant was dialyzed against water overnight. Equal aliquots (1/30) of the nondialyzable glycan fraction were digested with lysozyme (10 μg) before and after N-acetylation, and the increase of reducing groups was estimated. Curve 1, before N-acetylation; Curve 2, after N-acetylation.

The first indication about the factor responsible for the resistance of these cell walls to lysozyme was provided by the isolation of the disaccharide glucosaminyl-β(1-4)-muramic acid from acid hydrolysates of the peptidoglycan of AHU 1356 cell walls (10). The yield of this disaccharide accounts for over one

half of the amino sugar content in the peptidoglycan. The unusual yield of this disaccharide suggests that the majority of the β-glucosaminide linkages are acid-resistant probably because of the absence of the N-acetyl groups from the glucosamine residues. On the other hand, the muramic acid residues in the peptidoglycan appear to be almost fully N-acetylated since the alternative fragment muramyl-β(1-4)-glucosamine was not found in the acid hydrolysates.

The occurrence of the non-N-substituted glucosamine residues was confirmed by the treatment of the cell walls with fluorodinitrobenzene followed by hydrolysis and separation of the DNP derivatives on two-dimensional paper chromatograms as shown in Table 1. Significant amounts of DNP-glucosamine were given from the lysozyme-resistant cell walls of AHU 1355, 1356, and T, while a much smaller amount from the lysozyme-sensitive cell walls of AHU 1030. Similar results were obtained with the peptidoglycan preparations from these cell walls, too. In the content of diaminopimelic acid with a free amino group, on the other hand, any significant difference was not found among these strains. The parallel relationship between the content of the non-N-substituted glucosamine residues and the ability of resisting to lysozyme strongly suggests that the presence of the free amino groups in the glucosamine residues is responsible for the resistance of these cell walls to lysozyme.

Further evidence compatible with this suggestion was provided by conversion of the lysozyme-resistant preparations into sensitive ones by means of N-acetylation. After N-acetylation, thus, cell walls (Fig. 2), peptidoglycan, and muramyl L-alanine amidase-treated peptidoglycan (Fig. 1, B, Curve 2) were all degraded rapidly and completely on lysozyme treatment as indicated either by measuring the turbidity reduction, or by assaying the

TABLE 1

Determination of Amino Acids and DNP Derivatives in Hydrolysates of Dinitrophenylated Cell Walls

Compounds	Cell walls			
	AHU 1355	AHU 1356	T	AHU 1030
Glutamic acid	358	392	419	394
Diaminopimelic acid	251	258	286	213
Mono-DNP-diamino-pimelic acid	143	147	157	143
DNP-glucosamine	251	260	264	59

Cell walls (3 mg), treated at 30° for 5 hours in a mixture containing 0.5 ml of 5 % 1-fluoro-2,4-dinitrobenzene in ethanol, 0.5 ml of 4 % trimethylamine, and 0.5 ml of ethanol, were hydrolyzed in 4 N HCl at 95° for 12 hours, and the products were separated by two-dimensional paper chromatography in n-butanol-pyridine-water (6:4:3) and in 1.5 M potassium phosphate, pH 6. Amino acids were assayed by the ninhydrin reaction on paper (11), and DNP derivatives by measuring the absorbance at 360 mμ after extraction with 40 mM NH_4OH. The results are expressed as mμmoles per mg of walls.

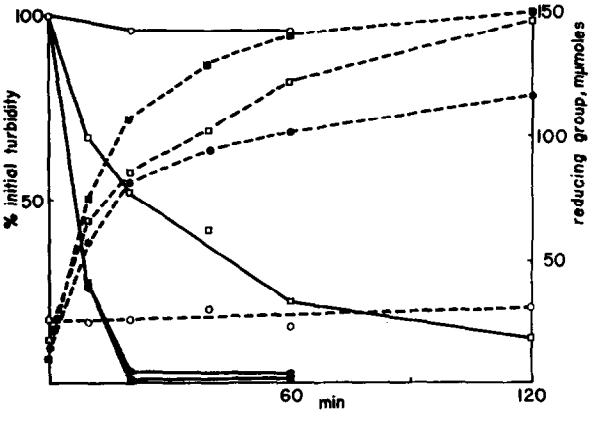

Fig. 2. Digestion of N-acetylated cell walls with lysozyme. Conditions for digestion and assay were the same as those in Fig. 1. Solid lines, turbidity; dashed lines, reducing groups liberated. ○, cell walls from B. cereus AHU 1356; ●, N-acetylated cell walls from AHU 1356; □, cell walls from AHU 1030; ■, N-acetylated cell walls from AHU 1030.

reducing group liberation.

Discussion. It seems to be believed that the amino sugar residues in peptidoglycan are fully N-acylated, although Mirelman and Sharon have provided evidence that some muramic acid residues are non-N-substituted in M. lysodeikticus cell walls (12). Acetylation of some bacterial cell walls with acetic anhydride has been found to increase their sensitivity to lysozyme, and blocking of the free amino groups of the peptide portion in the peptidoglycan has been considered to account for the lysozyme-susceptibility induced (3, 13). The evidence described above indicates that a predominant proportion of glucosamine residues in the lysozyme-resistant cell wall peptidoglycans from B. cereus have free amino groups. The resistance of these peptidoglycans to lysozyme is consistent with the function of the acetamido groups of the glucosamine residues which has been proposed on the basis of the X-ray crystallographic model of lysozyme in regard to the reaction mechanism of this enzyme (14). Therefore, it becomes of interest to determine whether the non-N-substituted glucosamine residues are of general occurrence in bacterial cell wall peptidoglycans, or not.

Acknowledgements. The authors wish to thank Dr. Y. Sasaki and Dr. J. L. Strominger for strains of B. cereus, and Dr. S. Kotani for Flavobacterium L-11 enzyme.

REFERENCES

1. Brumfitt, W., Wardlaw, A. C., and Park, J. T., Nature, 181, 1783 (1958).
2. Krause, R. M., and McCarty, M., J. Exp. Med., 114, 127 (1961).
3. Perkins, H. R., Biochem. J., 95, 876 (1965).
4. Strominger, J. L., and Ghuysen, J-M., Science, 156, 213 (1967).
5. Cummins, C. S., and Harris, H., J. Gen. Microbiol., 14, 583 (1956).

6. Knox, K. W., and Hall, E. A., Biochem. J., 96, 302 (1965).
7. Heymann, H., Manniello, J. M., and Barkulis, S. S., J. Biol. Chem., 239, 2981 (1964).
8. Park, J. T., and Johnson, M., J. Biol. Chem., 181, 149 (1949).
9. Kato, K., and Kotani, S., Bikin J., 5, 155 (1962).
10. Araki, Y., Nakatani, T., Makino, R., Hayashi, H., and Ito, E., Biochem. Biophys. Res. Commun., submitted for publication.
11. Primosigh, J., Pelzer, H., Maas, D., and Weidel, W., Biochim. Biophys. Acta, 46, 68 (1961).
12. Mirelman, D., and Sharon, N., J. Biol. Chem., 242, 3414 (1967).
13. Shockman, G. D., Thompson, J. S., and Conover, M. J., Biochemistry, 6, 1054 (1967).
14. Phillips, D. C., Proc. Nat. Acad. Sci. U. S., 87, 484 (1967).