ACTION OF LYSOZYME ON OLIGOSACCHARIDES FROM PEPTIDOGLYCAN N-UNACETYLATED AT GLUCOSAMINE RESIDUES

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Summary. On N-acetylmuramyl-L-alanine-amidase treatment followed by lysozyme digestion, peptidoglycan N-unacetylated partially at glucosamine residues yielded three oligosaccharides with N-unacetylated glucosamine residues, GlcN-MurNAc-GlcNAc-MurNAc*, GlcN-MurNAc-GlcN-MurNAc-GlcNAc-MurNAc, and GlcNAc-MurNAc-GlcN-MurNAc-GlcNAc-MurNAc. Lysozyme can not hydrolyze the former two saccharides, whereas it cleaves the latter one into GlcNAc-MurNAc and GlcN-MurNAc-GlcNAc-MurNAc. This confirms requirement for the acetamido group of the N-acetylglucosamine in the interaction with subsite C of lysozyme.

The X-ray crystallographic model of lysozyme (1, 2) predicts importance of the acetamido groups in the binding and catalysis of substrates by lysozyme. Although various data have accumulated in supporting the predicted participation of the acetamido groups in the formation of nonproductive complex between substrates and lysozyme (1-4), there has been disagreement as to the requirement for the acetamido groups in the catalytic process (3-7). In previous papers, we described that considerable proportions of glucosamine residues have free amino groups in cell wall peptidoglycan from Bacillus cereus (8) and other bacilli (9), and that resistance of these peptidoglycan preparations to lysozyme depends on the extent of N-unacetylation at N-acetylglucosamine residues. Therefore, lysozyme digestion of peptidoglycan N-unacylated to a proper extent at N-acetylglucosamine residues is expected to yield oligosaccharides, which resist further digestion on account of the occurrence of the free amino

* The abbreviations used are: GlcN, glucosaminyl; MurN, muramyl.

groups in particular glucosamine residues of the molecules. Studies on the structure of such oligosaccharides and on their reactivity as substrate were undertaken to obtain more information about the role of the acetamido groups in catalytic hydrolysis of natural substrates by lysozyme.

Materials and Methods. The peptidoglycan, in which the majority of glucosamine residues had free amino groups, was prepared from cell walls of Bacillus cereus AHU 1356 as described previously (8). Hen's egg-white lysozyme, N-acetylmuramyl-L-alanine amidase, and exo-β-N-acetylglucosaminidase were the same as those described previously (8). Authentic disaccharide GlcNAc-β(1→4)-MurNAc was kindly given by Dr. R. W. Jeanloz. Tetrasaccharide GlcNAc-MurNAc-GlcNAc-MurNAc was prepared from Micrococcus lysodeikticus cell walls as described by Sharon et al. (10). Amino acids and amino sugars were analyzed in a Shibata AA100 autoanalyzer. Prior to the analysis, oligosaccharides were hydrolyzed in 4 N HCl for 4 hours at 100° in sealed tubes. From these hydrolysates, N-unsubstituted glucosamine residues in the oligosaccharides were recovered as a disaccharide GlcN-MurN which could be assayed in the analyzer as described previously (8). Analysis was also carried out with the hydrolysates of samples which had been reduced with $NaBH_4$ as described by Tipper et al. (11). The glucosamine with a free amino group was determined by dinitrophenylation techniques (8). The values of N-unsubstituted glucosamine were corrected for the loss during analysis using a factor (1.43) calculated from analysis of GlcN-MurN (9). Reducing groups were determined by the method of Park and Johnson (12) with N-acetylglucosamine as a reference standard. N-Acetylation of oligosaccharides and assay of N-acetyl groups were carried out as described previously (8).

Results and Discussion. The peptidoglycan (0.9 g) containing 574 nmoles of N-unacetylated glucosamine per mg was treated with 5 ml of acetic anhydride at 25° for 18 hours in 100 ml of 50 mM potassium phosphate, pH 7.0. The resulting material, which contained 384 nmoles of N-unacetylated glucosamine per mg, was incubated with 1800 units of N-acetylmuramyl-L-alanine amidase at 38° in 1800 ml of 10 mM Tris-Cl, pH 8.2 until the turbidity of the mixture was reduced to 5 % of the initial value. The clarified mixture was dialyzed against distilled water overnight. The non-dialyzable fraction was digested with 180 mg of lysozyme in 450 ml of 50 mM Tris-Cl, pH 7.2 for 18 hours at 37°, and then concentrated to 12 ml. One-third portion of the digest was subjected to chromatography on a column of Sephadex G-25 (1.5 X 96 cm) which was connected with a column of Sephadex G-50 (1.5 X 98 cm). Reducing material resolved into three peaks as shown in Fig. 1. The fractions under Peak B from three columns were pooled, concentrated, and subjected to chromatography on a Sephadex G-25 column (1.5 X 96 cm). Two major peaks of reducing material (Peaks B-1 and B-2) were individually pooled and further fractionated. On paper chromatography in 1-butanol-acetic acid-water (2:1:1), Peak B-1 gave seven bands (B-1-1 to B-1-7), which reacted as amino sugar by the method of Sharon et al. (13) and were numbered in order of increasing R_F values, while Peak B-2 gave five bands (B-2-1 to B-2-5). Main components of Fractions B-1-5, B-1-7, and B-2-5 were further purified by paper chromatography in the same solvent and by paper electrophoresis in 80 mM pyridine-45 mM acetic acid, pH 5.5, giving Compounds B-1-5-1, B-1-7-1, and B-2-5-1, respectively. On the similar procedure, Fraction B-2-4 was separated into Compounds B-2-4-1 and B-2-4-2. The purified compounds were found to migrate as single spots on

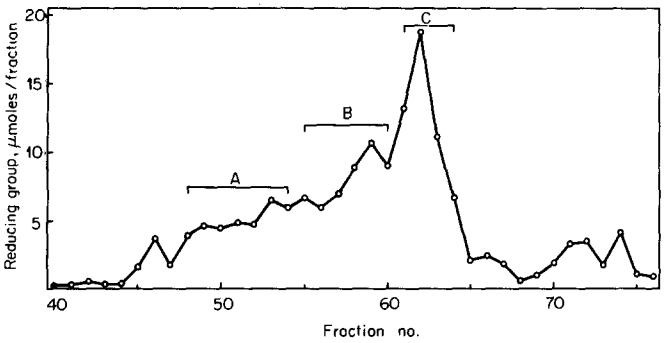

Fig. 1. Gel filtration of lysozyme digest of peptidoglycan. Elution was carried out with water and fractions of 4 ml were collected.

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paper chromatography in several solvents and on paper electrophoresis. The migration distances relative to that of N-acetylglucosamine on paper chromatography in 1-butanol-acetic acid-water (25:6:25) were: B-2-5-1, 0.75; B-2-4-2, 0.45; B-2-4-1, 0.34; B-1-7-1, 0.20; B-1-5-1, 0.12; N-acetylated B-1-5-1, 0.25. The migration distances (in cm) on paper electrophoresis (50 min, 118 volts/cm) in pyridine-acetate, pH 5.5 were: B-2-5-1, 10.4; B-2-4-2, 9.0; B-2-4-1, 4.1; B-1-7-1, 6.3; B-1-5-1, 3.6; N-acetylated B-1-5-1, 9.5.

B-2-4-2 was identified as $(GlcNAc\text{-}MurNAc)_2$ on the basis of comparison with the reference sample, while B-2-5-1 was identified as MurNAc-GlcNAc-MurNAc from the analytical data (Table 1) and its coincidence on paper chromatograms with an oligosaccharide which was formed on digestion of $(GlcNAc\text{-}MurNAc)_2$ with β-N-acetylglucosaminidase. B-2-4-1, B-1-7-1, and B-1-5-1, which showed positive reaction with ninhydrin on paper, were characteristic in that they contained glucosamine residues with free amino groups. On N-acetylation, B-2-4-1 gave an oligosaccharide coincident with $(GlcNAc\text{-}MurNAc)_2$, whereas B-1-7-1 and B-1-5-1 gave a compound which migrated on paper with the same R_F values as those

Table 1. Analysis of isolated compounds and reduction products

Compound	Composition of hydrolysates[a]			Yield[b]	Reducing power[c]	N-Acetyl group[d]	N-Unacylated glucosamine[d]
	GlcN	MurN	GlcN-MurN				
B-2-5-1	1.00	1.84	0.09	0.84	1.04		0.0
Reduced B-2-5-1	1.00	0.88	0.08				
B-2-4-2	1.00	0.99	0.08	1.38	1.28	4.48	0.0
Reduced B-2-4-2	1.00	0.54	0.00				
B-2-4-1	1.00	1.03	0.94	2.49	0.92	2.80	0.84
Reduced B-2-4-1	1.00	0.14	0.98				
B-1-7-1	1.00	0.98	0.52	2.28	1.09	5.37	0.87
Reduced B-1-7-1	1.00	0.49	0.52				
B-1-5-1	1.00	0.91	2.15	6.03	0.74	4.35	1.84
Reduced B-1-5-1	1.00	0.18	2.31				

[a] The values are expressed in molar ratios assuming glucosamine as 1.00. The values for glucosamine and muramic acid were corrected for the loss during hydrolysis, but those for the disaccharide were not corrected. The integration constant for the disaccharide was calculated from direct analysis of this compound.
[b] The yields, expressed in μmoles of oligosaccharides, were calculated from total glucosamine contents, which include this sugar recovered as the free sugar and disaccharide, assuming that B-2-5-1 is trisaccharide with one glucosamine; B-2-4-2 and B-2-4-1, tetrasaccharides with two glucosamine; and B-1-7-1 and B-1-5-1, hexasaccharides with three glucosamine residues.
[c] The values are expressed in moles of reducing groups as N-acetylglucosamine per mole of oligosaccharide.
[d] The values are expressed in moles per mole of oligosaccharide.

reported for hexasaccharide $(GlcNAc\text{-}MurNAc)_3$ (14). The analytical data are consistent with a tetrasaccharide N-unacylated at one of N-acetylglucosamine residues for B-2-4-1, and with hexasaccharides N-unacylated at one and two of N-acetylglucosamine residues for B-1-7-1 and B-1-5-1, respectively.

On analysis in the autoanalyzer, hydrolysates of B-2-4-1 gave disaccharide GlcN-MurN, glucosamine, and muramic acid in a molar ratio of 1:1:1, whereas hydrolysates of this compound which had been reduced with $NaBH_4$ gave only equimolar amounts of the disaccharide and glucosamine. Inasmuch as a glycosidic linkage of N-unsubstituted glucosamine is known to be highly resistant to acid hydrolysis, the above evidence is in accordance with the structure GlcN-MurNAc-GlcNAc-MurNAc for B-2-4-1. This compound was

insensitive to β-N-acetylglucosaminidase, whereas, after N-acetylation, it was hydrolyzed by the same enzyme yielding N-acetylglucosamine and an oligosaccharide identified as MurNAc-GlcNAc-MurNAc.

Hydrolysis of B-1-5-1 yielded the disaccharide, glucosamine, and muramic acid in a molar ratio of 2:1:1, whereas hydrolysates of this compound which had been reduced with $NaBH_4$ gave the disaccharide and glucosamine in a molar ratio of 2:1, but scarcely muramic acid. This result suggests that in B-1-5-1 the glucosamine residue linked to the muramic acid, which formed the reducing end of the molecule, had an acetamido group. This compound was stable toward β-N-acetylglucosaminidase. Thus, the most likely structure for B-1-5-1 is GlcN-MurNAc-GlcN-MurNAc-GlcNAc-MurNAc. The data from the similar experiments (Table 1) are compatible with the structure GlcNAc-MurNAc-GlcN-MurNAc-GlcNAc-MurNAc for B-1-7-1. The possibility of the occurrence of N-unsubstituted glucosamine as the nonreducing end of B-1-7-1 was excluded since this compound was hydrolyzed by β-N-acetylglucosaminidase.

As shown in Fig. 2, GlcN-MurNAc-GlcNAc-MurNAc or GlcN-MurNAc-GlcN-MurNAc-GlcNAc-MurNAc was not hydrolyzed by lysozyme at all, while GlcNAc-MurNAc-GlcN-MurNAc-GlcNAc-MurNAc was hydrolyzed very slowly. After N-acetylation, these compounds were hydrolyzed at the rates expected for the fully N-acetylated tetra- and hexasaccharides. The products resulting from lysozyme digestion of GlcNAc-MurNAc-GlcN-MurNAc-GlcNAc-MurNAc was identified as GlcNAc-MurNAc and GlcN-MurNAc-GlcNAc-MurNAc on the basis of their behavior on paper chromatography, paper electrophoresis, and on treatment with β-N-acetylglucosaminidase or lysozyme. It is concluded from this experiment that lysozyme could not hydrolyze the glyco-

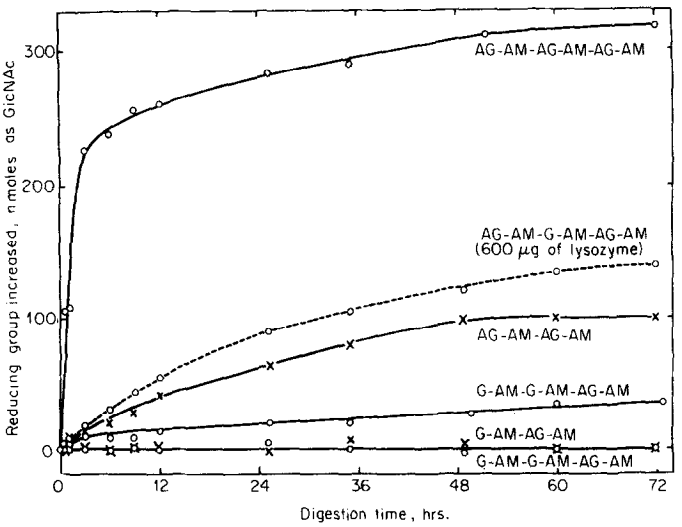

Fig. 2. Hydrolysis of oligosaccharides by lysozyme. The reaction mixture contained 60 μg of lysozyme and each saccharide (GlcN-MurNAc-GlcNAc-MurNAc, 109 nmoles; GlcNAc-MurNAc-GlcNAc-MurNAc, 78 nmoles; GlcNAc-MurNAc-GlcN-MurNAc-GlcNAc-MurNAc, 92 nmoles; GlcN-MurNAc-GlcN-MurNAc-GlcNAc-MurNAc, 135 nmoles; hexasaccharide prepared from B-1-5-1 by N-acetylation, 84 nmoles) in 200 μl of 40 mM ammonium acetate, pH 5.25. After incubation for various time intervals at 37°, aliquots were withdrawn for assaying reducing groups. The increase in reducing groups is expressed in nmoles as N-acetylglucosamine.

sidic linkage between GlcN-MurNAc and the disaccharide unit GlcNAc-MurNAc which formed the reducing end.

In the productive complex with lysozyme, the tetrasaccharide $(GlcNAc\text{-}MurNAc)_2$ is believed to interact with subsite C at the N-acetylglucosamine residue of the nonreducing end (2). The present evidence indicates the requirement for the acetyl group of this N-acetylglucosamine residue in the catalytic cleavage of the glycosidic linkage between the two disaccharide units. The lysozyme resistance of GlcN-MurNAc-GlcNAc-MurNAc moiety in GlcN-MurNAc-GlcN-MurNAc-GlcNAc-MurNAc and GlcNAc-MurNAc-GlcN-MurNAc-GlcNAc-MurNAc are also in accordance with the requirement for the acetyl group of the N-acetylglucosamine residue which interacts with subsite C of the enzyme in the productive complex. Thus,

the partially N-deacetylated peptidoglycan would be hydrolyzed by lysozyme at the linkages indicated below:

....AG-AM↑-G-AM-G-AM-AG-AM↑-AG-AM↑-G-AM-AG-AM↑-AG-AM....,

where AG, AM, and G stand for GlcNAc, MurNAc, and GlcN, respectively. This conclusion accounts for the resistance of the N-unacetylated peptidoglycan to lysozyme (8, 9).

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