ISOLATION OF 4-O-β-N-ACETYLMURAMYL-N-ACETYLGLUCOSAMINE AND 4-O-β-N,6-O-DIACETYLMURAMYL-N-ACETYLGLUCOSAMINE AND THE STRUCTURE OF THE CELL WALL POLYSACCHARIDE OF STAPHYLOCOCCUS AUREUS*

Donald J. Tipper and Jack L. Strominger

Department of Pharmacology
University of Wisconsin Medical School
Madison, Wisconsin

Received November 22, 1965

A powerful staphylolytic protein preparation, termed lysostaphin, has been obtained from the culture filtrate of a member of the genus Staphylococcus (designated K-6-WI) by Schindler and Schuhardt (1964, 1965). This substance lysed cells and and cell walls of all species of Staphylococcus aureus tested, but none of a large number of other microorganisms. Subsequent investigation (Browder, Zygmunt, Young and Tavormina, 1965) has revealed that lysis of staphylococcal cells by this substance is due to hydrolysis of the cell wall, catalyzed by a peptidase. The preparation also contains a glycosidase. These two activities were separated.

The peptidase catalyzed the cleavage of several peptide linkages in the cell wall of S. aureus resulting in liberation of both N-terminal glycine and N-terminal alanine (Browder et al., 1965). A polyglycine bridge between glycopeptide chains in the cell wall appears to be a genus-specific characteristic of S. aureus (Mandelstam and Strominger, 1961; Ghuysen, Tipper, Birge and Strominger, 1965). The limited range of bacteriolytic action of this enzyme is presumably due to the fact that its initial point of attack is within this bridge.

---

*Supported by research grants from the U.S. Public Health Service (AI-06247) and National Science Foundation (GB-1823).

The glycosidase, acting in conjunction with the peptidase, liberated a peptide-free oligosaccharide fraction, the reducing end group of which was acetylglucosamine, thus characterizing the enzyme as an endoacetylglucosaminidase (Browder et al., 1965). This enzyme thus cleaves a bond different from that cleaved by the acetylmuramidases which act on the bacterial cell wall, such as egg white lysozyme, the "32" enzyme from Streptomyces albus G, or the B enzyme of Chalaropsis. The purpose of the present communication is to report the isolation and characterization of the oligosaccharide fraction, obtained from the cell wall of S. aureus strain Copenhagen, after lysis with lysostaphin. It is a mixture of two disaccharides, isomeric to those previously isolated after lysis of the same cell walls with other hydrolytic enzymes (Ghuysen and Strominger, 1963; Tipper, Ghuysen and Strominger, 1965).

Cell wall (500 mg) was incubated with 5.2 mg of lysostaphin[1] for 16 hours at 37°. Previous small scale experiments indicated that at this time the liberation of new N-terminal amino groups had reached a maximum with liberation of about 0.7 mole of N-terminal glycine and 1.0 mole of new N-terminal alanine per mole of glutamic acid in the wall. Similarly, the liberation of reducing groups had reached a maximum of 0.7 mole (relative to an N-acetylglucosamine standard) at this time. These data confirm the conclusion of Browder et al. (1965) regarding the nature of the bonds cleaved by this enzyme.

The lysate was fractionated on a column of Ecteola-cellulose (Ghuysen, Tipper and Strominger, 1965). Water eluted a peptide and oligosaccharide fraction, and a gradient of increasing concentration of lithium chloride was used to elute the teichoic acid fraction. The peptide was selectively adsorbed on a column of CM-cellulose, and the oligosaccharide fraction was eluted with water. The yield (330

---

[1] We are greatly indebted to the Mead Johnson Research Laboratories for providing us with a sample of this enzyme.

μmoles of hexosamine after hydrolysis, 84 mg) was 70% of the theoretical yield[2] from 500 mg of cell wall. By paper chromatography in n-butanol:acetic acid:water (3:1:1), 53 mg of this mixture was fractionated into two compounds, disaccharide 3 and disaccharide 4, with R_f=0.29 and 0.43, the yields being 16 and 27 mg, respectively. It will be demonstrated that, as in the case of the isomeric disaccharides 1 and 2 (respectively 4-O-β-N-acetylglucosaminyl-N-acetylmuramic acid and 4-O-β-N-acetylglucosaminyl-N,6-O-diacetylmuramic acid) previously obtained, these two compounds differed from each other only in that the faster moving disaccharide 4 was substituted by an O-acetyl group.

Samples (50 mμmoles) of disaccharides 3 and 4, with and without prior reduction in 0.01 M $NaBH_4$ (50 μl) for 3 hours at 23°, were hydrolyzed in 3 N HCl at 95° for 4 hours, lyophilized, and chromatographed in n-butanol:acetic acid:water (3:1:1). Detection with ninhydrin revealed strong spots of muramic acid ($R_{glucosamine}$=2.2) in all four hydrolysates and of glucosamine in the unreduced materials. The reduced samples contained no glucosamine and gave weak spots of glucosaminitol ($R_{glucosamine}$=0.8) which reacts poorly with ninhydrin, but no spots of muramitol ($R_{glucosamine}$=1.6) which reacts moderately well with ninhydrin. Similarly, samples of the disaccharides, with and without reduction, were hydrolyzed and fractionated on the long column of a Beckman-Spinco amino acid analyzer. Standards of muramic acid and glucosaminitol gave peak areas (measured at 570 mμ) 71% and 49%, respectively, of that given by an equimolar amount of glucosamine, and had elution times 35% and 112%, respectively, of that of glucosamine. The quantitative data (Table 1) confirm that both materials contain equal amounts

---

[2] Assuming the repeating unit of the wall to be $(GlcNAc)_3(MurNAc)(Ala)_3(Glu)(Lys)(O\text{-}Ac)_{0.6}(Gly)_5(phosphoribitol)_2$, F.W.=2075.

Table 1

Analyses of Disaccharides 3 and 4 Before and After Reduction with $NaBH_4$

Analyses of acid hydrolysates were carried out on the Beckman-Spinco amino acid analyzer. Data are expressed as mμmoles per hydrolysate, each originating from 100 mμmoles of disaccharide.

Compound	Glucosamine	Glucosaminitol	Muramic Acid
Disaccharide 3	95	0	93
Reduced Disaccharide 3*	0	71	70
Disaccharide 4	107	0	105
Reduced Disaccharide 4	0	89	101

*A small amount of this sample was lost during preparation. The yield is therefore lower than that of the other samples.

of glucosamine and muramic acid and that all of the glucosamine but none of the muramic acid is susceptible to reduction. Thus both compounds are disaccharides of muramic acid and glucosamine with glucosamine as the reducing end. Neither the paper chromatogram nor the amino acid analyzer revealed traces of any amino acids, demonstrating that the peptidase had cleaved all the acetylmuramyl-L-alanine linkages. On paper electrophoresis both disaccharides were negatively charged at pH 4.8 and neutral at pH 2.0, thus indicating that their amino groups were still completely N-acetylated.

Disaccharide 3 was extremely inert to periodate oxidation, but after reduction with $NaBH_4$, 1.0 mole of periodate per mole of disaccharide was rapidly consumed with production of 1.0 mole of formaldehyde at the same rate. No further periodate consumption or formaldehyde production occurred on prolonged incubation (Figure 1). These data are compatible only with a 1,4-linkage of acetylmuramic acid to acetylglucosamine. The kinetics of formaldehyde production were identical

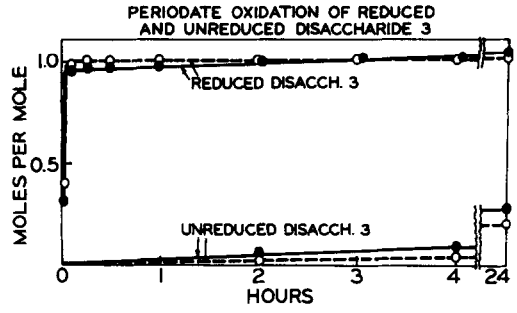

Figure 1. Periodate oxidation of disaccharide 3 and reduced disaccharide 3. Samples (160 mμmoles) were oxidized in 0.01 M periodate (45 μl) containing 0.01 M acetate, pH 4.5, at room temperature. At the indicated times, samples were withdrawn for the determination of periodate consumption (●————●) and formaldehyde production (O— — — —O).

---

to those obtained in oxidation of reduced disaccharide 1 (Tipper et al., 1965) under the same conditions (0.01 M periodate in 0.01 M acetate, pH 4.5, 22°). In each instance the formaldehyde results from oxidation of the C-5, C-6 glycol of the open chain moiety, in which C-4 is blocked by the glycosidic linkage.

Disaccharide 4 contained 1.0 mole of O-acetyl group per 2 moles of total hexosamine, while none was present in disaccharide 3. Data obtained on periodate oxidation after reduction of disaccharide 4, with and without subsequent removal of its O-acetyl substituent by mild alkaline hydrolysis, were identical to those obtained for disaccharide 3. The specific rotations of disaccharide 3 and disaccharide 4 were +12.4° and +15.4° (at equilibrium, C=0.5 in water), respectively. Thus, disaccharide 4 also has a 1,4 glycosidic linkage probably with the same anomeric configuration. The O-acetyl group is assigned to C-6 of its N-acetylmuramic acid residue on the basis of the previously determined structure of disaccharide 2 (Tipper et al., 1965).

Disaccharides 3 and 4 differ in several properties from disaccharides 1 and 2. Disaccharides 3 and 4 have a low reducing equivalent (0.64, relative to N-acetylglucosamine), and a low color yield in the Morgan-Elson reaction (0.07 after 30 minutes of heating in borate buffer, relative to the maximum color yielded by N-acetylglu-

cosamine, produced after 7 minutes of heating). In the Morgan-Elson reaction, 2-acetamido sugars with a 4-substituent yield little or no color (Kuhn, Gauhe and Baer, 1956; Jeanloz and Trémège, 1956). The corresponding values of 1.5 (reducing equivalent) and 0.51 (Morgan-Elson color at 30 minutes) for disaccharides 1 and 2 are due to their unusual alkali lability under the conditions of these reactions, a property which is not shared by disaccharides 3 and 4. Because of the low reducing power, negative Morgan-Elson reaction and inertness to periodate, it proved extremely difficult to detect disaccharides 3 and 4 on paper chromatography. They reacted very weakly with diphenylamine-TCA (Hough, Jones and Wadman, 1950), but not at all with alkaline silver nitrate reagent or aniline salts, except that disaccharide 3 gave a fluorescent derivative with aniline phosphate. They could, however, be detected on thin-layer chromatograms carried out on boric acid-buffered silica gel by the p-anisaldehyde-sulphuric acid reagent (Stahl and Kaltenbach, 1961) after prolonged heating at 150°, or by charring with H_2SO_4 at 200°.

In contrast to disaccharides 1 and 2, disaccharides 3 and 4 were not hydrolyzed by pig epididymal β-N-acetylglucosaminidase. Moreover, they were not hydrolyzed by three known acetylmuramidases (egg white lysozyme, the "32" enzyme from Streptomyces albus and the B enzyme from Chalaropsis), although their glycosidic linkage is presumably the point of attack of these enzymes in the intact cell wall.

The question of the α- or β-anomerism of the glycosidic linkage was investigated by nuclear magnetic resonance spectroscopy. The proton on the hemiacetal carbon of sugars is readily resolved from the other protons by NMR spectroscopy, and a clear correlation exists between the τ and J values of its absorption peak and the configuration of the anomeric carbon (Van der Veen, 1963; Lemieux, 1965). Spectra[3] of the acetylglucosaminyl-N-acetylmuramic acid disaccharides 1 and 2 were

[3] The NMR studies were made in collaboration with Mr. Joseph Mohlenkamp and Dr. Laurens Anderson. Details of the spectra will be presented in a forthcoming publication.

very similar to those of the N-acetylmuramyl-N-acetylglucosamine disaccharides 3 and 4 with respect to these proton peaks. Each had absorptions corresponding to 0.7 proton in the α-proton region and to 2 protons in the β-proton region, where the proton on C-2 of the lactic acid moiety of acetylmuramic acid also absorbs. It therefore remained to assign the α- and β-proton peaks between the anomeric carbons in the glycosidic linkage and at the reducing end of the disaccharides. Spectra of N-acetylglucosamine and N-acetylmuramic acid both had peaks corresponding to 0.7 proton in the α-region, while only the latter had absorption in the β-region, corresponding to 1.0 proton and due to the C-2 proton of the lactic acid moiety. It thus appears that both the free sugars exist in D_2O solution exclusively as the α-anomer, probably due in each case to intramolecular hydrogen bonding between the C-1 hydroxyl and the acetamido group (Van der Veen, 1963). This interaction should persist after glycosylation at C-4. Hydrolysis of the N-acetylglucosaminyl linkages of disaccharides 1 and 2 is catalyzed by a β-specific N-acetylglucosaminidase (Findlay and Levvy, 1960). It was therefore evident that the glycosidic linkage in disaccharides 1 and 2 had the β-configuration, and that the α-proton was at the reducing end, which thus resembled the free sugar. It seemed likely that the same assignments could be made fo disaccharides 3 and 4, placing the α-proton at the reducing end. This interpretation was supported by the low specific rotations of the disaccharides and by the NMR spectrum of the intact cell wall polysaccharide (Tipper, Ghuysen and Strominger, 1964). In this compound no absorption in the α-proton region was observed and proton absorption peaks corresponding to 2.6 protons per disaccharide unit were present in the β-proton and lactyl-proton regions. Thus disaccharide 3 is 4-O-(2-acetamido-2-deoxy-3-O-(D-1-carboxyethyl)-β-D-glucopyranosyl)-2-acetamido-2-deoxy-D-glucopyranose, and disaccharide 4 is the same compound with an O-acetyl group on C-6 of the N-acetylmuramyl residue.

It can, therefore, be concluded that all of the glycosidic linkages in the cell

wall polysaccharide are β-linkages. Since both of the linkages present are also 1,4-linkages, the major part, if not all, of the cell wall polysaccharide in S. aureus is a substituted chitin - i.e. a polymer of β-1,4-linked N-acetylglucosamine residues in which every other sugar is substituted by a 3-O-lactyl substituent and in which some of these are also substituted on the 6-position by an O-acetyl group. The fact that only the end groups of the intact polysaccharide can be oxidized by periodate (Tipper et al. 1964) is compatible with this structure.

Barkulis, Smith, Boltralik and Heymann (1964) have recently reported the isolation of an endoacetylglucosaminidase, muralysin, from phage lysates of Streptococcus hemolyticus. The disaccharide-peptides obtained from lysates of streptococcal cell walls by muralysin had properties which suggested that both 1,3 and 1,4-linkages were present. The data obtained with disaccharides 3 and 4 from S. aureus cell walls, especially periodate oxidation and the Morgan-Elson reaction, exclude more than 5% of 1,3-linkages in these preparations. Much comparative biochemistry remains to be done before the generality of the results reported in this paper can be assesed.

REFERENCES

Barkulis, S.S., Smith, C., Boltralik, J. J., and Heymann, H., Bact. Proc. 32 (1964) and J. Biol. Chem. 239, 4027 (1964).

Browder, H. P., Zygmunt, W. A., Young, J. R., and Tavormina, P. A., Biochem. Biophys. Res. Commun. 19, 383 (1965).

Findlay, J. and Levvy, G. A., Biochem. J. 77, 170 (1960).

Ghuysen, J. M., Tipper, D. J., and Strominger, J. L., Biochemistry 2, 1119 (1963).

Ghuysen, J. M., Tipper, D. J., Birge, C. H., and Strominger, J. L., Biochemistry 4, 2245 (1965).

Ghuysen, J. M., Tipper, D. J., and Strominger, J. L., Biochemistry 4, 474 (1965).

Hough, L., Jones, J. K. N., and Wadman, W. H., J. Chem. Soc. 1702 (1950).

Jeanloz, R. W. and Trémège, M., Fed. Proc. 15, 282 (1965).

Kuhn, R., Gauhe, A., and Baer, H. H., Chem. Ber. 87, 1138 (1956).

Lemieux, R. U., Canad. J. Chem. in press (1965).

Mandelstam, M. H. and Strominger, J. L., Biochem. Biophys. Res. Commun. 5, 466 (1961).

Schindler, C. A. and Schuhardt, V. T., Proc. Natl. Acad. Sci. U.S. 51, 414 (1964).
Schindler, C. A. and Schuhardt, V. T., Biochim. Biophys. Acta 97, 242 (1965).
Stahl, E. and Kaltenbach, U., J. Chromatography 5, 351 (1961).
Tipper, D. J., Ghuysen, J. M., and Strominger, J. L., Fed. Proc. 23, 379 (1964).
Tipper, D. J., Ghuysen, J. M., and Strominger, J. L., Biochemistry 4, 468 (1965).
Van der Veen, J. M., J. Org. Chem. 567 (1963).