LYSOSTAPHIN: ENZYMATIC MODE OF ACTION

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Lysostaphin has been reported (Schindler & Schuhardt 1964) as an antibiotic that lyses Staphylococcus aureus. The active principle is enzymic in nature with a molecular weight of about 30,000, an isoelectric point greater than 9, and a pH optimum of 7.5. Since the uniqueness of this agent resides precisely in its ability to destroy the staphylococcal cell enzymatically, we wish to report our observations concerning the enzymatic process involved. We have used concentrates in which the lytic principle was 300-fold purified on a protein basis relative to the culture filtrate, and as substrates, either cell wall or glycopeptide prepared from Staphylococcus aureus, strain Copenhagen. These particular substrates were selected because of the extensive knowledge that has been accumulated concerning their composition.

Although the ultimate details of the structure of the staphylococcal cell wall remain somewhat obscure, papers by Strominger, Park, and Thompson (1959), Mandelstam and Strominger (1961) and Ghuysen and Strominger (1963) suggest the glycopeptide portion of the cell wall of S. aureus, strain Copenhagen, to be composed of alternating molecules of N-acetylglucosamine and N-acetylmuramic acid. Attached to the lactyl moiety of the latter is a peptide whose structure (Strominger, 1959) is related to the amino acid portion of the Park nucleotide (1952): uridine-pyrophosphate-N-acetylmuramyl-L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine.

The peptide chains are considered to be cross-linked by

polyglycine which gives the structure additional rigidity. The stoichiometry of the amino acids and amino sugars is such that the molar ratios of the constituents in cell wall to glutamic acid are: lysine (1), alanine (3), glycine (4.8), glucosamine (2), and muramic acid (1).

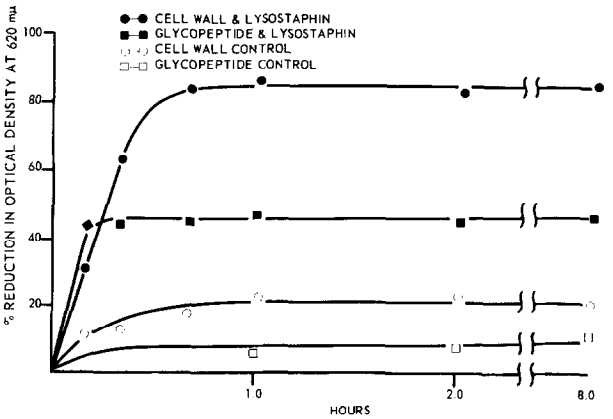
The intact cell wall differs from glycopeptide in that it has, in addition, a third polymer consisting of ribitol phosphate coupled with N-acetylglucosamine and D-alanine. This polymer is removed in the preparation of glycopeptide.

Cell walls used in these studies were prepared from S. aureus, strain Copenhagen (kindly supplied by Dr. J. L. Strominger). Cells were grown in Trypticase Soy broth (Baltimore Biological Laboratory), harvested in the logarithmic phase of growth, washed twice with distilled water, and suspended in 0.02M, pH 7.0 phosphate buffer. The washed cells were mechanically disrupted in a Nossal Disintegrator (McDonald Engineering Co., Cleveland, Ohio) with No. 12 Ballotini glass beads. Further purification was accomplished by digesting the cell wall with trypsin, deoxyribonuclease and ribonuclease. Electron microscopy showed the absence of whole cells in the preparation and only trace amounts of electron dense materials. Glycopeptide was prepared by heating cell wall in 10% trichloroacetic acid (TCA) for 8 hours at 60°C. or by incubating cell wall in 10% TCA in the cold (4°C.) for two weeks. While either treatment effectively removes teichoic acid from the cell wall, it also causes some degradation. This is particularly true when hot TCA is employed.

Incubation of lysostaphin preparations with either cell wall or glycopeptide results in rapid lysis as shown in Figure 1. Samples of the incubation mixture were taken at various time intervals and assayed for the release of amino groups (unpublished procedure of O. H. Lowry, cf. Ghuysen & Strominger, 1963), reducing groups (Park and Johnson, 1949), and N-acetylhexosamine (Reissig et al., 1955;

FIGURE 1

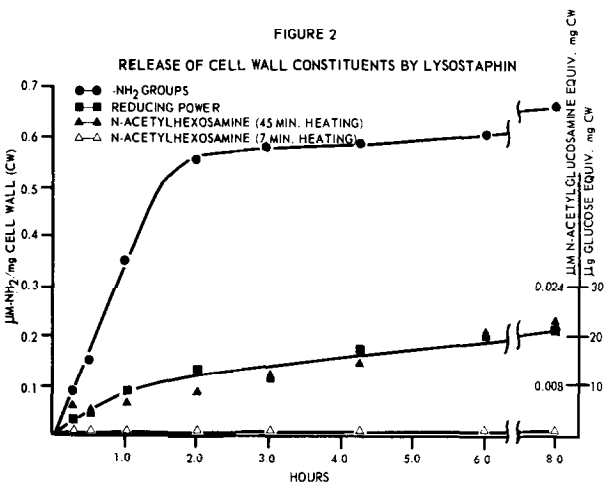
LYSIS OF S. AUREUS CELL WALL AND GLYCOPEPTIDE BY LYSOSTAPHIN



THE DECREASE IN OPTICAL DENSITY OF CELL WALL AND GLYCOPEPTIDE CONTROLS IS PROBABLY A NON-SPECIFIC SOLUBILIZATION OF SMALL AMOUNTS OF THE SUBSTRATES, SINCE IT IS NOT ACCOMPANIED BY THE RELEASE OF AMINO GROUPS, N-ACETYLHEXOSAMINE, OR REDUCING POWER.

FIGURE 2

RELEASE OF CELL WALL CONSTITUENTS BY LYSOSTAPHIN



Ghuysen and Salton, 1960). The release of cell wall constituents is represented in Figure 2. Similar results are obtained with glycopeptide. Reduction in optical density parallels a rapid release of amino groups from both substrates and furnishes presumptive evidence at least that lysis of the staphylococcal cell by lysostaphin is primarily associated with the loss of integrity of the

peptide structure. It is interesting to note that release of amino and reducing groups continues even after one hour, when the rapid fall in optical density has ceased.

Identification of the N-terminal amino acids liberated from cell wall was accomplished by treating lysates with 2,4-dinitrofluorobenzene (DNFB), followed by overnight acid hydrolysis (6N HCl), removal of acid *in vacuo* over KOH, and two dimensional chromatography with markers of authentic dinitrophenyl-(DNP)-amino acids. DNP-glycine and DNP-alanine were found in cell wall and glycopeptide lysates. Whereas DNP-alanine from cell wall may arise from the ester-linked D-alanine of teichoic acid, the occurrence of DNP-alanine in DNFB-treated glycopeptide lysates must indicate cleavage of alanine peptide bonds in addition to cleavage of glycine peptide bonds. It has also been observed that pentaglycine and other synthetic glycine peptides are hydrolyzed by this preparation.

In contrast to the peptide cleavage, the release of reducing groups and N-acetylhexosamine proceeds initially at a much slower rate. The parallelism of the curves for the release of N-acetylhexosamine and reducing groups (Fig. 2) suggests that both assays are measuring the cleavage of the same carbohydrate bond. The N-acetylhexosamine assay of Reissig *et al.* (1955) which with simple N-acetylhexosamines gives maximum chromogen formation after 7 minutes heating in borate buffer gives negative results with these lysates. A positive test for N-acetylhexosamine is obtained only after the prolonged heating recommended by Ghuysen and Salton (1960). These observations suggest that the carbohydrate fragments resulting from the enzymic cleavage of cell wall or of glycopeptide are not simple monomers.

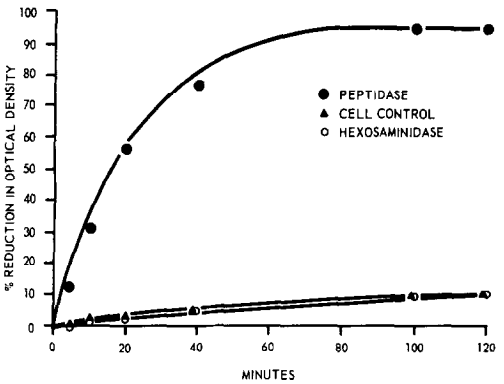
To determine which glycosidic bond is cleaved, a sample of cell wall was incubated with the lysostaphin preparation in buffer for

24 hours at 37°. The resulting low molecular weight fragments were separated by dialysis. Carbohydrate fragments free of ninhydrin-positive materials were obtained by electrophoresis (Ghuysen and Salton, 1960), and were located by spraying marker strips with Salton's N-acetylhexosamine reagent (Salton, 1956). The oligosaccharide was recovered by elution with water and the free carbonyl reduced to the alcohol with sodium borohydride. The borohydride-treated sample and a non-treated sample were hydrolyzed, and chromatographed in two directions on Whatman No. 1 paper in n-butanol:acetic acid:water (3:1:1) and pyridine:water (4:1) (Ghuysen and Salton, 1960). The amino sugars and the amino sugar alcohols were located with ninhydrin. The new spot which appeared in the reduced hydrolysate migrated with the same Rf value as an authentic sample of 2-amino-2-deoxy-D-glucitol, suggesting that the glycosidic bond cleaved is the glucosaminyl-muramic acid linkage.

The results obtained indicate that two distinct enzymes are present in this antibiotic preparation. In order to identify the lytic principle, further resolution studies were undertaken, and yielded both a major fraction, the peptidase, which liberates amino groups but has no hexosaminidase activity, and, in smaller amounts, a hexosaminidase preparation that has no peptidase activity. Less than 1 mcgm./ml. of peptidase is sufficient to reduce the optical density of a suspension of S. aureus, strain Copenhagen (1 X 10^8 cells/ml.) by 95% after 2 hours at 37°C. (Figure 3). On the other hand, levels of hexosaminidase up to 15-fold higher have no lytic activity.

The peptidase reported here differs from the Streptomyces albus G amidase of Ghuysen and Strominger (1963) which liberates N-terminal alanine only. Another lytic enzyme, from a Flavobacterium sp., has been reported by Kato et al. (1962). Their enzyme appears to liberate from S. aureus cell wall one fragment containing only

FIGURE 3
ENZYMATIC LYSIS OF S. AUREUS COP



glycine residues, and other fragments containing all the cell wall amino acids. These fragments have no reducing power, suggesting that the peptide chains are split from the carbohydrate backbone. Despite this apparent similarity of the Flavobacterium sp. enzyme[1] to the peptidase described in this communication, the two are readily distinguishable by disc electrophoresis.

The hexosaminidase found in this antibiotic preparation is per se devoid of lytic activity for S. aureus, and is characterized by its cleavage of the glucosaminyl-muramic acid bond of cell wall or glycopeptide. Except for the streptococcal muralysin recently reported by Barkulis (1964) which also hydrolyses the glucosaminyl-muramic acid bond, the hexosaminidase reported here differs from all previously described enzymes of this kind such as lysozyme (Salton & Ghuysen, 1960), or the S. albus G acetylmuramidase of Ghuysen and Strominger (1963), or the Chalaropsis sp. acetylhexosaminidase (Tipper et al., 1964), all of which cleave the alternate linkage of the bacterial carbohydrate backbone.

[1] We are indebted to Drs. Kato & Kotani for a sample.

In summary, we have found that the lytic principle of lysostaphin is a peptidase which liberates N-terminal glycine and alanine from S. aureus cell wall. The hexosaminidase which is present in lysostaphin preparations does not lyse S. aureus and is specific for the glucosaminyl-muramic acid bond of the bacterial carbohydrate backbone.

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