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Substrate Requirements of Glycosidases for Lytic Activity on Bacterial Walls

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Following Fleming's discovery in 1922 (1) that hen egg-white lysozyme caused the dissolution of living cells of *Micrococcus lysodeikticus* and the investigations of Meyer *et al.* (2), Epstein and Chain (3), and Meyer and Hahnel (4) on the degradation of a "soluble mucopolysaccharide" by lysozyme, modern studies on the mode of action of this enzyme and other bacteriolytic and antibiotic agents started in the 1950's when Salton succeeded in isolating the cell walls from mechanically disrupted bacteria and showed that the isolated wall of *M. lysodeikticus* could be used as "the substrate" for lysozyme (5). The isolation of the protoplasts of *Bacillus megaterium* by Weibull (6) beautifully confirmed the idea that the action of lysozyme was solely restricted to the wall structure of the cell, and the formation of spheroplasts of *Escherichia coli* under the action of either lysozyme (7) or penicillin (8) pointed to the identity of the cell target (i.e., the wall) impaired by these two antimicrobial agents (for complete references, see 9).

Structure of the Walls of *Micrococcus lysodeikticus*

It took about 15 years of work in several laboratories to establish the exact primary structure of the polymer which forms the solid matrix of the

wall of *M. lysodeikticus* and is susceptible to solubilization by lysozyme. This polymer is a peptidoglycan network in which linear glycan strands are interconnected by peptide chains.* The glycan strands essentially consist of alternating $\beta(1,4)$ linked pyranoside *N*-acetylglucosamine and *N*-acetylmuramic acid residues, i.e., a chitin-like structure in which each alternate *N*-acetylglucosamine residue is ether-linked at C-3 to a lactyl group which has the D configuration. Small amounts of mannomuramic acid were recently reported to occur along with glucomuramic acid (11). Less than 50% of the *N*-acetylmuramic acid residues have their D-lactyl groups substituted by pentapeptide units having the sequence N^{α}-/[L-alanyl-γ-(α-D-glutamyl-glycine)]-L-lysyl-D-alanine. Most of these pentapeptide units are in turn interconnected through "bridges" which extend from the C-terminal D-alanine residue of one pentapeptide unit to the ϵ-amino group of L-lysine of another peptide unit. A few of these interpeptide bridges are extremely short and consist of direct N^{ϵ}-(D-alanyl)-L-lysine amide bonds between two pentapeptide units. Most of the interpeptide bridges, however, are much longer and composed of one or several peptides, each having the same sequence as the pentapeptide units which substitute the *N*-acetylmuramic acid residues. In this type of peptidoglycan both D-alanyl-L-alanine peptide linkages and N^{ϵ}-(D-alanyl)-L-lysine amide bonds are involved in peptide cross-linking and the hydrolysis of either of these linkages causes the dissolution of the wall and the lysis of the cell. The D-alanyl-L-alanine linkages are sensitive to the *Myxobacter* Al_1 enzyme whereas the N^{ϵ}-(D-alanyl)-L-lysine linkages are sensitive to the *Streptomyces* ML enzyme.

The total number of pentapeptide units of the wall peptidoglycan is roughly equivalent to the number of disaccharide units of the glycan strands. It is known, however, that UDP-*N*-acetylglucosamine and UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-L-lysyl-D-alanyl-D-alanine are the cytoplasmic nucleotide precursors and that the glycine residue is added to the α-carboxyl group of D-glutamic acid at a later stage of the biosynthesis (for references, see 12). Evidently, the observed one-to-one molar ratio between the peptidoglycan constituents and the accommodation of unsubstituted glycan fragments must result from the migration at a certain stage of the biosynthesis, of some pentapeptide units from the *N*-acetylmuramic acid residues into a bridging position with the formation of oligomeric units. The mechanism of these migrations and polymerizations is still entirely unknown (10).

The observed yields of peptidoglycan fragments obtained by various degradation procedures established that both the peptide and glycan moieties of the peptidoglycan are polydisperse systems. These yields are consistent with a structure in which 5% of the pentapeptide units occur as monomers,

*For details and illustrations, see Ref. 10.

4% as dimers, 15% as trimers, and 60% as hexamers. The hexamer, of course, represents a statistical average among oligomers of various sizes. Similarly, the glycan strands appear to be composed, on the average, of about 15–20 disaccharide units. It is known that at most 50% of the *N*-acetylmuramic acid residues are peptide-substituted but the exact distribution of the peptide-free disaccharide units along the glycan chains is still a matter of speculation. It seems likely, however, that large unsubstituted glycan segments up to the size of octasaccharides may occur.

The tightness of a peptidoglycan network depends upon the frequency with which the glycan chains are substituted by peptide units and upon the frequency with which these peptide units are, in turn, interlinked. The low order of cross-linking both at the junction between the glycan and the peptide constituents and in the peptide moiety itself makes the *M. lysodeikticus* peptidoglycan a rather loose network and explains why its dissolution can be brought about by the hydrolysis of only a few bonds located either in the glycan or in the peptide part of the peptidoglycan. For example, the exposure of about 120 neq of reducing groups (expressed as *N*-acetylglucosamine) through the cleavage of glycosidic bonds by lysozyme, or, alternatively, the hydrolysis of about 150–200 nEq of N^{ϵ}-(D-alanyl)-L-lysine linkages by the ML endopeptidase are sufficient to cause the dissolution of 1 mg of walls (containing about 600 neq of disaccharide-pentapeptide units). The looseness of the *M. lysodeikticus* peptidoglycan is also reflected by the high rate of degradation observed at low lysozyme concentrations. Complete solubilization of the walls occurs in about 30 min at a ratio wall/enzyme of 100/1 (w/w).

In addition to the peptidoglycan, the walls of *M. lysodeikticus* contain another polymer composed of equimolar amounts of glucose and 2-acetamido-2-deoxymannuronic acid residues (13). This polymer is devoid of insolubility and rigidity and is anchored into the wall peptidoglycan matrix via phosphodiester bonds extending to the C-6 position of some of the *N*-acetylmuramic acid residues. On the average, one such phosphodiester bond would occur for each glycan strand of 15–20 disaccharides in length (14).

Action of Hen Egg-White Lysozyme on Walls of *M. lysodeikticus*

From the studies of Salton on the products found in the digested walls, it was clear that lysozyme attacked glycosidic bonds in endoposition in the glycan strands (9). Later on, it was established that lysozyme specifically hydrolyzed $\beta(1\text{–}4)$ linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine so that *N*-acetylmuramic acid was always at the reducing end of the degraded fragments. Among these fragments, peptide-free disac-

charide β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid, tetrasaccharide and higher oligosaccharides were isolated and characterized (10). The choice of *M. lysodeikticus* for these early studies was very fortunate. Indeed the peptidoglycans of all bacteria, with the exception of *M. lysodeikticus* and some related *Micrococcaceae* (10, 14), contain peptide substituents on all of their *N*-acetylmuramic acid residues so that the action of a glycosidase alone cannot bring about the release of unsubstituted di- and oligosaccharides. Kinetics of the lysis of *M. lysodeikticus* cell walls and the release of glycan fragments by lysozyme are a complex phenomenon. This complexity is due to a number of factors which are discussed elsewhere in this volume. Lysozyme performs a transglycosidase activity and its lytic activity is product-inhibited, thus precluding complete hydrolysis of all sensitive bonds. At completion of the reaction of lysozyme on walls of *M. lysodeikticus,* only 30–40% of the peptidoglycan *N*-acetylhexosamine residues are found in the form of disaccharides (either free or peptide substituted), i.e., the end product of the degradation. However, through the combined use of lysozyme and a mixture of *Streptomyces exo-N*-acetylhexosaminidases (which further degrade those di- and oligosaccharide fragments liberated by lysozyme treatment), about 70% of the glycan chains were found to have undergone complete conversion into monomeric *N*-acetylhexosamine residues (15).

Structure of Bacterial Walls Other than Those of *M. lysodeikticus*

Although there is a remarkable consistency of the structure of the wall peptidoglycans throughout the bacterial world, variations occur which have been used to divide the bacterial species into different chemotypes (10). The tetrapeptide units have the general sequence L-alanyl-γ-D-glutamyl-L-R_3-D-alanine where the L-R_3 residue may be a neutral amino acid (L-alanine or L-homoserine), or a dicarboxylic amino acid (L-glutamic acid) or a diamino acid (L-ornithine, L-lysine, LL- or *meso*-diaminopimelic acid). The α-carboxyl group of D-glutamic acid can be either free, amidated, or substituted by a glycine amide or a C-terminal glycine residue (as found in *M. lysodeikticus*). Similarly, the carboxyl group of diaminopimelic acid not engaged in peptide bond may be substituted by an amide. In almost all bacteria, the interpeptide bridges extend from the C-terminal D-alanine of one peptide to the ω-amino group of the diamino acid at the L-R_3 position of another peptide. However, the situation where these interpeptide bridges are made up of one or several peptides each having the same amino acid sequence as the peptide units that substitute the *N*-acetylmuramic acid residues is exceptional and restricted to *M. lysodeikticus* and some related

Micrococcaceae (chemotype III). Usually, the bridges either consist of direct N^{ω}-(D-alanyl)-L-R_3 peptide bonds (chemotype I) or are mediated via one or several additional amino acids (chemotype II). Finally, the peptide cross linking of a few bacterial species (plant pathogenic *Corynebacteria, Butyribacterium rettgeri*) extends from the C-terminal D-alanine residue of one peptide unit to the α-carboxyl group of D-glutamic acid of another peptide unit (chemotype IV). This type of bridging links two carboxyl groups and, hence, necessarily involves a diamino acid residue or a diamino acid-containing peptide.

Variations also occur in the glycan strands. They include (*i*) the *O*-acylation on C-6 of some of the *N*-acetylmuramic acid residues; (*ii*) the replacement of *N*-acetylmuramic acid by *N*-glycolylmuramic acid (in *Nocardia* and *Mycobacterium* sp.) (16); (*iii*) the occurrence of a high proportion of the muramic acid residues in the form of the lactam derivative (in the spore peptidoglycan of *Bacillus*) (17); (*iv*) the presence of small amounts of mannomuramic acid instead of glucomuramic acid (11) (in *M. lysodeikticus*, see above).

Early ideas about the organization of the peptidoglycan were that the glycan strands completely extended around the cell (18). Accurate analyses, however, showed that many terminal groups were present in both the glycan and the peptide moieties of all bacterial peptidoglycans. The average length of the glycan chains vary from 20 to 100 *N*-acetylhexosamine residues. In all bacteria, with the exception of *M. lysodeikticus* (see above) and the spores of *Bacillus*, virtually all of the *N*-acetylmuramine acid residues are peptide substituted. In *E. coli* about 50% of the peptide units occur as uncross-linked monomers, and the other units as peptide dimers. In *S. aureus*, which has one of the most cross-linked peptidoglycans, the average size of the peptide moiety does not exceed 10 cross-linked peptide units (10).

In addition to the peptidoglycan layer, the envelope of gram-negative bacteria contains an outer membrane very similar in appearance in thin sections to the inner plasma membrane. This outer membrane has different permeability properties and contains lipopolysaccharides and lipoproteins. In *E. coli*, the lipoproteins have been shown to be covalently linked to the peptidoglycan (about 1 lipoprotein molecule for every 10th repeating peptide unit) through lysl-arginine dipeptides extending to some *meso*-diaminopimelic acid residues (19). The envelope of gram-positive bacteria lacks the outer membrane. However, an almost endless variety of polysaccharides that are frequently negatively charged, and of polyol-phosphates that are collectively called teichoic acids, are covalently linked to the peptidoglycan. One such covalent link, but probably not the only one between the peptidoglycan and these other wall polymers, is via phosphodiester bridges to the C-6 position of *N*-acetylmuramic acid (10).

Substrate Requirements of Hen Egg-White Lysozyme for Lytic Activity on Bacterial Peptidoglycans

Following the discovery of *O*-acetyl groups in walls of *Streptococcus faecalis* by Abrams in 1958 (20), the importance of these substituents in relation to sensitivity to lysozyme was demonstrated by Brumfitt *et al.* (21). Resistance to lysozyme exhibited by mutants of *M. lysodeikticus* was shown to be due to the substitution of the glycan strands by *O*-acetyl groups (in fact on C-6 of *N*-acetylmuramic acid). Cells and walls could be made resistant to lysozyme by chemical *O*-acetylation with acetic anhydride. Similarly, resistance to lysozyme of mutants of *B. megaterium* was shown to be related to acetylation of wall hydroxyl groups [for references, see Salton (9) and Ghuysen (10)]. We now know from the X-ray crystallographic studies of Phillips and co-workers that subsite D of hen egg-white lysozyme could not accommodate an acetyl group at the C-6 position.

The frequency with which the glycan strands are substituted by peptides is also of importance for lysozyme action. This idea rests upon the following observations: (*i*) Intact glycan strands from which the peptide substituents were enzymatically removed (through the action of *Myxobacter* Al_1 *N*-acetylmuramyl-L-alanine amidase) were obtained from several types of lysozyme-sensitive peptidoglycans (10). The rate of hydrolysis of these peptide-free glycan chains by lysozyme was only a few percent of the rate of hydrolysis of the peptidoglycans themselves and much higher enzyme concentrations were needed for the degradation. (*ii*) The peptidoglycan of *E. coli* in which all of the *N*-acetylmuramic acid residues are peptide substituted could be degraded (after removal of the lipoproteins which are covalently linked to it) almost quantitatively into disaccharide peptide monomers and *bis*-disaccharide peptide dimers by lysozyme (18, 22). Only very small amounts of peptide-substituted tetrasaccharide fragments were found among the degradation products. Such a complete degradation contrasts with the incomplete degradation of the walls of *M. lysodeikticus* (see above) in which case long unsubstituted glycan segments occur in the native peptidoglycan. These observations suggest that the presence of a substituted *N*-acetylmuramyl carboxyl group facilitates the hydrolysis of the neighboring glycosidic linkage.

A great number of peptidoglycans that are not *O*-acetylated and in which all the *N*-acetylmuramic acid residues are peptide substituted are totally or at least highly resistant to hen egg-white lysozyme. Elimination of the *O*-acetyl groups of the glycan chains and removal of the teichoic acids do not render such peptidoglycans, for examples those of *Staphylococcus aureus* and *Lactobacillus acidophilus*, significantly more sensitive to lysozyme than the native cell walls. There are many examples of this type

which all point to the fact that some intrinsic property of the peptidoglycan must be involved in the resistance (or sensitivity) to lysozyme. From a survey of the sensitivity (or resistance) of peptidoglycans of different chemotypes to lysozyme, it appears, however, that the primary structure of the peptide moieties is probably not in itself a feature that is important for lysozyme action. Both the *M. lysodeikticus* and *E. coli* peptidoglycans are very sensitive to lysozyme and yet the peptide moieties of these two peptidoglycans differ in many respects. The α-carboxyl group of D-glutamic acid is free in *E. coli* and substituted by a glycine residue in *M. lysodeikticus*; the L-R_3 residue is *meso*-diaminopimelic acid in the former and L-lysine in the latter; the interpeptide bridges in these two organisms are very different both in length and in amino acid composition. From the foregoing, one can hypothesize that the overall macromolecular structure of the peptidoglycan which is imparted by its primary structure—perhaps the tightness of the network—is one of the main factors involved in lysozyme sensitivity. Several molecular models of peptidoglycans have been constructed which suggest possible conformations (23–25). All of them involve extensive hydrogen bonding within the network. To date, however, there is no physical evidence in support of any of these, or other, molecular models. The three-dimensional organization of the peptidoglycan is still completely unknown.

Lysozyme-Like Enzymes

Hen egg-white lysozyme has been a tool of great value for the isolation and purification of membranes and other cellular organelles from a wide variety of bacteria via the transformation of the bacteria into osmotically fragile protoplasts and spheroplasts. In this process, not all the glycosidic bonds of the peptidoglycans have to be hydrolyzed to sufficiently weaken the tensile strength of the wall and to prevent it from playing its role as a cell supporting structure. By contrast and because of its incomplete action on many types of walls, hen egg-white lysozyme has been of very limited use for the determination of the chemical structure of a great number of bacterial peptidoglycans (10). Indeed, interpreting the structure of a peptidogylcan on the basis of the fragments liberated through the action of a lytic enzyme is an exceedingly difficult task if only a fraction of the bonds, which should be hydrolyzed owing to the intrinsic specificity of the enzyme for the chemical bonds hydrolyzed, are actually split. Therefore, a search for other lytic agents has been undertaken which has resulted in the discovery of other lysozyme-like glycosidases as well as of two other classes of lytic enzymes, *N*-acetylmuramyl-L-alanine amidases and endopeptidases.

The use of these enzymes in the elucidation of the primary structures of the bacterial peptidoglycans has been reviewed (10).

Both the F1 enzyme isolated from *Streptomyces albus* G and the enzyme B from *Chalaropsis* (26) are of great interest. Among the many glycosidases so far studied, these two *endo-N*-acetylmuramidases have the broadest lytic spectrum, lysing cells or solubilizing walls of virtually all gram-positive bacteria and degrading the isolated peptidoglycans from gram-negative bacteria. With these enzymes, the glycan strands are usually quantitatively degraded into peptide-substituted disaccharide units, irrespective of the possible presence of *O*-acetyl groups and the chemotypes of the peptidoglycans. Thus far, the only requirement is that the *N*-acetylmuramic acid residues must be substituted by peptides. Both enzymes hydrolyze the peptidoglycan of *S. aureus* into disaccharide-peptide and *O*-acetyl disaccharide peptide units. However, by acting on *M. lysodeikticus,* they release a mixture of substituted disaccharide units and unsubstituted di-, tetra-, and octasaccharides. They have no or very little action on intact peptide-free glycan chains.

Of equal interest has been the discovery of the streptococcal muralysin and the staphylococcal glycosidase which act as *endo-N*-acetylglucosaminidases, i.e., they hydrolyze the glycosidic linkages between *N*-acetylglucosamine and *N*-acetylmuramic acid so that *N*-acetylglucosamine is at the reducing end of the degradation fragments [for references, see Ghuysen (10) and Wadstrom (27)]. These two enzymes are not readily available and hence their lytic spectra have not been thoroughly investigated. In contrast to hen egg-white lysozyme and other *endo-N*-acetylmuramidases, however, the staphylococcal *endo-N*-acetylglucosaminidase seems to act preferentially on glycan chains that are free of peptide or on unsubstituted segments of them. *Micrococcus lysodeikticus,* which has many unsubstituted *N*-acetylmuramic acid residues, is one of the most susceptible organisms. The enzyme has very little if any action on the *S. aureus* peptidoglycan but completely degrades the intact peptide-free polysaccharides obtained by hydrolysis of the walls with the *Myxobacter* Al_1 enzyme.

The foregoing observations emphasize the strict specificity of the lytic glycosidases which act either as *endo-N*-acetylmuramidases (lysozymes; glycoside hydrolases 3.2.1.17) or *endo-N*-acetylglucosaminidases (chitinases; glycoside hydrolases 3.2.1.14) with regard to the bonds they attack in the wall peptidoglycans. However, hen egg-white lysozyme degrades purified chitin (28) and soluble oligosaccharides isolated from chitin (29). Tetra-*N*-acetylchetotetraose yields chitobiose and higher oligosaccharides after incubation with lysozyme (9, 30). The *Streptomyces* F1 "*endo-N*-acetylmuramidase" was also found to be able to degrade the above tetrasaccharide into chitobiose (9). Turnip lysozyme, which is an excellent

chitinase, was actually isolated by following its lytic action on *M. lysodeikticus* (31). Hence, when exposed to appropriate nonpeptidoglycan substrates, some of the lytic glycosidases appear not to have the seemingly strict lysozyme- or chitinase-type of specificity that is suggested by their activity on the isolated wall peptidoglycans.

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