Endo-N-acetyl-glucosaminidase from Clostridium perfringens, Lytic for Cell Wall Murein of Gram-Negative Bacteria

H. H. Martin and Stefanie Kemper
Institut für Mikrobiologie, Technische Hochschule, D 61 Darmstadt, Germany

Received for publication 5 February 1970

An endo-N-acetyl-glucosaminidase which degrades the murein (peptidoglycan) sacculi of the cell walls of Escherichia coli and Spirillum serpens, but not those of Micrococcus lysodeikticus and Sarcina lutea, is present as a contaminant in a "phospholipase C" from Clostridium perfringens. The specificity of enzyme action was elucidated by reduction of liberated glycosidic groups with NaBH₄ and identification of glycosaminol as the reduction product. This finding contradicts previous reports associating cell wall breakdown with specific phospholipase action.

Bacterial cell wall mureins (peptidoglycans) can be lysed by a variety of enzymes which specifically degrade the glycan chains within the polymer network. The glycan is a heteropoly-saccharide of alternating N-acetylglucosamine and N-acetyl-muramic acid subunits connected by β-1,4-glycosidic bonds. Hence, enzymatic hydrolysis of the glycan is possible at two different sites, involving the glycosidic groups either of N-acetyl-muramyl or N-acetyl-glucosaminyl residues. Egg white lysozyme and numerous other endo-N-acetyl-muramidases from plant, animal, and microbial sources attack only at the N-acetyl-muramyl groups, leaving the second type of glycosidic linkage intact. Susceptibility to lysis by N-acetyl-muramidases is widespread among mureins of gram-positive and gram-negative bacteria. In contrast, endo-N-acetyl-glucosaminidases, acting only at the N-acetyl-glucosaminyl residues of the glycan, are of rare occurrence. Moreover, the action of the few known enzymes of this type is restricted to streptococcal and staphylococcal cell walls. None has so far been found to act on cell walls of gram-negative bacteria (1, 7).

In this report, we describe the degradation of isolated "murein sacculi" [i.e., cell-shaped murein tubes (8)] of gram-negative bacteria by a hitherto unknown endo-N-acetyl-glucosaminidase. This enzyme is present in a commercial preparation of phospholipase C from Clostridium perfringens.

MATERIALS AND METHODS

Isolation of murein. Murein sacculi from Escherichia coli B were isolated by the procedure of Martin and Frank (4).

Enzymatic lysis of murein. Lysis of murein was carried out with phospholipase C from C. perfringens (Worthington Biochemical Corp., Freehold, N.J.; lot no. 8 BA). Substrate (18 mg) and enzyme (10 mg) were incubated at 37°C in 10 ml of 0.05 M NH₄HCO₃ solution at pH 7.0. In using a very high enzyme concentration, we followed the recommendation of Weinbaum et al. (9). Degradation of the murein sacculi was followed by photometry at 650 nm and was confirmed by electron microscopy.

Deproteinization. Soluble murein degradation products were deproteinized by precipitation of protein with chloroform-octanol (4:1) or by use of 4.5% (w/v) perchloric acid and removal of perchloric acid as KClO₄.

Assay of reducing sugars and amino sugars. Reducing sugar was determined by the method of Nelson (5) and Somogyi (6). The Elson-Morgan reaction for the identification of amino sugars was applied according to the modification described by Ghuysen et al. (2).

Reduction with NaBH₄. Reduction of glycosidic groups of amino sugar was carried out by the reaction of 2 mg of murein or murein degradation products with 10 mg of sodium borohydride in 3.5 ml of 0.02 N Na₂HPO₄ solution for 3 hr at 4°C.

Amino sugar and amino acid analysis. For quantitative analysis of amino sugars and amino acids, samples of murein and its split products were hydrolyzed for 14 hr in 4 N HCl at 100°C and were assayed on an automatic BC 200 amino acid analyzer (Biocal Instrument G.m.b.H., München-Gräfelfing, Germany). Glucosamine and basic amino acids were separated on a 0.9 × 24 cm column (A) eluted with 0.35 N sodium citrate-HCl buffer at pH 5.28. Resolution of muramic acid and neutral and acidic amino acids was performed on a 0.9 × 54 cm column (B) eluted with 0.2 N sodium citrate-HCl buffer, pH 3.00, followed by 0.2 N sodium citrate-HCl buffer, pH 4.10.
RESULTS
Lytic activity of the phospholipase-associated enzyme on purified murein sacculi of E. coli B is shown in Fig. 1. Electron microscopy of samples taken after the maximal decrease of turbidity revealed complete disappearance of the typical cell-shaped sacculi from the lysate. Isolated murein sacculi from Spirillum serpens (3) were also disintegrated by the enzyme under the same conditions, whereas mureins of two gram-positive bacteria, Micrococcus lysodeikticus and Sarcina lutea, with known high sensitivity to lysozyme, proved resistant to lysis by "phospholipase C."

We studied the mechanism of murein degradation by analysis of the solubilized fragments of E. coli B murein after deproteinization. Attempts to remove the phospholipase C protein from the reaction mixture by precipitation with either chloroform-octanol or perchloric acid were only partially successful. Small amounts of material containing several amino acids, presumably in the form of small peptides, persisted in the preparation. The original presence of these non-protein amino acids in the phospholipase C stock was confirmed by the observation of equally incomplete deproteinization of controls containing only phospholipase C in phosphate buffer. The resulting contamination of the E. coli B murein lysate with foreign amino acids prevented precise quantitative analysis of the peptide moiety of the solubilized murein. However, since the nonremovable portion of the phospholipase contained no amino sugars, it did not interfere with the characterization and quantitation of the glycan breakdown in the E. coli B murein. Degradation of the glycan was first recognized by measuring the appearance of reducing sugar in the lysate and comparing with a standard curve obtained with graded amounts of N-acetylglucosamine. Portions of the deproteinized lysate containing 180 µg of original E. coli B murein gave values corresponding to 34 µg of free N-acetylglucosamine. Control runs with intact murein sacculi of E. coli B and deproteinized phospholipase C solution in phosphate buffer were negative. We attempted direct identification of the liberated amino sugar residues by use of the Morgan-Elson reaction. Samples of 715 µg of murein lysate produced by the action of the phospholipase C-associated enzyme gave a weakly positive result, whereas strong color development was produced from the same amount of E. coli B murein after digestion with egg white.

![Graph](https://example.com/graph.png)

**Fig. 1. Lysis of murein sacculi of Escherichia coli by "phospholipase C" from Clostridium perfringens.**

| Table 1. Molar ratios of amino sugars and amino acids in intact murein sacculi and in murein degradation products before and after reduction with NaBH₄

<table>
<thead>
<tr>
<th>Prepn</th>
<th>Glucosamine</th>
<th>Muramic acid</th>
<th>Glutamic acid</th>
<th>Diaminopimelic acid</th>
<th>Alanine</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>0.90</td>
<td>0.85</td>
<td>1.02</td>
<td>1.00</td>
<td>1.82</td>
</tr>
<tr>
<td>(b)</td>
<td>0.83</td>
<td>0.78</td>
<td>0.95</td>
<td>1.00</td>
<td>1.69</td>
</tr>
<tr>
<td>(c)</td>
<td>0.98</td>
<td>0.81</td>
<td>(1.61)*</td>
<td>1.00</td>
<td>(1.99)*</td>
</tr>
<tr>
<td>(d)</td>
<td>0.31</td>
<td>0.81</td>
<td>(1.63)*</td>
<td>1.00</td>
<td>(2.08)*</td>
</tr>
</tbody>
</table>

* Uncorrected values for glutamic acid and alanine are given in parentheses. The lower values were obtained by subtracting the amounts of contaminating glutamic acid and alanine present in amino acid-containing material of the phospholipase preparation which could not be removed by deproteinization. These were determined by deproteinizing a sample of the phospholipase C preparation alone and analyzing the soluble residue. An amount corresponding to the amount of phospholipase used for the production of the murein split products contained the following quantities of amino acids and sugars as compared with the value of 1.00 for diaminopimelic acid: lysine, 0.28; histidine, 0.13; aspartic acid, 0.40; threonine, 0.28; serine, 0.34; glutamic acid, 0.59; glycine, 0.60; alanine, 0.23; isoleucine, 0.15; leucine, 0.35; tyrosine, 0.11; phenylalanine, 0.19; diaminopimelic acid, 0; muramic acid, 0; glucosamine, 0.
lysozyme. These results suggested that the glycan hydrolase present in the phospholipase C preparation acted as a glucosaminidase rather than muramidase (2). Direct proof of this assumption was obtained by quantitative amino sugar and amino acid analysis of the murein degradation product before and after chemical reduction of enzymatically liberated glycosidic groups of amino sugars with sodium borohydride. Reduction of amino sugars into the corresponding sugar alcohols was measured by determining the decrease in the amount of unreduced amino sugar present in relation to the unchanged quantities of amino acids in the lysate. Results are given in Table 1 and Fig. 2. Control experiments did not reveal the presence of significant amounts of free glycosidic groups in the glycan of intact murein sacculi of E. coli B (Table 1, lines a and b; Fig. 2a). Molar ratios of the amino sugars, glucosamine and muramic acid, and the amino acids, glutamic acid, diaminopimelic acid, and alanine, were similar before and after reduc-

![Fig. 2. Diagrams of quantitative amino sugar and amino acid analyses.](http://jb.asm.org/)

---

**Table 1**

<table>
<thead>
<tr>
<th>Amino Sugar</th>
<th>Control</th>
<th>Reduced</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcN</td>
<td>0.05</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Lys</td>
<td>0.03</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Mur</td>
<td>0.02</td>
<td>0.02</td>
<td>0.00</td>
</tr>
</tbody>
</table>

**Abbreviations:** GlcN, glucosamine; GlcNol, glucosaminol; Lys, lysine; His, histidine; Asp, asparagine; Thr, threonine; Ser, serine; Mur, muramic acid; Glu, glutamic acid; Gly, glycine; Ala, alanine; Val, valine; Dap, diaminopimelic acid; Ileu, isoleucine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine.
tion of the sacculi with NaBH₄, allowing for the somewhat variable destruction of amino sugars during acid hydrolysis. Hydrolysates of the incompletely deproteinized murein degradation product obtained by the action of the glycan hydrolase of phospholipase C contained all typical amino acids and amino sugars of the murein in addition to nonremovable contaminating amino acids from C. perfringens. (Fig. 2b and line c of Table 1). Reduction of this product with NaBH₄ (Table 1, line d) caused a decrease of the amount of glucosamine to 33% of its content in the unreduced lysate.

Moreover, glucosaminol, the reduction product of glucosamine, could be recognized directly as a separate peak eluted from column A at 72 ml, following the peak of glucosamine eluted at 64 ml. In contrast, the amount of muramic acid was the same in reduced and unreduced samples of the lysate.

**DISCUSSION**

The results clearly prove that breakdown of the E. coli B murein in the presence of the phospholipase C preparation takes place as a specific degradation of the glycan moiety by the action of an endo-N-acetyl-glucosaminidase, which must also be present in this enzyme preparation from C. perfringens.

Weinbaum et al. (9) and Whiteside and Corpe (10) recently reported on the breakdown of cell wall rigidity in E. coli B and Chromobacterium violaceum by phospholipase C from C. perfringens. They concluded that the rigid part of the cell wall of these organisms consists of both murein and phospholipid. The finding that phospholipase C exerted no "lysozyme-like" activity against the murein of cell walls of M. lysodeikticus appeared to support this hypothesis (9). This point appears now to be clarified by our observation that phospholipase-associated endo-N-acetyl-glucosaminidase acts specifically on murein of gram-negative bacteria, but is inactive against the murein of the gram-positive M. lysodeikticus and S. lutea.

We wish to emphasize that our data reconfirm the specific structure and the function of the murein sacculus as the sole shape-conferring layer in the complex cell walls of gram-negative bacteria. There is, in our opinion, no evidence for a participation of phospholipid in the formation of the rigid part of the cell wall.

**LITERATURE CITED**