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Peptidoglycan Composition in *Neisseria*

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Abstract

The composition of *Neisseria* peptidoglycan has been of scientific interest for over four decades. Initial investigations focused on discovering the mechanisms causing rising rates of antibiotic resistance in *N. gonorrhoeae* by determining differences in peptidoglycan composition in penicillin susceptible and resistant strains. The discovery that cytotoxic peptidoglycan fragments are also released by *Neisseria* furthered the interest in peptidoglycan composition. This method describes the purification, enzymatic degradation, and separation of peptidoglycan fragments by High Performance Liquid Chromatography (HPLC). It also describes the preparation of samples so that they can be positively identified by mass spectrometry.

Keywords

Peptidoglycan; HPLC; Cell Wall; *O*-Acetylation

1. Introduction

The murein sacculus is composed of chains of repeating disaccharide units with the chains linked together by peptide stems. This structural component of the cell is nearly ubiquitous in bacteria. The general architecture of peptidoglycan and the addition of disaccharide pentapeptide units is similar in nearly all bacteria. Following the insertion of glycan strands, the peptidoglycan is continuously modified to allow for cell growth, division, and separation. Peptidoglycan needs to be degraded to allow for insertion of new peptidoglycan strands without disrupting the structural integrity of the cell. Despite iterative addition of disaccharide pentapeptides units, when broken down, the macromolecular structure is composed of dozens of unique constituents.

Gram-negative peptide stems contain a diaminopimelic acid (DAP) at the third position. DAP is used to link to other DAP residues or, more commonly, to the fourth position D-Alanines (Ala) on other peptide stems. DAP-containing peptidoglycan has long been known to be an adjuvant (1,2). More recently, it has become clear that human Nucleotide-binding oligomerization domain-containing protein 1 (NOD1) is capable of detecting peptidoglycan-derived muramyl tripeptides (3). Some *Neisseria* species are unusual in that they release a significant proportion of their peptidoglycan that would have been otherwise recycled. Host cells are then able to sense these released fragments generated from the peptidoglycan

sacculus. Understanding the structure of the sacculus can allow for a better understanding of how immunogenic peptidoglycan fragments are generated.

The previous method for identifying muropeptides relied on paper chromatography and large quantities of purified peptidoglycan that could be enzymatically digested and visualized using ninhydrin (4). This method was capable of separating eleven types of peptidoglycan fragments. Just prior to the development of this method, studying the nature of peptidoglycan required radiolabeled murein sugars and/or peptide stems to detect peptidoglycan fragments that could be separated by paper or size-exclusion chromatography (5,6), but they could only identify a small subset of the total complexity. The method for separating peptidoglycan fragments by High Performance Liquid Chromatography (HPLC) was developed by Glauner and Schwarz and first published in 1983 (7). The unexpected result from these experiments was just how complex macromolecular peptidoglycan structures are. Glauner and Schwarz could identify 80 different peptidoglycan-derived compounds in *Escherichia coli* which included amino acid substitutions and DAP – lipoprotein linkages (7).

Soon after this method was pioneered, the peptidoglycan composition of *Neisseria gonorrhoeae* was determined (8). *Neisseria* peptidoglycan was, and continues to be, of special interest because of its association to β -lactam resistance. Mutations in *penA*, encoding the biosynthetic transpeptidase PBP2, can cause resistance to penicillin and other β -lactam antibiotics. One of the initial findings that was made using this method was that *penA* mutants have different peptidoglycan composition in the presence and absence of antibiotics in *N. gonorrhoeae* (9). Similar results were also reported for *N. meningitidis* (10). The separation technique was later used in combination with mass spectrometry that has now mostly replaced the chemical identification of sugars and amino acids. The new mass spectrometry techniques, including MS/MS, confirmed the sequence of amino acids and the location of *O*-acetyl groups (11).

In order to determine the peptidoglycan composition, peptidoglycan first needs to be isolated from other cellular components by boiling cells in buffered sodium dodecyl sulfate (SDS). The insoluble peptidoglycan is then washed and treated with α -amylase to remove any bacterial glycogen present. A protease is then added to remove any peptidoglycan-associated proteins. Once the peptidoglycan is isolated, mutanolysin is used to cut the β -1,4 bond between *N*-acetylmuramic acid and *N*-acetylglucosamine. The peptidoglycan fragments are isolated from any insoluble peptidoglycan and treated with sodium borohydride to prevent anomerization of the reducing sugar. The peptidoglycan fragments are separated by reversed-phase HPLC using a phosphate buffered pH gradient and methanol. Separated fragments can be visualized as peaks on a chromatogram. The identity of these peaks can be positively identified by collecting corresponding fractions and determining the mass by mass spectrometry.

2. Materials

2.1. Preparation of Peptidoglycan

1. *Neisseria gonorrhoeae* strains or isolates stored in 15% glycerol in cryovials at -80°C or under liquid nitrogen.
2. Sterile polyester-tipped applicator swabs.
3. GCB agar plates: add 15 g/L of proteose peptone no. 3, 1 g/L of corn starch, 4 g/L of dipotassium phosphate (K_2HPO_4), 1 g/L of monopotassium phosphate (KH_2PO_4), 5 g/L of sodium chloride (NaCl) and 10 g/L of agar to 1 L of distilled H_2O . Stir to mix and autoclave at 121°C and 15 PSI for 30 minutes. Allow medium to cool to 55°C and then add 10 mL of Kellogg's Supplements.
4. 100X Kellogg's Supplements: add 40 g of glucose, 1 g of glutamine, 2 mg of cocarboxylase and 5 mg of iron(III) nitrate nonahydrate ($\text{Fe}(\text{NO}_2)_3 \cdot 9\text{H}_2\text{O}$) to 100 mL of distilled H_2O . Sterilize by filtration with through a $0.22\text{ }\mu\text{m}$ filter with a syringe.
5. Polyester-tipped applicator swabs.
6. UV-VIS Spectrophotometer.
7. Fernbach baffled culture flasks.
8. GCB Liquid medium (GCB L): add 15 g/L of proteose peptone no. 3, 1 g/L of corn starch, 4 g/L of dipotassium phosphate (K_2HPO_4), 1 g/L of monopotassium phosphate (KH_2PO_4) and 5 g/L of sodium chloride (NaCl) to 1 L of distilled H_2O . Stir to mix and autoclave at 121°C and 15 PSI for 30 minutes, Allow medium to cool to 55°C and then add 10 mL of Kellogg's Supplements.
9. Sorvall RC 6+ refrigerated superspeed centrifuge, or equivalent.
10. 500 mL Nalgene PPCO centrifuge bottles, or equivalent.
11. Sorvall SLA-3000 fixed angle rotor, or equivalent.
12. 50 mL PPCO Oak Ridge centrifuge tubes, or equivalent.
13. Sorvall SS-34 fixed angle rotor, or equivalent.
14. Phosphate Buffered Saline (PBS): add 8 g/L of NaCl , 0.2 g/L of potassium chloride (KCl), 1.44 g/L of dibasic sodium phosphate (Na_2HPO_4), 0.24 g/L of monobasic potassium phosphate (KH_2PO_4) and adjust pH to 7.4 with HCl .
15. 25 mM sodium phosphate buffer, pH6. Prepare by adding 3 mL of 1.0 M dibasic sodium phosphate (Na_2HPO_4) and 22 mL monobasic sodium phosphate (NaH_2PO_4) and bring the volume up to 1 L and autoclave at 121°C and 15 PSI for 30 minutes.
16. 25 mM sodium phosphate buffer, pH6.0 containing 4% (w/v) or 8% (w/v) sodium dodecyl sulfate (SDS).
17. Boiling water bath.

18. Pipettes and pipette tips.
19. α -amylase buffer: 10 mM Tris-HCl buffer, pH 8.0 containing 30 mM NaCl, 1 mM CaCl_2 , 320 mM imidazole.
20. α -amylase. Prepare a fresh 2 mg/mL (~20 units/mL) solution in 25 mM Tris-HCl pH-7.5 before adding to samples.
21. Pronase. Stored in aliquots at -20°C at a concentration of 2 mg/mL.
22. Quartz cuvette.
23. Humidified incubator set at 37°C with 5% (v/v) CO_2 .
24. Shaking platform inside an incubator set at 37°C .
25. Tube roller drum inside 37°C incubator
26. pH meter.
27. Sodium azide (NaN_3) solution in distilled H_2O . Dilute to final concentration of 0.02% (w/v) from stock solution for use.
28. *Micrococcus lysodeikticus* ATCC No. 4698.

2.2. Peptidoglycan Digestion and Sodium Borohydride Treatment

1. Microcentrifuge tubes.
2. Muramidase (EC 3.2.1.17) mutanolysin.
3. 10 kDa MWCO spin column.
4. 0.5 M sodium borate pH=8. Prepared by adding 7.729 g of boric acid to 150 mL water and adjust pH to 8 with 10 N sodium hydroxide (NaOH). Bring volume up to 250 mL and filter sterilize.
5. Sodium borohydride (NaBH_4). Add to 0.5 M borate buffer pH 8.0 immediately before adding solution to samples to a final concentration of 2 mg/mL.
6. 85% (w/w) phosphoric acid (H_3PO_4).
7. pH paper.
8. pH meter.
9. Vortex mixer.
10. Tube roller drum inside 37°C incubator.

2.3. HPLC Separation of Peptidoglycan Fragments

1. High-performance liquid chromatography (HPLC) system with UV detection ability, a 500 μL loop, a column oven and a fraction collector.
2. 4.6 mm x 250 mm, 5 μm particle, ODS-Hypersil chromatography column.
3. 50 mM sodium phosphate Buffer A, pH4.33. (see Note 1).

4. 50 mM sodium phosphate Buffer B, pH5.10 with 15% (v/v) methanol.

3.4. Mass Spectrometry

1. Microcentrifuge tubes.
2. SpeedVac.
3. HPLC system with UV detection ability, a 500 μ L loop, a column oven and a fraction collector.
4. 4.6 mm x 250 mm, 5 μ m particle, ODS-Hypersil chromatography column.
5. 0.05% (v/v) trifluoroacetic acid in distilled H₂O: Desalting Buffer A.
6. 50% (v/v) acetonitrile with 0.05% (v/v) trifluoroacetic acid in distilled H₂O: Desalting Buffer B.
7. Access to a Positive electrospray ionization time-of-flight mass spectrometer.

3. Methods

3.1. Preparation of Peptidoglycan

Macromolecular peptidoglycan is separated from the majority of cellular components by boiling in buffered SDS. Covalently attached proteins are then digested with a proteinase, and the peptidoglycan is purified again using boiling SDS.

1. Scrape some *Neisseria gonorrhoeae* from a cryovial of bacteria stored at -80°C or under liquid nitrogen onto 8 GCB agar plates using sterile polyester-tipped applicator swabs. Incubate overnight at 37°C in a humid atmosphere with 5% (v/v) CO₂.
2. Use a sterile swab to remove the growth from 8 GCB agar plates and suspend into 25 mL of GCBL and measure optical density $\lambda_{540\text{nm}}$ (OD₅₄₀). Add the appropriate amount of suspended cells to start a 1 L of GCBL medium at OD₅₄₀ of 0.25 in a Fernbach baffled culture flask. Incubate with shaking at 125 rpm at 37°C with aeration until cells grow to an OD₅₄₀ of 1.0 (see Note 2).
3. Harvest the bacteria by centrifugation at $7,000 \times g$ for 10 min at 4°C in 500 mL centrifuge bottles.
4. Suspend the bacteria in 25 mL of cold PBS.
5. Pellet the bacteria by centrifugation at $7,000 \times g$ for 10 min at 4°C . At this point, the bacteria can be stored at -80°C for later use.

Due to the strong pH dependency of this technique and small variations that can occur making these buffers, make sure there is enough buffer for all needed runs before starting. Before running samples, prepare Buffers A and Buffer B. These buffers are made by first adding sodium hydroxide (NaOH) to create a 50 mM solution, then adding phosphoric acid to obtain the desired pH. To prevent growth, 1 $\mu\text{g/mL}$ sodium azide can be added to Buffer A. Filter and degas all buffers.

To accurately compare peptidoglycan profiles it is important to grow cultures to the same optical density. During different growth phases the proportion of peptide stems changes (12). An alternative method is to take cells directly from 40 GCB plates, but the profiles from plates will be different than those from liquid.

6. Suspend the pelleted bacteria in 10 mL of 25 mM sodium phosphate buffer, pH 6.0.
7. Place 10 mL of 25 mM sodium phosphate buffer, pH 6.0 containing 8% (w/v) SDS in an Oak Ridge tube and place the tube upright in a boiling water bath.
8. Add the suspended bacteria drop-wise using a pipette into the Oak Ridge tube with boiling SDS and boil for 30 min.
9. Remove the tubes from the boiling water bath and let them cool to room temperature.
10. Centrifuge the samples at $45,000 \times g$ for 30 min at 15°C and discard the supernatant with biohazardous liquid waste.
11. Wash the pellet by suspending it in 10 mL of 25 mM sodium phosphate buffer, pH 6.0, followed by centrifugation at $45,000 \times g$ for 30 min at 15°C and discard supernatant. Repeat this step until all the SDS has been removed (see Note 3).
12. After the last wash, discard the supernatant and suspend the pellet in 1 mL of α -amylase buffer.
13. Add 200 μ g (or approximately 5 units) of α -amylase and incubate on a roller drum at 37°C for 1 h.
14. Add 200 μ g (~200 units) of pronase and incubate on a roller drum at 37°C for 2 h or overnight.
15. Place 10 mL of 25 mM sodium phosphate buffer, pH 6.0 with 4% (w/v) SDS in an Oak Ridge tube in a boiling water bath.
16. Repeat steps 7 to 11.
17. Suspend the insoluble peptidoglycan in 1 mL of 25 mM sodium phosphate buffer, pH 6.0. Peptidoglycan can be stored at -20°C or at 4°C with 0.02% (w/v) sodium azide solution.
18. Quantify the sample concentration by measuring the absorbance in a UV spectrophotometer at $\lambda_{206\text{nm}}$ in a quartz cuvette by creating a standard curve with either lyophilized *Micrococcus lysodeikticus* or lyophilized peptidoglycan to determine sample concentration.

3.2. Peptidoglycan Digestion and Sodium Borohydride Treatment

Purified macromolecular peptidoglycan is broken down using the muramidase mutanolysin that has both 1,4-*N*-acetylmuramidase-like lysozyme and *N,O*-diacetylmuramidase-like activity, which are necessary to cleave *O*-acetylated peptidoglycan. The muramic acid of the resulting disaccharides is reduced to muramitol with NaBH₄, in order to eliminate the confusion of having two anomers with different dissociation constants on an ODS column.

A simple and effective, although somewhat crude, way to determine if there is SDS in the 20mM sodium phosphate buffer, pH 6.0 is to take advantage of the detergent property of SDS and look for bubbles after vortexing samples. Although it is not necessary, a more exact concentration of SDS can be determined using colorimetric assays with methylene blue (13) or the carbocyanine dye stains-all (14).

1. Add 200 µg of mutanolysin to 500 µL of peptidoglycan suspended in 25 mM sodium phosphate pH 6. Incubate with rotation at 37°C for 2 h or overnight.
2. Separate soluble peptidoglycan fragments by filtering them through a 10 kDa MWCO spin column and collecting the flow-through.
3. Add 500 µL of freshly prepared NaBH₄ solution (0.5 M sodium borate pH 8 with 2 mg/mL NaBH₄) to soluble peptidoglycan fragments and leave for 20 min at room temperature in uncapped tubes. Briefly cap and vortex the samples after 10 min. (see Note 4).
4. Quench the reaction by adding approximately 20 µL of 85% (w/w) phosphoric acid.
5. Check the pH and add additional 85% (w/w) phosphoric acid to bring the samples to between pH 3 and 4, if necessary.
6. Store the samples at 4°C.

3.3. HPLC Separation of Peptidoglycan Fragments

1. Set the column oven to 30°C and allow the column and detector lamp to warm up.
2. Create or select the following HPLC method: Detector at $\lambda_{206\text{nm}}$, 0.5 mL/min flow rate, 10 min of Buffer A, a linear gradient of 0 to 100% of Buffer B over 120 min, 100% of Buffer B for 20 minutes.
3. Equilibrate the column to Buffer A and then inject approximately 200 µL of sample and begin collecting fractions.
4. After the run, use the HPLC software to compare chromatograms. The area under the curve can be calculated to compare peaks corresponding to different peptidoglycan species.

3.4. Mass Spectrometry

Mass spectrometry is recommended for identification of HPLC peaks (Fig.1, see Note 5). Analyze the chromatographs and collect the fractions corresponding to peaks of interest and pool fractions if necessary (see Note 6).

1. Use a SpeedVac concentrator to remove liquid from the samples.
2. Suspend the samples in 200 µL of H₂O.

This reaction violently releases hydrogen gas; do not cap the solution.

By comparing chromatograms the identity of some peaks should be apparent. In some cases, mutants can be used to identify some peaks, like those of acetylated fragments in an *O*-acetylation mutant (Fig. 1). The certainty of other peaks may be in question due to small variations in HPLC setup or by overlapping peaks. Other factors such as buffer composition and column temperature can cause variations. It is best practice to always confirm the identity of any peaks of interest by mass spectrometry. Samples will likely need to be desalted before mass spectrometry because phosphate buffer is incompatible with many systems.

It is beneficial to take peaks from multiple HPLC runs, especially if minor peaks are being collected. Sufficient material is required to obtain an accurate mass.

3. Create or select an HPLC method: Detector at $\lambda_{206\text{nm}}$, 1 mL/min flow rate, 10 min of Desalting Buffer A, a linear gradient of 0 to 100% of Desalting Buffer B for 10 min, 10 min of Desalting Buffer B.
4. Inject the concentrated sample.
5. Collect the peak(s) of interest by comparing the chromatogram to a blank run and/or a high salt run.
6. Concentrate the samples into a single microcentrifuge tube by SpeedVac.
7. Run the sample on a mass spectrometer.
8. Identify peaks by comparing the acquired mass to that of the anticipated peptidoglycan fragment or to other published values.

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Fig. 1:

Chromatograms showing the peptidoglycan composition of two strains of *N. gonorrhoeae*. A comparison of strain FA19 and an MS11 *pacA* mutant defective in *O*-acetylation. An asterisk (*) shows the locations where peaks containing acetylated PG fragments would appear in a wild-type strain. Some peaks contain two different peptidoglycan fragments, such as the one containing Penta(OAc) and the one containing crosslinked Tetra-tetra monomers and Tri(Anh). FA19 is known to contain more dipeptide than other strains, but it is otherwise similar to other wild-type strains. Labels indicate disaccharides with two (di), three (tri), four (tetra), or five (penta) amino acids. OAc, *O*-acetylation; Anh, 1,6-anhydromuramyl moiety.