# Simple protocol to purify cell wall polysaccharide from Gram$^+$ bacteria and assess its structural integrity

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**Abstract**

Cell wall polysaccharides (CWPS), which are usually covalently bound to the peptidoglycan and are closely associated with the cell wall, are considered as ubiquitous components of a cell envelope of Gram positive bacteria and play an important role as mediators of bacterial interactions with the environment. Here, we describe a simple method for purifying CWPS by extraction of bacterial cells with consecutive acid treatments. Purified CWPS are obtained by gel-filtration chromatography following treatment with HF. We also provide the methodology to easily assess the integrity of CWPS using High-Resolution Magic-Angle Spinning (HR-MAS) NMR.

## 1 Introduction

Cell wall polysaccharides (CWPS) are considered as ubiquitous components of a cell envelope of Gram positive bacteria. Unlike capsular polysaccharides (CPS), forming a thick outermost shell of the cell wall, and exopolysaccharides (EPS), loosely bound to bacterial cells and released in the growth medium, CW PS are usually covalently bound to the peptidoglycan and are closely associated with the cell wall. Because of their localization at the bacterial surface, they are mediators of bacterial interactions with the environment such as adhesion to abiotic surfaces, specific interactions with other microorganisms, eukaryotic host cells, and infecting bacteriophages [1].

Knowledge of the detailed chemical structure of CWPS of Gram positive bacteria is essential for understanding of molecular mechanisms of their various functions. Structurally different CWPS form the basis of some serological typing systems, such as Lancefield classification of *streptococci* [1]. Precise structures of CWPS acting as co-aggregating receptors for the formation of the dental plaque have been described for several oral bacteria, both Gram positive and Gram negative [1, 2]. It was shown that structural differences of CWPS of *Lactobacillus helveticus*, used in cheese manufacturing, are affecting its autolytic properties [3]. Another example is a recently described mechanism of action of Glycerophosphodiester phosphodiesterase (GDPD) of bacteriophage 17 infecting *Lactobacillus delbrueckii* subsp. *bulgaricus* Ldb17, which hydrolyzes the Gro3P decoration of the major CWPS (Gro3P-substituted linear D-galactan) of Ldb17 [4].

It is important to point out that often several CWPSs in variable quantities are present in one bacterial strain, some more surface-exposed, others more tightly bound to the peptidoglycan (PG). As an example, the cell surface of *L. delbrueckii* subsp. *bulgaricus* Ldb17 was shown to contain at least two different cell surface polysaccharides, in addition to a CWTA [5]. In *Lactococcus lactis* MG1363, a CWPS referred to as PSP, "polysaccharide pellicle" forms a compact pellicle on the outer surface of the cell [6]. A rhamnan, another CWPS of *L. lactis*, is trapped inside the peptidoglycan and is covalently bound to it. It appears to be conserved component of the lactococcal cell wall and plays an essential role in cell division and morphogenesis in *L. lactis* cells [7].

The majority of other *L. lactis* strain studied to date have a CW architecture similar to MG1363 and have two distinct CWPSs: a PSP which is often composed of oligosaccharide repeating units linked with a phosphodiester bonds, and a neutral rhamnan [8–10]. The two polysaccharides are often covalently linked. Crude trichloroacetic acid (TCA) extracts *(see* **Note 1***)* contain both polysaccharides in different proportions, which often co-migrate as a broad peak. HF treatment, cleaving the phosphodiester bonds in PSP leads to its depolymerization and liberation of free rhamnan and oligosaccharide (OS) fragments of PSP (Fig. 1).

The extraction method described here is adapted essentially from Prakobpol et al. [11], and allows preparing samples of purified CWPS strongly bound to PG after a pre-extraction of other surface polysaccharides. Briefly, the extraction is carried out by hot HCl solutions (0.01 N and 0.1 N, consecutively). Proteins and nucleic acid debris are precipitated by addition of TCA (*see* **Note 2**). Crude CWPS is obtained after dialysis and lyophilisation. Treatment of the crude CW PS with 48% HF and following gel-filtration chromatography allow efficient depolymerisation and removal of contaminating nucleic acids, as well as teichoic and lipoteichoic acids (Fig. 2). However, for structural analysis of CWPS carrying phosphate groups, a different strategy should be adopted. This simple method afforded pure preparations, suitable for structural analysis, in case of various gram positive bacilli and cocci [7, 12]. A panel of conventional methods including monosaccharide composition analysis, methylation analysis, mass-spectrometry and NMR may then be used to elucidate the structure of purified polysaccharides.

Not only that extraction procedure should be simple and consistent, but it should also keep the extracted polysaccharide as intact as possible. However, considering that the vast majority of structural analysis methodologies require polysaccharides to undergo extensive purification processes, it is very difficult to assess to what extent the final structure reflects the native cell-surface expressed polysaccharide. This can be achieved using High-Resolution Magic-Angle Spinning (HR-MAS) NMR that enables the acquisition of NMR spectra from small amounts of viscous or solid samples, with a resolution equivalent to liquid NMR analysis of soluble molecules. Indeed, by spinning at the magic angle, line broadening effects due to dipolar interactions and susceptibility differences within the sample are removed resulting in high-resolution quality spectra. In the field of glycobiology, this technique enables an easy and sensitive read out of surface components on intact cells. Among other applications, it was previously effectively used to analyze cell wall polysaccharides from yeasts and bacteria without proceeding to any potentially denaturing purification step [13–15]. We will provide here the methodology to easily assess the integrity of polysaccharides using HR-MAS NMR and show that the easy extraction method of CWPS

from Gram+ bacteria does not modify the structure of purified polysaccharides by comparing the structural features of the polysaccharides before and after extraction procedure.

## 2 Materials

### *2.1 Extraction of the CWPS*

1. Centrifuge and centrifuge tubes.

3. Dialysis tubing (Visking), dialysis reservoirs, closures.

4. Lyophilizer and adapted glassware.

5. Trichloroacetic acid (TCA) solution, 50% (w/w): in a glass beaker, weigh 100 g of TCA, and add 100 mL of water. Stir in a magnetic stirrer till the TCA is completely dissolved, transfer into glass bottle (*see* **Note 3**).

6. 0.01 M and 0.1 M HCl.

### *2.2 Chromatography*

1. Empty columns for gel permeation chromatography: 1 x 40 cm; 1 m x 2.6 cm for larger quantities.

2. Chromatography media Sephadex G-50 fine (GE Healthcare).

3. 0.01% AcOH (acetic acid) in milli-Q water.

### *2.3 HR -MAS*

1. 4 mm $ZrO_2$ MAS rotors (CortecNet, Paris, France).

2. Disposable inserts for 4 mm rotor with sealing screws and plugs (CortecNet, Paris, France).

## 3 Methods

### *3.1 Extraction of CWPS*

Extraction and purification steps of CWPS are summarized in Fig. 2.

1. Cells (7-10 g) are washed twice with water (*see* **Note 4**).

2. Suspend cells in 5% TCA (~8 mL per 1 g of cells) in a 250 mL or 500 mL glass bottle. Stir on a magnetic stirrer for 48 h at 4-6 °C.

3. Transfer into appropriate 35 mL centrifuge tubes. Centrifuge (10 000 *g*, 10 min). Clear supernatant (TCA extract) usually contains surface PS more loosely bound to the CW (if present) and CWPS in different proportions. Dialyze (*see* **Note 5**) and lyophilize to obtain the crude TCA extract.

4. Suspend the cells in 0.01 M HCl (~100 mL) and transfer them into a glass bottle. Stir for 20 min in a boiling water bath.

5. Cool down and transfer into centrifuge tubes. Centrifuge (10 000 *g*, 10 min). Clear supernatant contains the crude 0.01 M HCl extract.

6. Suspend the cells in 0.1 M HCl (~100 ml) and proceed as in step 4. Clear supernatant contains crude 0.1 N extract. Discard the pellets.

7. Measure the volume of the clear supernatant and transfer it into a glass beaker. Add 1/10 volume of the 50% TCA, in order to precipitate eDNA and proteins (*see* **Note 2**).

8. Centrifuge (10 000 x g, 10 min). Discard the pellet and collect the supernatant.

9. Prepare dialysis tubing by cutting an appropriate length, soaking and closing one edge with a closure or by making a knot. Fill approx. two thirds of the volume of the tubing with the supernatant and close the tubing at the top.

10. Dialyze against water for at least 48 hrs (*see* **Note 5**).

11. Freeze-dry. The obtained material corresponds to a crude preparation of CWPS (*see* **Note 6**).

### *3.2 Purification of CWPS*

1. Under the fume-hood, add 48% HF (50 µL) to the crude HCl extract (10 mg in an Eppendorf tube, *see* **Note 3**). Make sure the extract is dissolved completely. Keep 24 h at 4°C.

2. Evaporate HF under a stream of nitrogen at room temperature.

3. Take the extract in distilled $H_2O$ (1 mL) and fractionated on a Sephadex G-50 column, eluted with 0.1% acetic acid. Fractions are collected and assayed for neutral [16] and, if necessary, amino sugars (Fig. 1).

4. Fractions corresponding to CWPS are pooled and lyophilized.

### *3.3 HR-MAS NMR analysis of intact cells*

1. Cells are washed twice with water and pelleted down by centrifugation in a 1 mL microcentrifuge tube (*see* **Note 7**).

2. Bacterial pellets are washed once with two volumes of $D_2O$ then incubated for 2 hours in two volumes of $D_2O$ with 0.5% (v/v) acetone as internal standard.

3. HR-MAS rotor is loaded as described in Fig. 3. Bacterial paste is first loaded into 4 mm rotor mm $ZrO_2$ MAS rotors by low speed centrifugation (5 min at 600 g) through a 1 mL pipette tip in a plastic 4 mL tube. The pipette tip is removed and the rotor is transferred into a 1 mL plastic microcentrifuge tube. The bacteria are then compacted in the rotor by higher speed centrifugation (5 min at 10.000 g). Supernatant $D_2O$ is removed by suction using a 100-200 µL automatic pipette and the process is repeated until the rotor is filled up with

compacted bacterial paste. Depending on the consistency of the bacterial suspension, it may take two to four rounds to fill up the rotor.

4. Rotor is inserted into HR-MAS NMR probe, tilted at 54,7° and spun at 8kHz. NMR experiments are run using dedicated HR-MAS-NMR pulses. The most useful pulses for analysis of intact cells are 1D $^{1}$H, 2D $^{1}$H-$^{1}$H COSY and $^{1}$H-$^{13}$C HSQC experiments, with or without water suppression.

5. HR-MAS NMR analysis of intact bacteria generates spectrum very similar to liquid NMR analysis of the purified CWPS isolated from the same bacterial strain. As exemplified in Fig. 4, the liquid NMR $^{1}$H-$^{13}$C HSQC spectrum of CWPS isolated from *Lactococcus lactis* MG1363 by HCl extraction shows NMR signals (A-F) associated to the hexasaccharide repeat unit [-6)βGlcNAc1-3αRha1-3(αGlc1-6)βGlcNAc1-2βGal*f*1-6αGlc-P-]$_n$, as previously identified [6]. In addition to the signals associated to the intact polysaccharide, two minor signals A1'a and A1'b were identified as terminal reducing αGlc and βGlc residues resulting from the partial cleavage of αGlc-P bond. The HR-MAS NMR $^{1}$H-$^{13}$C HSQC spectrum of the intact *Lactococcus lactis* MG1363 bacteria show identical signals than the purified CWPS, albeit the absence of A1'a and A1'b signals.

## 4 Notes

1. It is recommended to treat bacterial CWs with hot SDS prior to extraction of CWTAs and CWPS [17] in order to eliminate LTA and other lipophilic compounds. However, in some strains, hot SDS was shown to extract also relatively hydrophobic CWPS, such as surface-associated polysaccharides sPS1 from *Lactobacillus delbrueckii* subsp. *bulgaricus* Ldb17, composed of repeating units containing Glc, Gal, Rha, and Rha$_2$OAc [5]. Teichoic acids of *Listeria monocytogenes* were also partially extracted with SDS [18]. We therefore recommend to omit the SDS treatment step to prevent losses of CWPS.

2. In our experience, precipitation with TCA is an efficient method of removal of protein and nucleic acids. It can be used instead of enzymatic digestions.

*3.* TCA and HF are very aggressive agents causing burns. Always wear gloves and work in the fume hood. Please keep in mind to never use glass when manipulating HF.

4. The suspension of cells in water should not be very thick. We typically suspend the cells in water in large (350 mL) centrifuge tubes with a glass rod. 6,000 g for 20 min are usually sufficient to spin down the cells. However if the pellet is “slimy”, add more water to the suspension and /or increase centrifugation time.

5. Water should be changed several times during the first hours of dialysis, then twice a day. The efficiency of dialysis can be checked with the simple pH paper placed on the bottom of the dialysis reservoir, detecting the accumulation of acid. The volume of dialysate increases significantly during dialysis. Neutral pH of the solution indicates that dialysis is complete.

6. Sometimes the crude extract is not completely soluble in water. Insoluble material usually represents protein and lipid impurities. Take the material in water and remove the insoluble material by centrifugation (14,000 g, 5 min).

*7.* 100 μL of packed bacteria is required to fill up a 4 mm rotor. This volume may be reduced down to 25 μL when disposable inserts are used with 4 mm rotors. Filling up the rotors or the insert, requires about 2-3 times the final volume of bacterial pellets in order to ease the handling of biological material.

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**Figure legends**

**Figure 1.** The majority of *Lactococcus lactis* strain studied to date have two cell wall polysaccharides : a surface polysaccharide pellicle, PSP which is often composed of oligosaccharide repeating units linked with a phosphodiester bonds, and a neutral rhamnan CW PS [6, 8]. The two polysaccharides are often covalently linked. TCA extracts contain both polysaccharides in different proportions, which often migrate as a broad peak. HF treatment, cleaving the phosphodiester bonds in PSP leads to its depolymerization and liberation of free rhamnan and OS fragments of PSP (A). The figure shows a representative elution profile of the TCA extract of *L. lactis* SK11 on a Sephadex G-50 column (1 x 40 cm) before and after HF treatment (B).

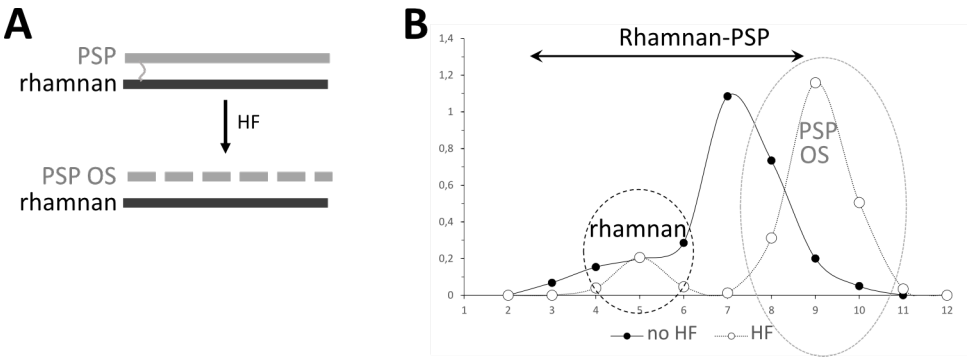


**Figure 2.** Purification pipeline of CWPS from Gram$^+$ bacteria. Purification steps described in section 3.2.1 to 3.2.4 may be applied either to CWPS1 and CWPS2 independently or to the pooled CWPS extracts from 0.01 (section 3.1.4; 3.1.5) and 0.1M (section 3.1.6 to 3.1.10) HCl

extraction steps. TCA extract may also contain a certain amount of loosely bound CWPS and may be submitted to purification steps described in section 3.2.1 to 3.2.4.

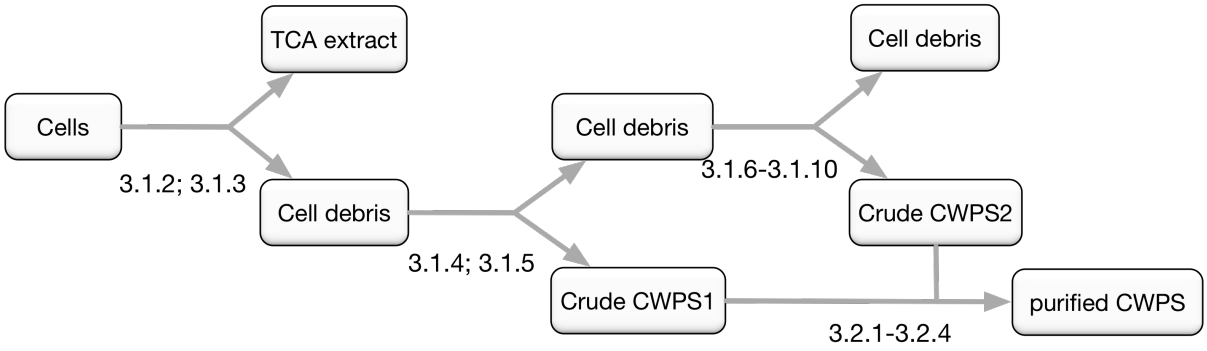


**Figure 3.** HR-MAS rotors or inserts for HR-MAS rotor are loaded with bacteria as previously described by Hanoulle and collaborators [19]. Briefly, (1) 1mL pipette tips are shorten and (2) inserted into a 4 mm $ZrO_2$ MAS rotors; (3) about 100 μL of bacterial paste is loaded into the tip with automatic pipette; (4) the rotor and tip are inserted into a 4 mL plastic tube and centrifuged at low speed (600 g, 5 min); (5) the tip is removed and (6) the rotor is spun at high speed (14000 g, 10 min) into a 1mL microcentrifuge tube; (7) the supernatant $D_2O$ is removed by suction and the process is repeated from (2) until the rotor (8) or the insert is filled up (9).

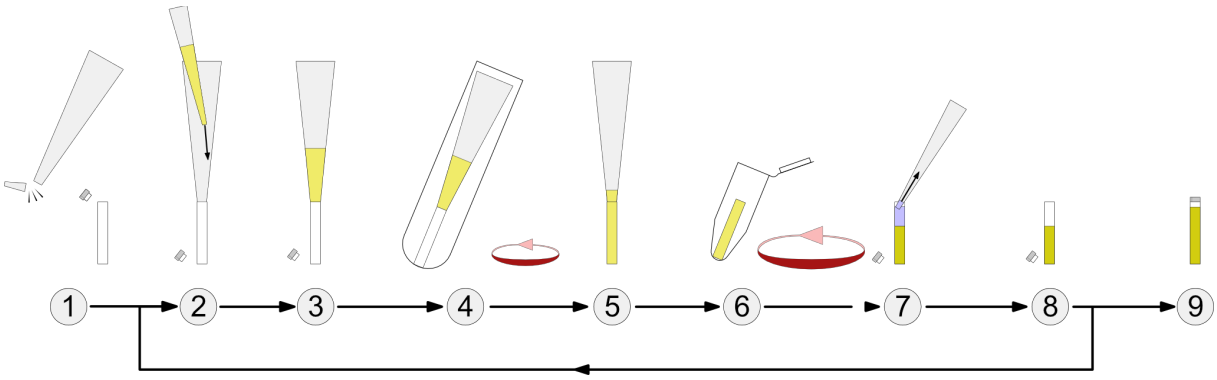

**Figure 4**. Comparison of the $^{1}H$-$^{13}C$ HSQC spectra from (left panel) the liquid NMR analysis of purified CWPS purified from *Lactococcus lactis* cell wall and (right panel) the HR-MAS NMR analysis of total *Lactococcus lactis* [8].