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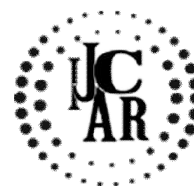

Research Article

ANALYSIS OF IMPURITIES BY X-RAY DIFFRACTION AFTER PURIFICATION PROCESS OF THE BACTERIAL POLYSACCHARIDE STREPTOCOCCUS AGALACTIAE

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A B S T R A C T

Through this study it is clear that each purification process of the bacterial polysaccharide is different, obtaining different results of X-ray diffraction patterns. The MRI (magnetic resonance imaging) methodology for the characterization of polysaccharides during the production of vaccines is currently used and there are few studies conducted by X-ray diffraction for molecular characterization in the vaccine area. The use of X-ray diffraction can contribute to a better description of the processes and thus the reduction / removal of impurities caused by the decomposition of the product or the manufacturing process, improving the discussion of the potential impact on quality, safety and efficacy.
Polysaccharides are high molecular weight polymers with repeating units composed of glucose, galactose, N-acetylglucosamine and sialic acid. Some bacterial capsular polysaccharides possess virulence factors and are responsible for pathogenicity. The bacterial strain of Streptococcus agalactiae ATCC 12386, corresponding to serotype Ia, was cultivated and four different polysaccharide purification processes with different reagents were performed. X-ray diffraction was carried out to evaluate the identification of molecules of the polysaccharide. Through this study, could be observed that, each purification process of the bacterial polysaccharide is different, obtaining different results of X-ray diffraction patterns. The magnetic resonance imaging methodology for the characterization of polysaccharides during the production of vaccines is currently used, wherein it is proved that the X-ray diffraction could be a useful tool as a complementary molecular characterization methodology for identifying impurities in the purification process of a biological product.The present article focuses on the possibility of using the X - ray diffraction technique as an analytical methodology to evaluate the purification process, free of impurities. It can be useful as methodology of process route analysis and identification of contaminants. At work, we are not characterizing the monosaccharides and their composition, but it would be interesting more studies to compare the purity of materials.

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INTRODUCTION

Streptococcus agalactiae (group B streptococci, GBS) is a commensal bacterium, being part of the healthyhuman microbiota. Besides beasymptomatically in the gastrointestinal and genitourinary tract. *S. agalactiae* was recognized as a leading cause of neonatal sepsis and meningitis (BELLAIS *et al.* 2012; CI, BAKER; EDWARDS, 1988).Recently as an important pathogen in adults with underlying medical conditions (DUTRA *et al.* 2014, AUPÉRIN *et al.* 2005). Its taxonomy is shown in table 1. Bergey and colleagues (1934) present *Streptococcus* spp. as Gram-positive, catalase-negative bacteria, they are in the form of spheres, with a diameter less than 2 µm (TORRES, 2012; BERGEY, 1934).

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Group B streptococcal (GBS) are classified into ten (10) serotypes based on different type of polysaccharides (types Ia, Ib, II, III, IV, V, VI, VII, VIII, IX). About 40% of invasive diseases are caused by the capsular polysaccharide (CPS) types Ia or Ib, of GBS isolates (GLASER *et al.* 2002).

Polysaccharides are high molecular weight polymers with repeating units composed of glucose, galactose, N-acetyl glucosamine and N-acetylneuraminic acid (sialic acid) (JENNINGS *et al.* 1984).Each serotype produces a specific polysaccharide with a regularly repeating sequence of at least two saccharide units (ISAAC *et al* 1978).Most of the bacteria produce polysaccharide with high molecular weight with acidic isoeletric points. These polysaccharides are highly charged and their polar nature provides multifunctional role of them (ISAAC *et al* 1978).The capsular polysaccharide (CPS)

is an important virulence factor of GBS, a major antigenic factor and confers immunological specificity.

The physicochemical properties of polysaccharides are configured by their sugar sequences derived through biosynthetic enzymes and results from many bacterial polysaccharides. They can be identified as having original chemical structures, produced with different composition and consequently, different molecular weights. They are water soluble, usually biocompatible, often increase the viscosity of the solvent. The knowledge of CPSs chemical structure and physical properties enable the improvement of bacterial engineering for the development of the biopharmaceutical products, vaccines and others. (DUMITRIU, 2004).

Therefore, understanding the molecular conformation of CPS is relevant because the different compositions are responsible for the different bacterial antigenicity. The understanding of the molecular structure and how the processes of production and purification affect its conformation, help in the optimization of the vaccine production process.

Biological Properties

Bacterial polysaccharides are described as "slimes" and, their present conformation as gel-like structure provide protection against bacteriophages, shock absorption and mechanical damage (ISAAC *et al.* 1981; ISAAC *et al* 1978; CERNING 1990; DAIN *et al.* 1956; CHRISTENSEN, 1989). This gel-like feature allows the binding of large quantities of water and ions, preventing dehydration of the cell and provides accessible storage, in which the cell is easily able to absorb water and ions (ISAAC *et al* 1978).

The biological properties and repeating unit structures of polysaccharides are diverse, and several species of streptococci produce extracellular polysaccharides in the form of secreted exopolysaccharides or cell associated capsules (CIESLEWICZ *et aI* 2001; ISAAC, 1981; CHRISTENSEN, 1989, VU, 2009).These extracellular polysaccharides are a complex mixture of monosaccharide and disaccharide biopolymers as well as proteins and nucleic acids. It has as main functions, mediation in adhesiveness in different substrates and protection against phagocytosis and dehydration (ISAAC, 1981; CHRISTENSEN, 1989, VU, 2009).

The capsular polysaccharide (CP) of *Streptococcus agalactiae* type Iais a high molecular weight polymer, consisting of a pentasacchariderepeating unit with the molecular formula described as4)-[α-D-Neu*p*NAc-(2-> 3)-β-D-Gal*p*-(1->4)-D-Glc*p*NAc-(1->3)]-β-D-Gal*p*-(1->4)-β-D-Glc*p*-(1
(YAMAMOTO *et al.* 1999).Subunit structures of CPs from S. agalactiae type Ia, type III and S. pneumoniae serotype 14. Glc, glucose; Gal, galactose; GlcNAc, N-acetylglucosamine; NeuNAc, N-acetylneuraminicacid (YAMAMOTO *et al* 1999).
Analysis of type IX polysaccharide from GBS revealed a structure similar to type V and VII and repeating unit structures of polysaccharides (BERTI *et al*, 2014).

A variety of bacteria produce extracellular polysaccharides that surround the cell wall or are more dispersed in the culture fluid.The study of extracellular structure of polysaccharides has been a field of great interest in food, pharmaceutical, biomedical, supplementation and bioremediation. This fact is due to its structural diversity, its physical properties, rheology and other unique properties. For example, the use of microorganisms in the treatment of environmental effluents produced by the mining and metallurgical industries, to oxidize solid compounds, resulting in soluble and extractable elements. The extracellular chemical structure of the polysaccharide varies according to the type of substrate in which the cells are cultured. The mode of binding also presents differences as a function of the substrate and therefore a differentiation occurs in the expression of polysaccharide capsule genes (VU, 2009).

The capsular polysaccharide from type II of *Streptococcus agalactiae* contains D-galactose, D-glucose, 2-acetamido-2-deoxy-D-glucose and sialic acid in the molar ratio of 3:2:1:1. GBS type II antigen contains terminal β-D-galactopyranosyl residues in addition to the terminal sialic acid residues and is not sensitive to neuraminidase (JENNINGS*et. al.*1983; VAN CALSTEREN*et al*. 2010). The different antigenic types of GBS capsular polysaccharide are chemically related, but they are different from an antigenic standpoint,due to the different linking's of the main trisaccharides (Β-D-GlcpNAc(1->3)β-D-Galp(1->4)β-D-Glcp) (VAN CALSTEREN *et al*. 2010).

Structural Conformation of Capsular Polysaccharides

The conformation of capsular polysaccharides consists of a large variety of polymers. Their chemical structures and physical properties vary considerably according to these structural conformations. The putative function of the enzymes involved in the assembly of the type IX CPS is indicated by *arrows*. Different monosaccharides are added to the conformational structure according to the enzymes involved in the reaction of glycosyltransferases (BERTI *et al* 2014).

Thus, the structural conformation of the polysaccharide capsule is a very important factor since it determines the binding affinity for the cellular epitopes. For example, *Streptococcus agalactiae* or B-type *Streptococcus* (GBS) type VIII and *Streptococcus pneumoniae* type 23F share the sequence Neu5Ac-Gal-GlcNAc-Gal in common with the CPS of GBS Ia, Ib, II, III and IV, but differ inthe presence of rhamnose. The presence or absence of rhamnose confers antigenicity and different virulence (BERTI *et. al* 2014; VAN CALSTEREN, *et al.* 2010). BERTI *et al*. 2014 demonstrated the importance of structural conformation by comparing type IX $_{SPC}$ with types VII and V of GBS.

From the X-ray diffraction studies, the polysaccharide of pneumococcal streptococcal type II was identified as having crystalline conformation with the composition: poly [(1β3) -β-D-Glc*p*A- (14) -β- D-Glc*p*]. Possibly the conformation is helical, with helix-like extensions and gel structure, protecting against bacteriophage (MARCHESSAULT *et al*. 1980).

X-ray diffraction analysis of the *Escherichia coli* K29 capsular polysaccharide serotype and the two mutants, M13 and M41 showed that the chemical structure consists of a disaccharide backbone with hexamer repeats with side branches of disaccharides. (MOORHOUSE, 1977).The molecule of the capsular polysaccharide of *Klebsiella* serotype K25has a tetrasaccharide repeating structure consisting of a disaccharide backbone and a disaccharide side chain. The analysis of the diffraction patterns gives rise to a triple helical conformation of polysaccharide (ISAAC, 1981).

The chemical and physical conformations of each bacterial polysaccharide serotype, present differences between them (ISAAC*et al.*,1981; VAN CALSTEREN, *et al.* 2010). For ISAAC 1978, *Klebsiella* capsular polysaccharide serotype

K57/ K57 is a polytetrasaccharide that contains galactosyluronic acid, mannosyl, and galactosyl residues in the backbone, and an additional mannosyl group as a side-appendage. Possibly has a crystalline structure of three-fold helix. Bacterial polysaccharides have double helix structures, and the study of the conformation of single and parallel and antiparallel chains is essencial for conformational identification and analysis OKUYAMA, K. *et al*. SLETMOEN, *et al.* 2003).

Currently, nuclear magnetic resonance (NMR) is extensively applied to investigate and analyze the polysaccharide structure (BUNDLE *et al*., 1974) and the X-ray diffraction technique is not commonly used (BUNDLE *et al*., 1974).

A technique widely applied to investigate and analyze the polysaccharide structure is nuclear magnetic resonance NMR (BUNDLE *et al*., 1974). However, this is not the case with the X-ray diffraction technique.

Purification of Capsular Polysaccharides

Vaccine manufacturing designs normally present significant challenges, because they depend on processes based on factors such as biochemical diversity and variety of purification protocols. The product specificity affects significantly the design of facilities, product purification platform, ahigh capital requirement due to this specificity, process validation and quality control (BALL *et. al* 2009).

The manufacturing process of vaccines can be separated into three important steps: upstream processing, downstream processing (purification / clarification) and formulation. Purification and clarification process is an essential step to remove large and small particles, whole cells, debris, residual cells, DNA and RNA. Improper optimization of purification can affect substantially not only the process but the purification of the final product as well as the results of characterization (TANIZAKI, 1996).

Several bacterial pathogens have developed new ways to escape immune detection by mimicking host cell surface carbohydrates that are crucial for self/non-self recognition and which confer important properties upon the cell surface (WESSELS *et al.*1989, FINLAY *et al* 2006). Sialic acid is a terminal residue of these carbohydrates. Sialylation of capsular polysaccharide (CPS) is a significant factor for virulence of GBS(WESSELS *et al,*.1989).Many pathogenic bacteria have also evolved to cover their cell surfaces with sialic acid, which results in different phenotypes in their ability to resist the host's innate immune response and their ability to interact with different host-cell surfaces. The most abundant and best-studied sialic acid is N-acetylneuraminic acid (Neu5Ac), although there are numerous naturally occurring variations (CHAFFIN 2005).

For CHAFFIN (2005), sialylation is critical for the physicochemical properties of CPS and is also critical for the biosynthetic process. Capsular sialic acid is a virulence determinant for type III group B *Streptococcus* and supports the general hypothesis that surface sialylation aids pathogenic microorganisms in evading host defenses. Culturing and purification processes can generate differences in the structural conformation of CPS, and consequently, the characterization analyzes will provide diversity in the data due to such differences. All GBS capsules have a sialic acid [N-acetylneuraminic acid [Neu5Ac]] attached to the α2-3 terminal, which interferes with complement-mediated death (LEWIS*et al.,* 2004; LEWIS *et al.,* 2006). Sialic acid occupies the terminal position within the glycan molecules on the surfaces of the bacterium.

The polysaccharide *Streptococcus agalactiae* has a variable molecular weight between 800 kDa and 1800 kDa. The culture medium and nutrients influence the production of microorganisms and the size of the polysaccharide chain. The method of extraction, purification and recovery of the polysaccharide causes the breaking of the polysaccharide repeating units longer in shorter fragments. Depending on the size of the fractional unit, the composition of the monomers diverges, and this information is important for the characterization and development of related products, such as polysaccharide vaccines, ensuring a safer and more effective product (BALDUCCI *et al*. 2017).

X-ray diffraction of polysaccharides

The conformation of capsular polysaccharides consists of a great variety of polymers, a fact that explains the great variability of their chemical structures and physical properties. Powder X-ray diffraction (XRD) experiments provide information on the crystalline forms in the samples and may be associated with polymorphisms with data obtained from XRD (GUBICA *et al.* 2009).The x-ray difference pattern serves as a molecule identifier under certain conditions.

The biological properties of polysaccharides are related to their composition and structure. Many factors, such as sugar, binding, molecular weight or biopolymer sulfate content, influence the relationship between structure and biological function (GÓMEZ-ORDÓÑEZ *et al*., 2012).The conjugation of a protein in the formulation of polysaccharide vaccines is important to stimulate the immune response. Thus, the study of X-ray diffraction and its analysis of composition are critical to planning the best method for conjugation with a protein.

X-ray diffraction analysis, for the characterization of different phases of crystalline materials, collaborates to identify the chemical components and the morphological structure through the identification of the interplanar distances (SLETMOEN *et al.*, 2003)..The diffraction patterns of different purification methods may be different, since the presence of different components during the process, generate different geometries of the unit cell

MATERIAL AND METHODS

Bacterial strain: The bacterial strain of *Streptococcus agalactiae* ATCC 12386, corresponding to serotype Ia, was supplied by IAL (Adolfo Lutz Institute-São Paulo, Brazil).
Seed culture preparation: Seed cultures of *Streptococcus agalactiae* were grown in 100 mL of Trypticase Soy Broth= TSB medium (Merck, Germany), in incubators with shaking at 37°C / 250rpm for 10-12h.

Fermentation process: Modified TSB medium, with the addition of inosine, 1,5g/L, was used for fermentation. The pH was adjusted to 7.0 using 1M NaOH. The medium was sterilized at 121°C for 15 minutes. Fermentation was performed at 37°C, pH range between 7.2-7.4 and 250 rpm. Cetaflon detergent, 1% w / v, was added to the culture after reaching the plateau of optical density.

Purification process:Biomass was separated by centrifugation.

Was performed four different purification processes (Table 1) with thecrude polysaccharide.

Table 1 Protocolof purification and analytical methods

Type	Protocol	Analytical methods	Reference
A1	• Concentration – diafiltration 100 kDa • 30%Ethanol precipitation • 80%Ethanol precipitation • Enzymatic treatment • Storage on Buffer PBS	1. Optical density – presence and absence of bacteria 2. Polysaccharide determination – modified Bial method using ribose as standard 3. Protein determination – Lowry´s method 4. Nucleic Acid determination – absorbance at 260nm (OD 260nm = 1.0 corresponds to 50µg NA / ml) 5. Phosphorus determination- ascorbic acid method 6. Rocket imunoelectrophoresis – analysis for the specific antibody recognition 7. Molecular mass determination – size exclusion chromatography	ALBANI*et al.* 2015
A2	• Concentration – diafiltration with SDS 30 kDa • Precipitation with TCA • 20%Ethanol precipitation • 60%Ethanol precipitation • Concentration – diafiltration with SDS 50 kDa • Concentration – diafiltration with DOC 0,5%/EDTA 2mM - 30 kDa	1. Polysaccharide concentration – ELISA method using rabbit serum 2. Protein determination – Lowry´s method 3. Nucleic Acid determination – absorbance at 260nm (OD 260nm = 1.0 corresponds to 50µg NA / ml)	ZANARDO*et al.* 2016
A3	• Concentration with 300 kDa tangential flow ultra filtration membranefollowed by diafiltration with normal saline • Sodium deoxycholate (0.3% w/v) and absolute ethanol were added to thecrude concentrate. Ethanol precipitation • Aluminium phosphate adsorption • Precipitation with absolute ethanol • Diafiltration with tangential flow filtration 300kDa	1. Polysaccharide content determined by colorimetric method using rhamnose as standard 2. Protein determination – Lowry´s method 3. Nucleic Acid determination – absorbance at 260nm (OD 260nm = 1.0 corresponds to 50µg NA / ml) 4. Endotoxins determined using Limulus Ameobocyte Lysate test 5. Relative purity calculated based on the ratio of CPS input at crude concentrate to the CPS obtained at the end of purification 6. Molecular size of protein impurities: SDS-Page 7. Molecular size of nucleic acid impurities – Agarose Gel electrophoresis	MACHA *et. al*2014 (adapted)
A4	• Centrifugation with 2M NaCl • Digestion and centrifugation of macromolecules with papain • 30%Ethanol precipitation • 80%Ethanol precipitation with • Lyophilization and storage	1. The. Identification and dosage of sugar: Dubois method (Phenol sulfuric) 2. Gas chromatography: identification of sugar monomers 3. Bradford's test: protein dosage 4. Absorbance/spectrometry: nucleic acid dosage	In House methodology (USP and UFRJ, 2017)

The X- Ray diffraction:X- Ray diffraction was conducted in a Rigaku Theta-theta geometry diffractometry model Ultima^{+}(The Cu radiation (1.5418A^{o}) was monochrome using a crystal of graphite and the power of the beam was 40KV, 30mA. The measurement was takingstepsof 0.05^{o},and acquisition time of 8s. The diffraction of the acrylic sample tray(Figure 1), where the PS is deposited during the analysis, was also carried out to evaluate the measurement pattern.

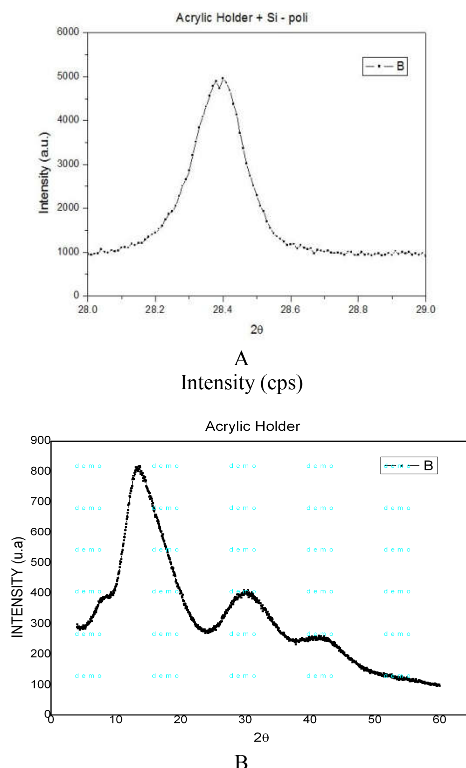

Figure 1 (A) Accuracy of support and measurement (B) Acrylic sample tray and its X-ray diffractogram

RESULTS

It can be seen in figure 2 that sample A1 relating to the first purification analysis still had a large amount of PBS (saline solution), which possibly interfered with the reading of the peak intensity. Thus, it was not possible to identify the presence of polysaccharide sugars.

Saline solution was withdrawn from the A2 sample. However, the diffraction pattern obtained, corresponded to the adapted sample tray, due to the insufficient amount of sample. Therefore, new analyses would be necessary to identify the crystalline structures of the sugars.

The small peaksin the third purification A3 correspond to the residues of impurities such as aluminum, which was used in the purification process. The high intensity peak possibly indicates the presence of the polysaccharide chain.

In the fourth purification A4 of in house methodology, there was no detection of contaminants such as saline and metal impurities. It was possible to identify a high peak in the same intensity we could see in A3 purification.

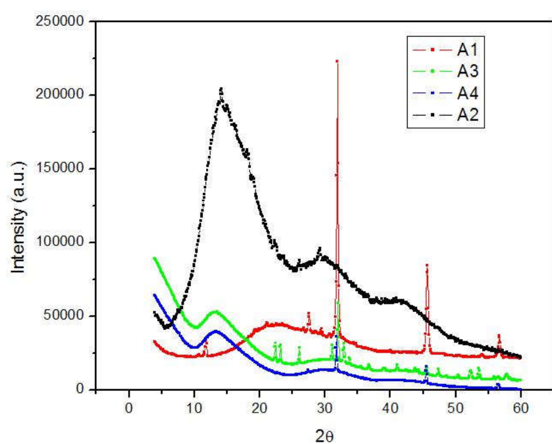

Figure 2 X-ray diffraction (type 1 ALBANI) of different polysaccharide purification protocol samples of the microorganism *Streptococcus agalactiae.*

CONCLUSION

The analysis of X-ray diffraction is a useful tool for a preliminary identification of the polysaccharides at the molecular level and for the product characterization.

Buffer conditions and reagents used in the purification process affect the polysaccharide final products and consequently their physical chemical characterization (BALDUCCI, *et al.* 2017; ICH Q3A R2, 2006). HADIDI and colleagues, 2016 demonstrated how the ionic strength and pH impactthe characterization test. It agrees with SOLDI (2005), who stated that the polysaccharide production depends on the chemical and thermal conditions.

Some research studies are trying to establish a standard polysaccharide purification platform (COLLINS *et al.* 2015), however it is a difficult task due to the complexity and diversity of microorganisms. The purification stage is very important and essential for the biological products because it determines the yield, product consistency, and reproducibility. Purification process influences directly on the structures because different reagents and temperatures can break the polysaccharide in different places of the molecule (SOLDI, 2005). The residues of purification processes, such as saline and metallic ions, can affect the physical properties of the final product and consequently its process characterization. Thus, according to our research, X-ray diffraction patterns would help in the identification and analysis of the purified material and detection of possible impurities of the purification process. Through this study, it is clear the importance of experimental planning and development methodological preparation for obtaining a well purified biological product (polysaccharide). One of the great challenges is to obtain a purified product free of contaminants and impurities. As with synthetic products, there is a need for the evaluation of contaminants and impurities from starting materials and excipients. The use of X-ray diffraction can contribute to a better description of the processes and thus the reduction / removal of impurities caused by the decomposition of product or process, improving the discussion of the potential impact on quality, safety and efficacy.

List of Abbreviations

ATCC American Type Culture Collection
GBS Group B streptococcis
ICH International Conference on Harmonisation
MRI magnetic resonance imaging

Declarations

Ethics approval and consent to participate - “Not applicable”
Consent for publication - “Not applicable”
Availability of data and material - “Not applicable”
Competing interests - “Not applicable”
Authors' contributions- “Not applicable”

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