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Preparation and evaluation of polysaccharide sulfates for inhibiting *Helicobacter pylori* adhesion

Weijuan Song[a,b,1], Yalong Wang[a,1], Liyan Zhang[c], Shengnan Fu[a], Ying Zeng[c], Haiyan Hu[a,*]

[a] School of Pharmaceutical Sciences, Sun Yat-sen University, University Town, Guangzhou 510006, PR China
[b] Department of Clinical Pharmacy, The Fifth Affiliated Hospital of Zhengzhou University, Zhengzhou 450002, PR China
[c] Guangdong General Hospital, Guangzhou 510080, PR China

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ABSTRACT

In treatments of *Helicobacter pylori* infections, recrudescences were common because of an unfavorable bacterial eradication rate due to the ever increasing resistance to antibiotics. In this study, we chose pectin, guar gum and chitosan to synthesize their sulfates to inhibit adhesions of *H. pylori* and thus enhance the eradication rate. The introduction of sulfates was characterized using FT-IR and elemental analysis. Data from zeta-potential, hydrodynamic diameter, hydrolysis and rheological property demonstrated the sulfates were physicochemically stable. Inhibition assay of hemagglutination and adhesion indicated sulfates prevented *H. pylori* from adhering to erythrocytes and AGS cells. In binding assay, affinities of sulfates to *H. pylori* suggested sulfates could compete with target cells for bacteria and moderated the bacterial adhesion to hosts. A higher content of galactoses and 2,3-O-linked sulfates benefited this action. Thus polysaccharide sulfates can serve as potential adjuvants to raise the bacterial eradication rate by inhibiting adhesions of *H. pylori*.

1. Introduction

Helicobacter pylori (*H. pylori*), a spiral-shaped and gram-negative bacterium, was first isolated in 1982 (Marshall & Warren, 1984), and is a causative agent of chronic active gastritis, peptic ulcer disease and gastric cancer (Blaser, 1990; Marshall & Warren, 1984; Parsonnet et al., 1994). It is estimated to infect 50% of the world populations (Kobayashi, Lee, Nakayama, & Fukuda, 2009). A standard triple therapy combining a proton pump inhibitor (PPI) and two kinds of antibiotics, especially clarithromycin and amoxicillin has been widely used for the treatment of *H. pylori* infections (Malfertheiner et al., 2012). However, making use of a large dose of antibiotics has caused the ever increasing resistance to clarithromycin and the reduction in eradication rates (74–78%). Recently quinolones and rifabutin are used instead of clarithromycin, but the results have been mixed (Qian et al., 2012; Zullo et al., 2012). Hence new drugs are in demand.

Recent studies have indicated some native acidic polysaccharides can inhibit the adhesion of *H. pylori* on cells such as gastric cells or macrophages (Lutay, Nilsson, Wadström, & Ljungh, 2011; Sim et al., 2011; Wittschier, Faller, & Hensel, 2009; Xu et al., 2010). Other researches in adhesion mechanisms have demonstrated the adhesion of *H. pylori* to hosts is mediated by bacterial outer membrane proteins (OMPs) and particular oligosaccharide epitopes on gastric mucins (Kenny et al., 2012). The oligosaccharide epitopes always contain acidic groups (sialylated or sulfated), which are usually attached to lacNAc (Galβ1-4GlcNAcβ1-) or lacdiNAc (GalNAcβ1-4GlcNAcβ1-). Thus we deduce acidic polysaccharides rich in sialylated or sulfated galactoses may display a strong affinity to *H. pylori*, which compete with host cells or mucins for *H. pylori* and thus the adhesion of *H. pylori* to hosts is inhibited. The aim of this study was to synthesize polysaccharide sulfates with characteristics of binding affinity to *H. pylori* and inhibiting bacterial adhesion to hosts, which could raise the eradication rate of *H. pylori* and ultimately reduce the dose of antibiotics.

Polysaccharides are perfect adjuvants for *H. pylori* related diseases. Firstly, polysaccharides are difficult to be absorbed in the alimentary tract and thus avoid possible side effects caused by absorption. Secondly, polysaccharides can increase the viscosity of stomach contents, prolong the time of gastric emptying and reinforce the effect of time dependent antibiotics. In this paper, we choose three types of polysaccharides, pectin, guar gum and chitosan for sulfating. Pectin (PEC) is a linear chain of α-(1-4)-D-galacturonic acids. As for guar gum (GUA), the backbone is a

* Corresponding author at: Sun Yat-sen University, No. 132 Waihuan East Road, Guangzhou 510006, PR China. Tel.: +86 20 39943118; fax: +86 20 39943118.
E-mail address: lsshhy@mail.sysu.edu.cn (H. Hu).
[1] These authors contributed equally to this work.

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linear chain of β-(1-4)-mannoses to which galactose residues are 1-6-linked at every two mannoses. Chitosan (CHI) is composed of β-(1-4)-D-glucosamines and N-acetyl-D-glucosamines. The sulfation occurs on hydroxides or aminos on the polysaccharides. Sulfates only can be grafted on 2,3-OH on galacturonic acids of PEC, while GUA, besides 2,3-O-linked sulfates, 4,6-O-linked sulfates can also be obtained. As for CHI, 2-N-linked and 3,6-O-linked sulfates can be gained by sulfation. The structural differences were employed to identify the distinctive effects of linkage modes of sulfates and galactose amounts on the adhesion inhibition.

Over the years, although anti-adhesion effects of polysaccharide on *H. pylori* have been reported, there are little reports on the binding affinity of polysaccharides to the bacteria, limited by the existing detecting techniques. The most widely employed method is thin layer radiochromatography (Takamura et al., 2012; Teneberg, 2009), but mechanical strength of silica gel thin layer plates are too poor to be appropriate for immobilization and isolation of polysaccharides. Isotope labeling also requires demanding operations and lab conditions. We modified the classic method using nylon membranes to immobilize polysaccharides and fluorescein isothiocyanate (FITC) to label bacteria instead of isotopes. The novel method met the qualification of immobilization and quantitative detection.

In this work, we obtained pectin sulfate (PECS), guar gum sulfate (GUAS) and chitosan sulfate (CHIS), and evaluated their physicochemical properties such as zeta potential, hydrodynamic diameter, rheological property and stability. Erythrocytes and AGS cells were exploited for *H. pylori* adhesion inhibition assay. To certify whether the adhesion inhibition is caused by competitive bindings of polysaccharide sulfates to *H. pylori*, a binding assay between polysaccharide sulfates and bacteria was conducted on nylon membranes. From the results above, we found out favorable polysaccharide structures for adhesion inhibition.

2. Experimental

2.1. Chemicals

PEC, GUA and CHI were obtained from Sigma–Aldrich (USA). All other chemicals unless otherwise stated, were of analytical grade.

2.2. Sulfation of polysaccharides

The sulfation of PEC, GUA and CHI was carried out according to the previous method (Vogl, Paper, & Franz, 2000). In brief, polysaccharide was soaked in dry DMF overnight and then mixed with SO_3-pyridine in DMF under agitation. For every mole of SO_3-pyridine, 1 mol pyridine was added to the mixture. After stirring under nitrogen atmosphere (120 °C, 4 h), the reaction was terminated in an ice bath and adding distilled water. To obtain soluble polysaccharides, the pH of solutions was adjusted to 6.0–7.0 using 1 mol/L NaOH. The sulfated polysaccharides were dialyzed against distilled water for 4 days with dialysis bags (MWCO: 3500 Da) and freeze-dried.

2.3. Structural characterization

The structure of polysaccharide sulfates was characterized using a FT-IR spectrometer (TENSOR 37, Bruker, Germany). An elemental analyzer (Vario EL cube, Elementar, Germany) was applied to determine the elemental composition of PECS, GUAS and CHIS.

2.4. Zeta potential and hydrodynamic diameter

The polysaccharide solutions (0.1 mg/mL) were used for zeta potential (Z_p) and hydrodynamic diameter (*Z*-average) determination. The experiment was conducted at 25 °C using a Malvern Zetasize (Zetasizer Nano ZS90, Malvern Instruments, UK).

2.5. Hydrolysis stability

The sulfated polysaccharides were dissolved in 0.1% HCl solution (pH 1.2) or acetate buffer (pH 4.0), shaken in water bath (37 °C, 100 rpm) and sampled at 1 h, 2 h, 4 h and 6 h. Content of sulfate was measured using an elemental analyzer (Vario EL cube, Elementar, Germany).

2.6. Rheological property

The polysaccharide solutions were prepared in distilled water at a concentration of 1% (w/v). All rheological measurements were conducted with a rotational rheometer (DV-III ULTRA, Brookfield, US) at 25 °C.

2.7. Bacterial and cell culture

H. pylori SS1 and *Lactobacillus bulgaricus* ATCC 10638 were used in the study. *H. pylori* was grown on fetal bovine serum (Gibco, USA) coated Columbia agar with 5% sheep blood (BD, USA) for 48–72 h under microaerophilic condition at 37 °C. *L. bulgaricus* was grown in MRS broth for 12–24 h under anaerobic condition at 37 °C. AGS cells were cultivated in culture dish using RPMI 1640 medium (Corning, USA) with 10% fetal bovine serum at 37 °C and subcultivated every 2 days at a ratio of 1:3.

2.8. FITC-labeling to bacteria

Bacteria were suspended in 10 mL PBS (6×10^8 cfu/mL) and incubated with 6 μL FITC (10 mg/mL in DMSO) at 37 °C in the dark for 12 h. The FITC-labeled bacteria were harvested by centrifugation (3500 rpm, 10 min) and washed three times in PBS containing 0.1% Tween-20 (Xu et al., 2010). The labeling efficiency of the bacteria was detected by a flow cytometer (EPICS XL, Beckman Coulter, USA) equipped with a 488 nm argon laser for excitation. The events collected were ten thousands and the data were analyzed through EXP032 software.

2.9. Binding assay

Nylon membranes (pore size 0.22 μm) were soaked in polysaccharide solutions (1 mg/mL) and incubated at 50 °C for 24 h. Then polysaccharides were coated on nylon membranes (pore size 0.22 μm) by pressure filtration to obtain PECS chips, GUAS chips and CHIS chips respectively, which were placed into a 96-well opaque plate. In direct binding assay, 100 μL FITC-labeled *H. pylori* or *L. bulgaricus* solution (6×10^8 cfu/mL) was added into each well, incubated for 1 h at 37 °C, and then washed three times in PBS containing 0.1% Tween-20. In presaturated binding assay, 100 μL unlabeled *H. pylori* solution (6×10^8 cfu/mL) was first added into each well and incubated to saturate the polysaccharides on the chips and then washed to remove the excess bacteria. After that, 100 μL FITC-labeled *H. pylori* solution (6×10^8 cfu/mL) was added. The following steps were the same as in the direct binding assay. Fluorescence intensity was determined by a microplate reader (Flex Station 3, Molecular Devices, US) using an excitation wavelength at 485 nm and a detection wavelength at 528 nm.

2.10. Hemagglutination and hemagglutination inhibition assay

Hemagglutination (HA) assay was improved based on the previous study (Belogortseva, Yoon, & Kim, 2000). Twenty-five microliter