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Structural studies of the exopolysaccharide from *Lactobacillus plantarum* C88 using NMR spectroscopy and the program CASPER

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ABSTRACT

Some lactic acid bacteria, such as those of the *Lactobacillus* genus, have the ability to produce exopolysaccharides (EPSs) that confer favorable physicochemical properties to food and/or beneficial physiological effects on human health. In particular, the EPS of *Lactobacillus plantarum* C88 has recently demonstrated in vitro antioxidant activity and, herein, its structure has been investigated using NMR spectroscopy and the computer program CASPER (Computer Assisted Spectrum Evaluation of Regular polysaccharides). The pentasaccharide repeating unit of the O-deacetylated EPS consists of a trisaccharide backbone, →4)-α-D-Gal*p*-(1→2)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→, with terminal D-Glc and D-Gal residues (1.0 and 0.8 equiv per repeating unit, respectively) extending from O3 and O6, respectively, of the →4)-α-D-Gal*p*-(1→ residue. In the native EPS an *O*-acetyl group is present, 0.85 equiv per repeating unit, at O2 of the α-linked galactose residue; thus the repeating unit of the EPS has the following structure: →4)[β-D-Glc*p*-(1→3)][β-D-Gal*p*-(1→6)]α-D-Gal*p*2Ac-(1→2)-α-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→. These structural features, and the chain length (~10^3 repeating units on average, determined in a previous study), are expected to play an important role in defining the physicochemical properties of the polymer.

1. Introduction

Many strains of lactic acid bacteria (LAB) are able to produce exopolysaccharides (EPSs) on the bacterial cell wall to form a capsule or to be secreted into the surrounding growth medium to form loose slime.[1] EPSs produced by LAB have received increasing attention mainly because of the generally-regarded-as-safe (GRAS) status of the EPS-producing LAB and the remarkable physical and physiological functions of the EPSs.[2,3] These natural biopolymers, which often are composed of oligosaccharide repeating units of monosaccharides such as glucose, galactose, or rhamnose, have been widely used as viscosifying, bioflocculating, stabilizing, gelling, and emulsifying agents in the food industry.[4,5] Some EPSs produced by LAB can be employed as natural texturizers in dairy processing and help protect the bacteria against harsh environmental conditions; resistance to gastrointestinal acids and bile salts is, for instance, a prerequisite for probiotic activity.[6] Important health benefits such as immune stimulation, antitumor, cholesterol-lowering activity, and antioxidant activities of fermented dairy products prepared with EPS-producing LAB or EPS itself have been investigated.[7–9]

Among EPS-producing LAB, *Lactobacillus plantarum* has frequently been isolated from food products, and it is also one of the most studied LAB species, particularly because some strains are considered to be probiotics due to several of their properties.[10] *L. plantarum* 70810 isolated from Chinese Paocai was reported to produce an EPS with a high yield (0.859 g L^{-1}), composed of galactosyl residues, which could be used as a potential biosorbent for the removal of heavy metals from environment.[11,12] The EPS produced by *L. plantarum* KF5 was composed of mannose, glucose, and galactose in an approximate ratio of 1:5:7, and the physicochemical characteristics of the EPS from the KF5 strain were shown to differ from other commercially available gums, which imparted its potential applications in the food industry.[13] *L. plantarum* EP56 was shown to produce two EPSs, one with low-molecular-mass that was released into the culture medium and another one with high-molecular-mass loosely, partially, and temporarily linked to cells.[14] The structure and biocompatibility of α-D-glucan (dextran) from probiotic *L. plantarum* DM5 and its unique physical and rheological properties facilitated its application in the food industry as a viscosifying and gelling agent.[15]

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Previously, an EPS-producing strain (C88) of *L. plantarum* was isolated from traditional dairy Tofu in Inner Mongolia of China, and it showed prominent in vitro scavenging activity against hydroxyl and 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals, as well as in vivo antioxidant activity tested in a mice model.[16] Recently, the EPS produced by *L. plantarum* C88 was confirmed to be involved in the antioxidant activity of this strain, since the purified EPS exhibited strong in vitro radical scavenging activity and antioxidant activity against H_2O_2-induced injury in Caco-2 cells.[17]

Herein we report the structural elucidation of the EPS produced by *L. plantarum* C88 using NMR data and the computer program CASPER (Computer Assisted Spectrum Evaluation of Regular polysaccharides). The latter uses ^{1}H and ^{13}C chemical shifts of mono- to trisaccharides, stored on its database, for the prediction of chemicals shifts of polysaccharides. Furthermore, based on those chemical shifts predictions, the program is also capable of analyzing unassigned NMR data from 1D ^{1}H and ^{13}C NMR spectra, as well as ^{1}H–^{1}H and ^{1}H–^{13}C correlations from 2D experiments (such as ^{1}H,^{1}H-TOCSY, ^{1}H,^{13}C-HSQC (or ^{13}C,^{1}H-HETCOR), ^{1}H,^{13}C-H2BC and/or ^{1}H,^{13}C-HMBC), in order to suggest a list of possible structures ranked according to the deviation between experimental and predicted NMR data.[18–20] In addition, a module for component and absolute configuration analysis has been recently implemented, allowing the fully or semi-automatized analysis of glycans using solely NMR data as input information.[21,22]

2. Results and discussion

2.1. Sugar analysis and absolute configuration determination

The ^{1}H NMR spectrum of the native EPS of *L. plantarum* C88 showed a resonance at 2.211 ppm (3H, singlet) that disappeared after treatment with aqueous 0.1 M NaOH, indicating the presence of an *O*-acetyl group. The monosaccharide components of the EPS, and their absolute configuration, were determined using a methodology previously developed in our laboratory.[21,22] In this method, the pre-hydrolyzate of the O-deacetylated EPS was subjected to (+)-2-butanolysis and the unassigned NMR data, from both 1D ^{13}C and 2D multiplicity-edited ^{1}H,^{13}C-HSQC spectra, were used as input information in the 'component analysis' module of the web interface of the CASPER program (Supplementary Table S1).[18,19] The results (calculation time ~3 s) revealed two possible monosaccharide components: D-Glc and D-Gal (Supplementary Table S2). The resonances from the different pyranose and/or furanose forms of each monosaccharide derivative were identified by comparison of the anomeric region of the ^{1}H NMR spectrum of the (+)-2-butyl glycosides obtained from the EPS of *L. plantarum* C88 (Fig. 1a) with those of D-Glc (Fig. 1b) and D-Gal (Fig. 1c). In addition, the anomeric region of ^{1}H,^{13}C-HSQC spectrum of the (+)-2-butyl glycosides of the pre-hydrolyzate of the *L. plantarum* C88 EPS (Fig. 1d) is a perfect match to the overlay of the ^{1}H,^{13}C-HSQC spectra of D-Glc (gray color in Fig. 1e) and D-Gal (black color in Fig. 1e). Integration of the respective resonances in the ^{1}H NMR spectrum revealed a ratio of D-Glc and D-Gal of 2:1, respectively, that is consistent with previous reports.[17]

2.2. Structural analysis of the O-deacetylated EPS

The structural analysis of the O-deacetylated EPS of *L. plantarum* C88 was carried out by submitting selected unassigned NMR data to the 'determine structure' module of the CASPER program (cf. Supplementary Table S3). Thus, $^{3}J_{H1,H2}$ (two of 2–7 Hz and three >7 Hz) and $^{1}J_{C1,H1}$ (three <169 Hz and two >169 Hz) couplings were extracted from 1D ^{1}H (Fig. 2a) and 2D coupled multiplicity-edited ^{1}H,^{13}C-HSQC NMR spectra, respectively. ^{13}C chemical shifts were obtained from a 1D ^{13}C NMR spectrum (Fig. 2b), and ^{1}H-^{13}C correlations from a 2D multiplicity-edited ^{1}H,^{13}C-HSQC spectrum (Fig. 2d and e) were used to correlate the ^{13}C resonances to their directly attached protons. Additional data from a 2D ^{1}H,^{1}H-TOCSY spectrum (Fig. 2c) were also used to assist in the assignment of the respective ^{1}H spin systems of each monosaccharide component. One should note that the ^{13}C and ^{1}H,^{1}H-TOCSY spectra were obtained simultaneously using the parallel detection technique (PANSY),[23] thus reducing the total acquisition time of both spectra

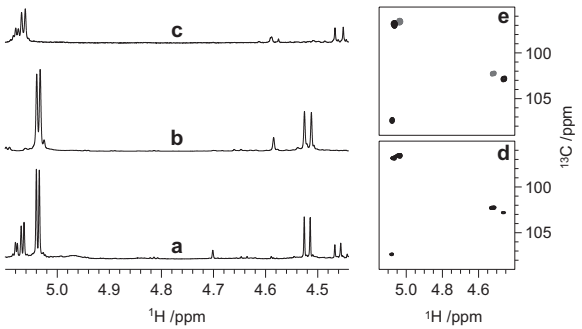

Figure 1. ^{1}H NMR spectra of the (+)-2-butyl glycosides of: (a) the pre-hydrolyzate of the EPS of *Lactobacillus plantarum* C88, (b) D-glucose and (c) D-galactose (the spectra were recorded at a ^{1}H frequency of 500, 600, and 700 MHz from a to c, respectively). The ratio of D-Glc and D-Gal in the ^{1}H NMR spectrum of panel a is 2:1, respectively. (d) The anomeric region of the ^{1}H,^{13}C-HSQC spectrum of the (+)-2-butyl glycosides of the pre-hydrolyzate of the EPS of *Lactobacillus plantarum* C88 recorded at a ^{1}H frequency of 700 MHz. (e) Overlay of the anomeric region of the ^{1}H,^{13}C-HSQC spectra of the (+)-2-butyl glycosides of D-glucose and D-galactose (in gray and black color, respectively).

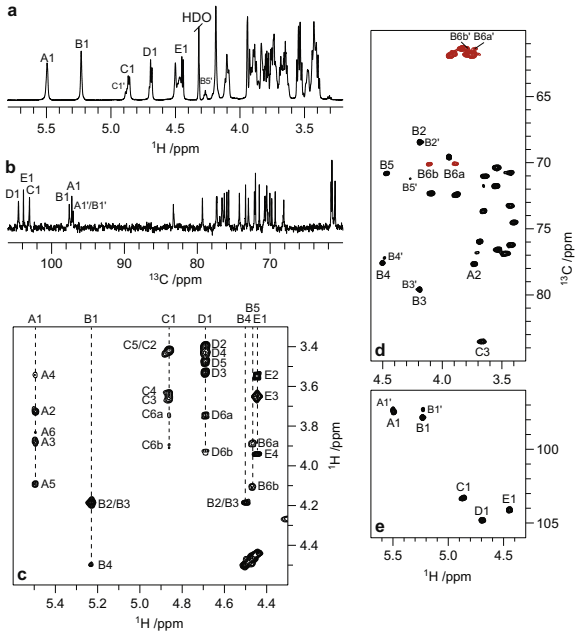

Figure 2. The ^{1}H and ^{13}C NMR spectra of the O-deacetylated EPS of *L. plantarum* C88 (a and b, respectively) and selected region of the ^{1}H,^{1}H-TOCSY spectrum (c) showing correlations from anomeric protons and the H4 and H5 protons in residue **B**. The ^{13}C NMR spectrum with proton decoupling was acquired during the spin-lock of the ^{1}H,^{1}H-TOCSY experiment (τ_m = 120 ms) using a PANSY experiment. Selected regions of the multiplicity-edited ^{1}H,^{13}C-HSQC NMR spectrum showing the region for the ring atoms and hydroxymethyl groups (in which the cross-peaks from the latter appear in red) (d) and the anomeric region (e). Resonances from anomeric and substitution positions are annotated, as well as all the resonances of residue **B**. Resonances from minor spin systems are indicated with primed characters.