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Water-soluble (1 → 3),(1 → 4)-α-D-glucan from mango as a novel inducer of cariogenic biofilm-degrading enzyme

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ABSTRACT

A water-soluble polysaccharide (WSP) extracted from mango (*Mangifera indica* L.) fruits has been suggested as a new alternative to mutan for mutanase induction in *Trichoderma harzianum*. Structural analyses revealed that the purified WSP was a (1 → 3),(1 → 4)-α-D-glucan with the molecular mass of ca. 760 kDa in which the (1 → 4)-linked and (1 → 3)-linked α-Glc*p* residues were in a ratio of 1:2.4. When the strain *T harzianum* CCM F-340 was grown in the presence of WSP, the maximal enzyme productivity obtained after 3 days of cultivation was 34 mU/mL. The mango WSP proved to be a very effective stimulus of mutanase expression giving a 5.1-fold higher than without WSP, transcription. It was shown that the mixture of WSP-induced mutanase and commercial dextranase had a high hydrolytic potential in the reaction with streptococcal mutan, where maximal degrees of solubilization and saccharification of this biopolymer (93.4% and 80%, respectively) were reached within 9 h (solubilization) and 24 h (saccharification). The mixed enzymatic preparation was also effective in degradation of streptococcal mutan and its removal from cariogenic biofilms. After 3 h hydrolysis, only 18.2% of the biofilm remained adhered to the glass surface.

1. Introduction

Microbial mutanases (exo-(1 → 3)-α-D-glucanase: E.C. 3.2.1.84; endo-(1 → 3)-α-D-glucanase: E.C. 3.2.1.59), are enzymes capable of hydrolyzing (1 → 3)-α-glucosidic bonds in streptococcal mutans and removing of oral biofilms [1]. Therefore, they could be applied as active ingredients in oral hygiene measures. Mutanases have also been successfully used for obtaining fungal protoplasts, improvement of filterability of wine obtained from grapes infected with the mold *Botrytis cinerea*, and improvement of filterability and injectability during the secondary and tertiary petroleum recovery by waterflooding [2–4]. Moreover, applications derived from the use of these enzymes are related to their antifungal effect against phytopathogenic fungi containing (1 → 3)-α-D-glucan in their cell wall, like *Fusarium oxysporum* [5].

Mutanases are mainly inducible extracellular enzymes. It must be emphasized that in the chemical structure of potential inducers, only (1 → 3)-α-linkages can be responsible for specific induction of mutanase. One of the most effective inducers of mutanase production is mutan, synthesized by cariogenic streptococci (1 → 3),(1 → 6)-α-D-glucan. Unfortunately, this biopolymer is not available in bulk quantities due to the pathogenicity of its producers, the necessity to use complex and expensive media, multistage production process, low product yields, and high structural heterogeneity [6]. Therefore, identification of alternative and inexpensive carbon sources as mutanase inducers would facilitate production of the enzyme on a larger scale and at relatively low costs. Besides the fungal cell wall, which is also a rich reservoir of (1 → 3)-α-D-glucans [7,8], glucans containing (1 → 3)-α-linkages within the molecule structure are found in a few plant fruits and mosses [9,10]. For example, Das and Rao [11,12] described a neutral polysaccharide isolated from mango fruits, which was a glucan consisting of repeating units, containing about nine (1 → 3)-linked and nine (1 → 4)-linked α-Glc*p* residues. Mango (*Mangifera indica* L.) is the most important tropical fruit in the Anacardiaceae family, with high nutritional and medicinal value. It originated from the Asia Indo-Burmese region approximately 4000 years ago and it is now commercially grown in more than 87 countries [13]. Hence, mango fruits can become an inexpensive, easily available, and safe source of inducer for mutanase production. To the best of our knowledge, there are no reports dealing with their use in this matter.

In the present work, the structural features of water-soluble polysaccharide (WSP) isolated from mango fruits and its application as a new inducer of mutanase synthesis in *T. harzianum*, were investigated. The aim was also to hydrolyze streptococcal mutan effectively by WSP-induced mutanase or a mixture of mutanase and

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dextranase and to remove artificial oral biofilms formed in vitro by pathogenic microorganisms.

2. Materials and methods

2.1. Plant material

Mango fruits (*Mangifera indica* L.), cultivar Dole (Brazil), were obtained from a commercial source in Poland in June 2006. The voucher specimen was deposited at the Department of Industrial Microbiology, Maria Curie-Skłodowska University, Lublin, Poland, under no. ZMP MF1/2006. Before use, the mango fruits were allowed to ripen at room temperature for 5 days.

2.2. Isolation of water-soluble polysaccharide (WSP)

The water-soluble polysaccharide (WSP) was carefully isolated from mature mango fruits using a modification of the procedure of Das and Rao [11]. Briefly, the outer skin and the seeds of the mangoes were removed and the sliced mesocarp (400 g) was macerated during 2 min in a blender in the presence of 96% ethanol (100 mL) and allowed to stand overnight. The suspension was filtered through a 0.45 μm membrane of a Vacuum Driven Disposable Filtration System (Durapore, Millipore, USA) and air-dried at 45 °C to yield a fibrous material. The material obtained (50 g) was stirred with water (2 L) at boiling water-bath temperature for 5 h and the resulting slurry was allowed to stand overnight. This was filtered through a 0.45 μm membrane and the water extract was clarified by centrifugation (17,001 × *g* for 30 min). The clear solution was added to acidified (pH 4) 96% ethanol (4 L), when a white gelatinous precipitate formed. The precipitate was collected by centrifugation (17,001 × *g* for 30 min), washed with 96% ethanol, and lyophilized to give a white powder.

2.3. Microorganisms

Trichoderma harzianum strain CCM F-340 (The Czech Collection of Microorganisms, Brno, Czech Republic) was used as a starting culture for mutanase induction by WSP from mango fruits. The cariogenic streptococci used in this study included *Streptococcus mutans* CAPM 6067 and *S. sobrinus* CAPM 6070 (The Collection of Animal Pathogenic Microorganisms, Brno, Czech Republic); *S. sobrinus* DSM 20381 (The German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany); and *S. sobrinus*/*downei* CCUG 21020 (The Culture Collection, University of Göteborg, Göteborg, Sweden). The pathogenic yeast *Candida albicans* was a gift from Prof. A. Kędzia (Medical University of Gdańsk, Poland).

2.4. T. harzianum cultivation

Stock cultures of *T. harzianum* CCM F-340 maintained at 4 °C on potato dextrose agar slants were used for inoculations. Liquid medium A (pH 5.3), as described by Mandels et al. [14], supplemented by 0.25% of WSP from the mangoes, 0.05% proteose peptone, and 0.1% Tween 80 was used for mutanase production. Shaken cultures were performed in 500 mL conical flasks containing 100 mL of sterile medium. The flasks were seeded with conidia to a final concentration of about 2×10^5 conidia/mL and placed on an orbital rotary shaker at 300 rpm and 30 °C for 3 days.

2.5. Determination of the mutAW gene expression

T. harzianum RNA was purified by means of a Total RNA Mini Kit (A&A Biotechnology, Gdynia, Poland). Preliminary disintegration of cell walls was performed with 150 μL of 1,0 mm Zirkonia/Silica Beeds (BioSpec Products, Bartlesville, USA) in Ribolyser (HyBaid, Ashford, UK). cDNA from 1 μg of purified RNA was obtained by means of a Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, USA). The quantitative real-time PCR was performed to compare the level of mutanase expression. The applied primers were designed based on the comparison of the DNA and mRNA sequence of the *mutAW* gene encoding mutanase. The reaction mixture consisted of 1 μL of 10 μM TRforA primer (5′ TGCTGACGGCGAGTCTGGCAACTACAT 3′), 1 μL of 10 μM primer TRrevB (5′ ACAGGCCCAGATAACCATCGCCTTCTC 3′), 10 μL of 2xPCR MasterMix SYBR A (A&A Biotechnology, Gdynia, Poland), 2 μL of synthesized cDNA and 6 μL of deionized water. The reaction was performed using a LightCycler1,5 (Roche, Indianapolis, USA). The time-temperature profile started with 5 min preliminary denaturation in 95 °C, followed by 45 cycles of 10 s denaturation in 95 °C, 10 s annealing in 60 °C and 15 s elongation in 72 °C. The analysis of the melting point of the PCR product obtained was performed in the following conditions: heating to 94 °C and increasing temperature from 65 °C to 94 °C (ramp 0.1 °C/s). The Cp values obtained were normalized against the Cp value obtained for the qRTPCR for cDNA of the control sample (*T. harzianum* cultured in the absence of the WSP inducer).

2.6. Carbohydrate analysis

For sugar analysis, the polysaccharide was hydrolyzed with 2 M CF_3CO_2H (100 °C, 4 h). The absolute configuration of monosaccharides was established by an analysis of acetylated *R*-(−)2-butylglycosides, according to Gerwig and co-workers [15]. The sugars were converted into alditol acetates [16]. WSP was methylated according to the method of Hakomori [17], and the methylated polysaccharide was purified on a Sep-Pak C_{18} cartridge [18]. The resulting material was hydrolyzed in 2 M CF_3CO_2H (100 °C, 4 h) and reduced with $NaBD_4$. The partially methylated alditols were converted to acetate derivatives. Alditol acetates as well as partially methylated alditol acetates were analyzed using combined gas chromatography–mass spectrometry (GC–MS). The average molecular mass of the α-glucan from mango was determined by gel permeation chromatography with a Sepharose CL-6B column (0.7 cm × 90 cm). The α-glucan (5 mg) was dissolved in a 0.7% Na_2SO_4 water solution (0.5 mL). The separation was done at room temperature and eluted with 0.7% Na_2SO_4 at a flow rate of 0.4 mL/min. Carbohydrates in the eluate were determined by the phenol-H_2SO_4 assay [19]. Dextrans of known molecular masses were used as standards to calibrate the column. GC–MS analyses were carried out on a Hewlett-Packard gas chromatograph (model HP5890A) equipped with a capillary column (HP-5MS, 30 m × 0.25 mm) and connected to a mass selective detector (MSD model HP5971). Helium was the carrier gas and the temperature program was 150 °C initially for 5 min, then raised to 310 °C at a ramp rate of 3 °C/min, final time 20 min ^{1}H NMR spectra of the mutan preparations dissolved in 1 M NaOD in D_2O as well as WSP from mangoes (dissolved in D_2O) were recorded with a Bruker Avance (300 MHz) spectrometer at 60 °C. The ^{1}H chemical shift was obtained using acetone (δ_H – 2.225 ppm) as the internal standard. An infrared absorption spectrum (FT-IR) between 400 and 4000 cm^{-1} was recorded using a Perkin Elmer FT-IR spectrophotometer (Model 1725X). A specimen was prepared by the KBr-disk method. Specific rotation $[\alpha]_D^{25}$ (*c* 1.0, H_2O) was measured at 589 nm in a Perkin Elmer Automatic Polarimeter (Model 341 LC). The viscosity of polysaccharides (*c* 1.0, H_2O) was measured with a Brookfield (Model DV 3) viscometer at 20 °C.