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Ultrasonic and free-radical degradation of mannan from *Candida albicans*

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ABSTRACT

Mannan from pathogenic *Candida albicans* serotype A was degraded by means of ultrasound and/or $OH^{\bullet}$ generated in situ by Fenton reaction. The kinetics of degradation was monitored by HPLC analysis and the weight-average molecular weights (M_w) and index of polydispersity (PDI) were compared. A well-defined low-molecular-weight mannan (~30 kDa) with narrowed PDI of 1.8 was obtained after 120 min of ultrasonication. Similar or even lower M_w (up to 16 kDa) was achieved upon free-radical exposure depending on Fe^{2+} concentration used; however, this was accompanied by overall broadening of PDI and distinct changes in polymer structure as indicated by NMR analysis.

1. Introduction

Bacterial and yeast cell-wall polysaccharides are preferably used in construction of conjugated vaccines. Recent study concerning mechanism of immune response to a glycoconjugate vaccine [1] revealed that in antigen-presenting cells carbohydrate epitope in conjunction with carrier protein or peptide is presented to T-cells upon significant degradation (down to ~10 kDa) in endolysosomes. This is induced by oxidation with reactive oxygen (ROS) and/or reactive nitrogen species [2,3]. Based on this knowledge, glycoconjugate construct consisting of depolymerised group B streptococcal type III polysaccharide epitope induced several times higher immune response [1].

Besides the aforementioned oxidative degradation by ROS, several other methods (chemical, physical and mechanical) of partial degradation of bacterial and non-bacterial polysaccharides have been reported, e.g. acid hydrolysis [4], enzymatic hydrolysis [5], ozonolysis [6], radiolysis by γ-rays [7], electron beam radiation [8], microwave [9] and ultrasonic degradation [10–13]. Among these methods, ultrasound belong to the most gentle in terms of chemical structure preservation and side reaction elimination. Moreover, separation and further purification necessary in case of chemical treatment could be avoided.

A number of mechanisms for ultrasonic degradation of polymers have been proposed by now [14]. It is generally accepted that providing low-frequency (20 kHz) ultrasound, degradation is caused by mechanical effect of cavitation rather than radical formation [15]. The existence of limiting molecular weight (M_{lim}) observed after prolonged ultrasound exposure for various polysaccharides, such as cellulose [16], dextran [15], starch [17], chitosan [18], hyaluronic acid [19], sodium alginate [20], and bacterial polysaccharides [11], indicates that ultrasonication is a molecular-weight dependent process [21], mechano-chemical in nature and not a result of temperature and/or pressure fluctuation [19,22].

Preparation of low-molecular-weight mannan from pathogenic *Candida albicans* serotype A is described here. It is a branched cell-wall polysaccharide consisting of α-1,6-linked mannose backbone with different frequency of α-1,2; -1,3 and β-1,2-linked mannooligomer branches [23]. Regarding the intention of future use in construction of glycoconjugate vaccine, preservation of epitope structure after degradation was crucial. To meet the request for a simple, gentle and accessible fragmentation method, ultrasonic degradation was used. Free-radical degradation mimicking the real mechanism of polysaccharide processing in endolysosomes was discussed and both methods were compared.

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2. Materials and methods

Mannan was isolated as a mannan–copper complex [24] from the yeast cell wall of *C. albicans* serotype A (CCY 29-3-32) (Culture Collection of Yeasts, Institute of Chemistry, Bratislava, Slovakia).

2.1. Degradation of mannan

2.1.1. Ultrasonic degradation

Ultrasonic degradation was performed using BRANSON 450A Sonifier Analog Cell Disruptor (20 kHz; BRANSON Ultrasonics Corporation, Danbury, CT, USA) equipped with Ø19 mm high grain titanium probe. 30 mL of aqueous solution of mannan (2.5 and 33.3 mg mL^{-1}) was ultrasonicated at 60 W ultrasonic power for a total of 120 min. The temperature of the sample was kept below 50 °C during the ultrasonic treatment using ice-bath and by applying batch exposure to ultrasound. After defined periods of ultrasonication, a portion of sample solution was withdrawn, diluted and subjected to molecular weight analysis as described below. Any bits of titanium shed by the probe because of long exposure times were removed by centrifugation prior to further analysis. Solution of degraded mannan was freeze-dried and the product (ManUS) was obtained in a form of white solid.

2.1.2. Free-radical degradation

Both the original and the 80 min ultrasonicated mannan (15 mg mL^{-1}) in deionized water were exposed to OH$^{\bullet}$ produced in situ by means of Fenton reaction [25]:

$$Fe^{2+} + H_2O_2 \rightarrow OH^{\bullet} + OH^{-} + Fe^{3+} \quad (I)$$

Briefly, 400 μL of 3 or 6 mM aqueous solution of $FeCl_2{\cdot}4H_2O$ for two concentrations of Fe^{2+} (0.935 and 1.87 mM, respectively) followed by 1.2 or 2.4 μL of 30% H_2O_2 ($n(Fe^{2+}):n(H_2O_2) = 1:10$), respectively, were added and the reaction mixture was stirred at ambient temperature for 3.5 h. After defined periods of OH$^{\bullet}$ exposure, a portion of sample solution was withdrawn, diluted and subjected to molecular weight analysis as described below. Finally, the reaction mixture was filtered and washed with distilled water using 3 kDa centrifugal filter (Amicon® Ultra 0.5 ml, Millipore). Remaining Fe^{2+}/Fe^{3+} ions were removed by incubation (30 min) with ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-Na_2) and subsequent filtration. Degraded original (ManFR) and ultrasonicated mannan (ManUSFR) was recovered by freeze-drying as a white solid.

2.2. Analytical methods

High performance liquid chromatography (HPLC) using two HEMA-BIO 100 and 300 columns (8 mm × 250 mm) connected in series was used to monitor changes in molecular weight during degradation. The mobile phase used was 0.1 M $NaNO_3$. The carbohydrate content in the eluent was monitored by differential refractometer RIDK-102 (Laboratorní přístroje Praha, Czech Republic). A set of dextrans (American Polymer Standard Corporation, Mentor, OH, USA) was used for calibration of the HPLC system. Number-average (M_n) and weight-average (M_w) molecular weights and index of polydispersity (PDI) were calculated as follows:

$$M_n = \frac{\sum h_i}{\sum (h_i/M_i)} \quad (1)$$

$$M_w = \frac{\sum h_i M_i}{\sum h_i} \quad (2)$$

$$\mathrm{PDI} = \frac{M_w}{M_n} \quad (3)$$

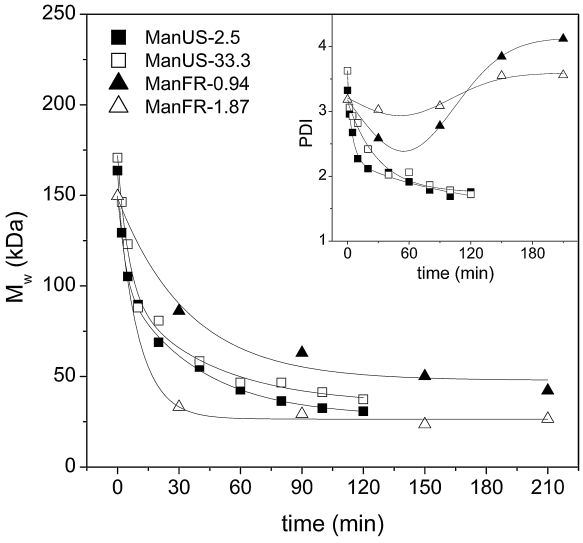

Fig. 1. Effect of ultrasonication time and mannan concentration (■ 2.5 mg mL^{-1}; □ 33.3 mg mL^{-1}) and free-radical exposure time and Fe^{2+} concentration (▲ 0.94 mM; △ 1.87 mM) on weight-average molecular weight (M_w) and polydispersity index (PDI; inset) of mannan.

where h_i is the height of HPLC chromatogram at the ith elution increment and M_i (Da) is the molecular weight of species eluting at this increment obtained from calibration curve.

Pseudo-first-order reaction model represented by Eq. (4) was used to characterize the degradation kinetics of mannan:

$$\frac{1}{M_t} = \frac{1}{M_0} + kt \quad (4)$$

where M_t and M_0 are weight-average molecular weights (Da) at time t and 0 min, respectively.

The rate coefficient k (Da^{-1} min^{-1}) was determined as a slope of regression line of the $1/M_t$ versus t (min) plot.

All ^{1}H, ^{13}C and ^{13}C–^{1}H HSQC NMR spectra were measured in D_2O at 25 °C on Varian 600 MHz VNMRS spectrometer on inverse 5 mm PFG cold probe and processed using MestReNova 7.0 software.

3. Results and discussion

3.1. Ultrasonic degradation of mannan

Cell-wall mannan from *C. albicans* serotype A is a branched polysaccharide with a broad molecular weight distribution (PDI ~ 3.3) and molecular weights ranging from 1 MDa to 10 kDa. Ultrasonication showed to be an effective way to degrade this polysaccharide as evidenced by the decrease of molecular weight (Fig. 1). In the first stage of ultrasonic treatment, reduction of molecular weight proceeded rapidly (down to 55% of original value within first 10 min) then slowed down and levelled off asymptotically approaching a constant value of approximately 30 kDa after 120 min of ultrasonication. Similar values (25–26 kDa after 120 min) were reported for cellulose [16] and chitosan [20]. This so-called limiting molecular weight (M_{lim}) is defined as a critical length of macromolecule that can diffuse the loaded stress without a breakage of covalent bonds [19]. In other words, reaching M_{lim} molecules are too small to be cleaved by ultrasound and further exposure to ultrasonic waves will not induce any degradation [21]. Moreover, mechano-chemical degradation of macromolecules is influenced by polymer architecture [26]. In contrast to linear polymers, physical length of polymeric chain of branched polymers is shorter at the same molecular weight. Therefore, when it comes to branched polysaccharide a condition that $M_{path} > M_{lim}$ should be