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Bacillus subtilis 168 levansucrase (SacB) activity affects average levan molecular weight

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ABSTRACT

Levan is a fructan polymer that offers a variety of applications in the chemical, health, cosmetic and food industries. Most of the levan applications depend on levan molecular weight, which in turn depends on the source of the synthesizing enzyme and/or on reaction conditions. Here we demonstrate that in the particular case of levansucrase from *Bacillus subtilis* 168, enzyme concentration is also a factor defining the molecular weight levan distribution. While a bimodal distribution has been reported at the usual enzyme concentrations (1 U/ml equivalent to 0.1 μM levansucrase) we found that a low molecular weight normal distribution is solely obtained al high enzyme concentrations (>5 U/ml equivalent to 0.5 μM levansucrase) while a high normal molecular weight distribution is synthesized at low enzyme doses (0.1 U/ml equivalent to 0.01 μM of levansucrase).

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1. Introduction

Levansucrases are enzymes that carry out transferase and hydrolysis reactions from sucrose as substrate, in which transferase results in levan, a fructan polymer joined mainly through β-2,6 linkages. Levansucrase activity has been described in bacterial species such as *Microbacterium laevaniformans*, *Rahnella aquatilis*, *Zymomonas mobilis*, and some Bacillus, Erwinia and Pseudomonas species. Hydrolysis to transferase activities in levansucrases may be modulated by several factors such as sucrose concentration, temperature or the presence of organic solvents in the reaction medium. In general, high sucrose concentration increases transferase activity and markedly reduce sucrose hydrolysis (Euzenat, Guibert, & Combes, 1997); low temperatures also promote transferase (Tanaka, Oi, & Yamamoto, 1980), while high temperatures favor hydrolysis (Chambert & Gonzy-Treboul, 1976); high concentrations of organic solvents also increase transferase activity (Castillo & López-Munguia, 2004; Chambert & Petit-Glatron, 1989)

Levan is the main product of levansucrase transferase activity and has a molecular weight (Mw) associated to the source of the enzyme. Some levansucrases synthesize oligosaccharides such as the enzymes from *Gluconacetobacter diazotrophicus*, *Z. mobilis* and *Lactobacillus sanfranciscensis* while levans produced by enzymes obtained from cultures of *Gluconoacetobacter xylinus*, *M. laevaniformans*, *R. aquatilis* and *Z. mobilis* have average Mw of 40, 710, 380 and 570 kDa, respectively (Yoo, Yoon, Cha, & Lee, 2004). According to Ozimek, Kralj, van der Maarel, and Dijkhuizen (2006) fructans may be synthesized by two distinct processes following either a processive or a non-processive mechanism, at least in the case of levansucrase and inulosucrase enzymes of *Lactobacillus reuteri* 121. Nevertheless, in some particular cases, levansucrases produce *in vitro* levan with a bimodal type Mw distribution. This is the case of *Z. mobilis* levansucrase which is able to synthesize high and low Mw levan with an average of 3000 and 5 kDa respectively (Byun, Lee, & Mah, 2014). Similarly, *Bacillus subtilis 168* levansucrase synthesizes levan with high (≥2000 kDa)) and low (8.3 kDa) average Mw distributions (Ortiz-Soto, Rivera, Rudiño-Piñera, Olvera, & López-Munguía, 2008), and *B. subtilis* TH4-2 which produces levans of 6 and 660 kDa (Ben Ammar, Matsubara, Ito, Iizuka, & Masaru, 2002), in all cases under the reaction conditions tested.

Levan Mw distributions may also be modulated by several factors all related to reaction conditions. Temperature for instance has an unclear effect, as *Bacillus sp.* TH4-2 levansucrase produces

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levan with average Mw of 8 kDa at 30 °C, while at 50 °C the bimodal distribution already described is observed. But contrary to other levansucrases, this enzyme is able to produce high Mw levan at 50 °C (Ben Ammar et al., 2002) which is the same effect observed for levansucrase from *Bacillus licheniformis* RN-01 which synthetises high Mw levan (612 kDa) as a major product at 50 °C while at low temperature (30 °C), an 11 kDa levan is the main product. The effect of temperature is different in levansucrase from *B. subtilis* C4 which produces high Mw levan more efficiently at 37 °C than at 60 °C (Euzenat et al., 1997). Other factors described in the literature include the ionic strength and the presence of solvents. In reactions with levansucrase from *B. subtilis*, the average Mw distribution is shifted toward high Mw levan in the presence of 40% ethanol or 20% polyethylene glycol. On the contrary, levan Mw distribution shifts from the bimodal type, to a single low Mw distribution increasing the ionic force from 0.08 to 0.8 mM phosphate buffer (Tanaka et al., 1980). A similar effect is observed in levansucrase from *B. licheniformis* RN-01 when 0.5 M NaCl is included in the reaction medium, with 11 kDa as the major levan size obtained at various reaction temperatures (Nakapong, Pichyangkura, Ito, Iizuka, & Pongsawasdi, 2013).

Levan offers a variety of applications depending on its Mw, particularly in the fields of health, cosmetics and foods (Belghith, Song, Kim, & Rhee, 1996). Levans can be used as industrial rubber, substitute for blood plasma, and as a prebiotic sweetener. Other proposed potential applications include its use as emulsifier, stabilizer, thickener and carrier of flavors and fragrances (Shih, Yu, Shieh, & Hsieh, 2005). Furthermore, levan from *B. licheniformis* has been proposed as biological sealant in highly permeable soils, reducing significantly the hydraulic conductivity (Ghaly, Arab, Mahmoud, & Higgins, 2007). In recent years, reports about the health benefits of levan have increased, most of them associated to a particular levan size. High Mw levan produced by levansucrase from *B. subtilis* NRC1aza and its sulfated derivative exhibits the strongest free radical scavenging activity with DPPH (Abdel-Fattah, Gamal-Eldeen, Helmy, & Esawy, 2012). The low Mw levans produced by levansucrase from *Z. mobilis* have a stronger *in vitro* antibacterial inhibitory effect than the high Mw levan (Byun et al., 2014). Levans produced by several *Z. mobilis* strains show anti-tumor activities depending on the polysaccharide size (Calazans, Lima, de Franca, & Lopes, 2000). It is also important to point out that levan has been part of a traditional fermented product known as natto, produced from cooked soybeans using *B. subtilis* natto, and consumed in Japan since early times (Shurtleff & Aoyagi, 2014). More recently, the prebiotic properties of levan-type fructooligosaccharides have been evaluated to explore its role as soluble fiber and/or prebiotics (Porras-Domínguez et al., 2014).

Therefore, the possibility to define reaction conditions leading to the synthesis of levan with a defined Mw is an interest subject to understand the mechanism of levan chain elongation, but also to ensure the efficient production and availability of levans required for industrial scale applications.

2. Materials and methods

2.1. Enzyme preparation

Recombinant *B. subtilis* 168 levansucrase (SacB) was obtained as previously described (Ortiz-Soto et al., 2008). *Escherichia coli* BL21 (DE3) transformants were grown in Luria-Bertani broth containing 100 μg/ml of ampicillin at 37 °C. Culture was induced during 8 h at 18 °C by addition of 0.2 mM IPTG when the optical density reached 0.5. Cells recovered by centrifugation were suspended in 0.1 M potassium phosphate buffer (pH 6.0) and broken with a French Press (Thermo Spectronic). Enzyme was purified by ion exchange chromatography (AKTA prime; Amersham Pharmacia Biotech). The enzymatic extract was charged in a CM Sepharose resin (Pharmacia) previously equilibrated at pH 6.0 with 100 mM phosphate buffer at 1 ml/min, and eluted in a 100–1000 mM phosphate buffer (pH 6.0) gradient.

2.2. Standard activity assay

Levansucrase activity was assayed at 37 °C in 50 mM phosphate buffer (pH 6.0) using 1% sucrose solutions as substrate in 500 μl reactions. Global initial rate was determined by measurement of the reducing sugar released by DNS method (Miller, 1959). This is a general activity measurement used only as reference as the reducing power release includes both glucose and fructose in the case of hydrolytic activity, but only glucose in the transferase activity. Moreover, when low Mw levans are synthesized, a significant amount of sucrose molecules are consumed as acceptors, so that the reaction rate determination requires the measurement of sucrose consumption. Therefore, global activities are only measured during initial rates where the enzyme is mainly hydrolytic and the resulting values used only as reference for reaction conditions. However, when required, the activity value was corrected by HPLC determination of sucrose, glucose and fructose concentrations (see later). One global enzyme unit (U) was defined as the amount of enzyme that releases 1 μmol of glucose per minute. The effect of temperature on the Mw of the reaction products was evaluated at 4 °C and 37 °C in reactions containing 5 U/ml of enzyme activity, while the effect of sucrose concentration was evaluated in the range of 200–600 g/l. The effect of enzyme concentration was evaluated using 0.1, 1.0 and 10.0 U/ml of levansucrase activity in assays performed at 37 °C. All measurements were carried out in duplicates.

2.3. Identification and analysis of products

2.3.1. Structural analysis of low and high Mw levans

Low and high Mw levans were purified from reaction by ethanol fractionation using 3.5 and 1.5 v/v respectively. The precipitated levans were washed three times and freeze dried.

Levan structure was characterized by nuclear magnetic resonance (NMR) in a Varian 400 instrument operated at 100 MHz for ^{13}C and at 400 MHz for ^{1}H. FT-IR Spectroscopy was also obtained for the low and high Mw levan in a Fourier NICOLET 6700 between 400 and 4000 wave number (cm^{-1}), with DTGS KBr detector.

2.3.2. Polymer analysis by GPC and HPAEC-PAD

The Mw distributions of levan produced in levansucrase reactions were determined by gel permeation chromatography (GPC), with an HPLC equipment (see later) equipped with an isocratic pump and a refractive index detector (Shodex). Size exclusion columns of Ultrahydrogel Linear (7.8 mm × 300 mm) and Ultrahydrogel 500 (7.8 mm × 300 mm) with a size exclusion index between 0.50 and 10,000 kDa, respectively, were serially connected on the HPLC equipment. Levans diluted 1/10 directly from reactions were analyzed in the GPC columns at 37 °C with 0.8 ml/min of 0.1 M sodium nitrate as eluent. .A 10 μL 1/10 diluted samples from the reaction medium were directly injected to the HPLC. Dextrans of 5.2, 11.6, 23.8 and 48.6 kDa at 1% (w/V) (Waters. Polymers Std. Service) were used as standards to determine the low Mw levan profile. For the high Mw levan, a 2000 kDa dextran standard was available (Sigma Chemical), which is eluted in the column void volume.

A 10 μl sample of the low Mw levan distribution diluted 1/10 from the reaction medium was also analyzed by high performance anion exchange chromatography (HPAEC), using a precolumn and a Carbopac PA 200 column (Dionex) (3 mm × 250 mm) at 30 °C. NaOH (150 mM) was used as the mobile phase at 0.5 ml/min.

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