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Partial purification of a *Bacillus licheniformis* levansucrase producing levan with antitumor activity

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ABSTRACT

The extracellular fructosyltransferase (FTase) of a novel strain of *Bacillus licheniformis* capable of producing fructooligosaccharides (FOS) and a polysaccharide type levan was obtained and partially purified. The purification was achieved by ammonium sulfate precipitation, DEAE cellulose and gel filtration chromatographies. The enzyme was partially purified as determined by SDS-PAGE, and the specific activity reached was 67.5, representing a purification factor of 177 and yield of 40%. Levan was isolated from the cultures of *B. licheniformis*. The levan was composed mainly of fructose residues when analyzed by TLC after acid hydrolysis and NMR analysis. In a previous study, the levan produced exhibited a hypoglycemiant activity. The present paper deals with the study of the antitumor and anti-cytotoxic effect of levan produced by *B. licheniformis* strain. In the *in vitro* antitumor activity test of levan against some tumor cell lines, relatively the significantly high activity was observed against the $HepG_2$.

1. Introduction

Levansucrases (LSs), which are fructosyltransferases (FTFs) (E.C. 2.4.1.9) belonging to family 68 of glycoside hydrolases, catalyzes the formation of fructooligosaccharides (FOS) and the synthesis of β-(2–6)-levan by transferring the fructosyl group of non-activated sucrose into the fructan chain. In general, FTFs can also transfer the fructose residue either to water (hydrolysis reaction) or to an acceptor molecule other than the sucrose or fructan present in the reaction medium (acceptor reaction) [1]. Purification and characterization of FTFs from various sources have been reported [2]; however, this accumulated knowledge is confusing [3]. The information differs from one source to another, from one microorganism to another, even from one strain to another, there by making it imperative to purify the enzyme from each source. Attempts to purify the fructosyltransferase enzyme from culture supernatants, following growth on sucrose or raffinose, is sometimes unsuccessful, due to the presence of polymer. Moreover the enzyme binds very tightly to its product. An additional application of FTF is derived from its potential to fructosylate a variety of molecules of industrial interest, particularly for food and pharmaceutical applications [4,5]. Levan-type fructooligosaccharides (FOS) and β-(2–6)-levan are of increasing interest because of their potential health benefits to selectively support the intestinal health. FOSs have broad applications in the food and pharmaceutical industries. Indeed, β-(2–6)-FOSs have demonstrated prebiotic effects that surpassed those of β-(2–1)-FOSs available for human consumption. Levan, a polymer of fructose, is employed in food and nonfood industries as a viscosifier, stabilizer, emulsifier, gelling or water-binding agent. The high solubility and low viscosity of aqueous levan solutions would make it a suitable substitute for arabic gum [6]. Not only as a bioactive substance, levan, may also, have a wide range of applications in medicine, food, printing and cosmetics [7,8]. Several applications have been developed for levan, such as an anti-inflammatory agent against skin irritation and as a cell-proliferative agent [9]. Levan polymers have a variety of potential applications in the food and pharmaceutical fields because of their physical properties and their biological functions such as the promotion of infection and necrosis, tumor inhibition, tumor stimulation, and increasing the permeability of cells against cytotoxic agents [10]. The properties of levans depend, to a considerable extent, on the molecular weight as well as their structure. It has been reported, by several authors, that levans produced by *Aerobacter levanicum* [11,12] and *Zymomonas mobilis* [13,14] displayed antitumor and immunomodulatory activities. In a previous study, we have isolated a novel levansucrase produced by *Bacillus licheniformis* strain [15]. We have also showed that the levan produced have a hypoglycemic effect [16]. The present study deals with the partial purification of the levansucrase enzyme of *B. licheniformis* and characterization of the levan produced. In this paper, we try

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also to test if the levan used has the ability to have an antitumoral activity.

2. Materials and methods

2.1. Source of microorganism

Several samples were collected from Tunisian thermal source and different microorganisms were screened from these samples for their levansucrase activity. One of these microorganisms, morphologically identified as *B. licheniformis*, was retained and used for the production of levansucrase presented in this study.

2.2. Screening medium and method

The basal medium used for screening the levansucrase bacteria contained sucrose 200 g/l, yeast extract 5.0 g/l, trypton 10 g/l, K_2HPO_4 2.5 g/l, and agar 15 g/l. The pH was maintained at 7.4. The slants and plates were incubated at 40 °C for 24 h. Prior to its cultivation on the petri dishes, bacteria were cultivated on a subculture medium containing sucrose 50 g/l, trypton 10 g/l, NaCl 5.0 g/l, and yeast extract 5.0 g/l, pH 7.4. The media were first autoclaved at 121 °C for 20 min and then cooled in test tubes before the inoculation. The incubation was carried out in a rotary shaker (200 rpm) at 40 °C for 18 h [1].

2.3. Enzyme production

The medium used for the enzyme production had the following composition (g/l): Trypton, 10; NaCl, 5.0; yeast extract, 5.0 and sucrose 5.0. The initial pH was 6.5. The preculture and main culture were incubated at 40 °C, and shaken for overnight and 36 h, respectively.

2.4. Enzyme purification

2.4.1. Production step

Three liters of culture were prepared, inoculated and shaken for overnight at 40 °C for 36 h. Then, the culture was centrifuged at 10,000 rpm for 20 min in order to remove cells. The resultant cell free supernatant from the 3 l of culture was used as the starting material for enzyme purification.

2.4.2. Ammonium sulfate precipitation

100 ml of the extracellular enzyme was subjected to ammonium sulfate precipitation according to standard methods. Levansucrase enzyme precipitates between 60 and 80%. Both pellet and supernatant were assayed for levansucrase activity. Pellets from 60 to 80% precipitation were with high enzyme activity. The pellet was suspended in the minimum volume of phosphate buffer (20 mM, pH 6.5), and centrifuged again. The supernatant was dialyzed against 3 changes of phosphate buffer (20 mM, pH 6.5) at 4 °C for overnight to remove the ammonium salt. Dialysis was carried out using a cellulose dialysis membrane (220 mm in diameter) with a cut off point of 10 kDa.

2.4.3. Anion-exchange chromatography

Mono-Q Sepharose column (Pharmacia) was used in this study. The resin was packed into a column (18 cm × 3 cm) after activation and then equilibrated with phosphate buffer. The dialyzed sample was loaded onto the column and eluted with phosphate buffer (20 mM, pH 6.5). Adsorbed proteins were eluted with a linear sodium chloride gradient of "0–1 M NaCl" at a flow rate of 1 ml min^{-1} using a peristaltic pump. Fractions were collected on a fraction collector. The fractions were assayed for levansucrase activity. The protein content was measured at the OD of 280 nm. The active fractions were pooled and lyophilized.

2.4.4. Gel filtration chromatography

The lyophilized sample was dissolved in 1.0 ml of phosphate buffer (pH 6.5) and then loaded on a Sephacryl S200 column (2.5 cm × 150 cm) previously equilibrated with 20 mM phosphate buffer (pH 6.5). Proteins were eluted with the same buffer at a flow rate of 30 ml/h and fractions were collected as earlier described. The levansucrase activity and protein content of each fraction were determined. The active fractions were pooled, lyophilized, and stored at 4 °C until further use.

2.5. Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [17] in a tris-glycine after buffer system; 12% acrylamide separating gel was used with a molecular weight markers applied.

2.6. Enzyme assay

Levansucrase activity was assayed by incubating 250 μl of enzyme with 750 μl of sucrose (20%, w/v) in 20 mM sodium acetate buffer (pH 5.6) at 37 °C for 1 h. The activity of the enzyme was determined based on the amount of glucose released using a glucose oxidase kit. Levansucrase activity was defined as the amount of enzyme required to liberate 1 μmol of glucose under the specified conditions [18,19].

2.7. Analysis of reaction products by thin-layer chromatography (TLC)

The sugars in the reaction mixtures were analyzed by thin-layer chromatography (TLC) on silica gel G-60 using chloroform/acetic acid/water (6:7:1 by volume) as a mobile phase system. After layer development and mobile phase evaporation under continuous warm air flow for 15 min, the spots on the chromatograms were visualized by spraying a mixture of sulphuric acid and ethanol (10:90) on the plate.

2.8. Chemical hydrolysis of produced levan

The levan was enzymatically produced by the partial purified enzyme. The latter was added to a solution containing 40% sucrose (w/v) in 20 mM acetate buffer (pH 5.6) and incubated for 72 h at 40 °C. The levan was, then, chemically hydrolyzed by the addition of oxalic acid. The effects of the oxalic acid concentration and hydrolysis reaction time were then investigated. A 2 ml of reaction mixture containing levan were precipitated by adding 2.5 volumes of ethanol. The pellet was collected by centrifugation and dissolved in a minimum amount of distilled water for 24 h. The final step involved the release of an acid hydrolysis with two different concentrations of oxalic acid, 0.125% and 0.25%, in boiled water after 5, 10, 15, 20, 25 and 30 min. The products of hydrolysis were, then, analyzed by thin-layer chromatography.

2.9. Polymer production and characterization

Polymer was produced by incubating the partial purified levansucrase in a sodium acetate buffer (20 mM, pH 5.6) with 40% sucrose at 40 °C for 72 h. Polymeric material was precipitated with 2 volumes of 96% ethanol followed by 10 min centrifugation at 10,000 rpm. The pellet was suspended in demineralized water at 4 °C during 12 h followed by dialysis overnight against demineralized water. Subsequently, the polymer was precipitated with