Levan And Levansucrase-A Mini Review

Bruna Caroline Marques Goncalves, Cristiani Baldo, Maria Antonia Pedrine Colabone Celligoi

Abstract: Levansucrase is a fructosyltranferase that synthesizes levan and present great biotechnological interest. It's being widely used in therapeutic, food, cosmetic and pharmaceutical industries. Levansucrase is produced by many microorganisms such as the Bacillus subtilis Natto using the sucrose fermentation. In this mini-review we described some properties and functions of this important group of enzymes and the recent technologies used in the production and purification of levansucrase and levan.

Index Terms: Levan, levansucrase, fermentation, sucrose

1 INTRODUCTION

The levansucrase (EC 2.4.1.10) is the best characterized fructosyltranferases and are synthesize by most microbial levans by transferring a β(2→1)-D-frutosyl residue to the acceptor molecule (sucrose or levan) [1]. The database "carbohydrate-active enzymes" (CAZY) grouped the microbial levansucrases into glycoside hydrolases 68 family (GH68) due to be able to act in specific substrate and share two catalytic, often acidic residues, acting as a protons donor and nucleophile or general base, respectively [2]. This mini-review focuses on the properties, functions and activity of these enzymes as wells as on the production and purification technologies used to obtain levan and levansucrase.

2 LEVANSUCRASE: PROPERTIES, FUNCTIONS AND ACTIVITY

Pascal and co-workers [3] studied the sucrase activity in Bacillus subtilis and concluded that this activity is supported by two distinct proteins: levansucrase and sucrase. The main differences between these two enzymes reside in their localization (intracellular for sucrase and almost completely extracellular for levansucrase) and their capacity to catalyse levan synthesis. The SacA enzyme from Z. mobilis is a monomer with a molecular weight of 58 kDa [4]. The deduced amino acid sequence of sacA gene product showed strong homology with the intracellular sucrose of B. subtilis and yeast invertases. In the absence of a transport mechanism for sucrose, the role of intracellular sucrase in Z. mobilis is not clear. An extracellular sucrase (SacC or InvB) has a high specific activity for sucrose hydrolysis.

- *Bruna Caroline Marques Gonçalves, Department of Biochemistry and Biotechnology, Centre of Exact Science, State University of Londrina, Parana, Brazil.*
- *Cristiani Baldo, Department of Biochemistry and Biotechnology, Centre of Exact Science, State University of Londrina, Parana, Brazil.*
- *Maria Antonia Pedrine Colabone Celligoi, Department of Biochemistry and Biotechnology, Centre of Exact Science, State University of Londrina, Parana, Brazil. E-mail: macelligoi@uel.br*

This enzyme contributes 60% of the extracellular sucrase activity, but it catalyses neither fructose polymerization into levan nor degradation of polyfructose such as levan or inulin. Thus, this enzyme differs from the B. subtilis SacC, which showed levanase activity in addition to sucrose hydrolysis. FOUET et al. [5] characterized the precursor form of Bacillus subtilis levansucrase and identified 3 structural genes induced by sucrose. One of them, sacB, codify extracellular levansucrase and 4 of 5 recognized regulatory loci are able to control Sac B expression. The 5' end sequence of the sacB gene was determinate, and the signal peptide aminoacid sequence was deduced. It contains 29 aminoacid residues and is characterized by the presence of a hydrophilic portion with 3 Lys residues followed by a hydrophobic sequence with an Ala residue in the position 1. The levansucrase proteic structure consists in an ellipsoid and compact polypeptide monomeric or dimeric form, and have the highest molecular weights. According to Ammar et al. [6] and La Gorrec et al. [7], levansucrase activity is engaged in: (i) soil bacterial survive (B. subtilis), (ii) phytopathogenesis (Erwinia and Pseudomonas) (iii) symbiosis (B. polymixia). Levansucrase is produced by innumerous microorganisms and, among them, B. subtilis has been studied due to the ability to degraded sucrose and catalyze high molecular weight levan chain formation [8]. Bekers et al., [9] related the most levansucrase producers have been B. subtilis e Z. mobilis, which synthesize inducible form of levansucrase followed by extracellular secretion, instead of Aerobacter levanicum, which present a constitutive and intracellular enzyme. Three differents levansucrase activities are recognized: (i) fructosyltranferase, (ii) hydrolase and (iii) polymerase. The fructosyltransferase activity is characterized by transferring fructosyl residues of sucrose to a variety of acceptor. According to adopted experimental conditions the acceptor can be: water, during sucrose hydrolysis, glucose, promoting the change of [^{14}C] glucose in the reaction of frutose-2,1-glucose + [^{14}C]glucose to fructose-2,1-[^{14}C]glucose + glucose and sucrose, during the synthesis of mono-, di-, oligosaccharide or levan, during the polymerization reaction and alcohols [10]. Dedonder [11] studied the levansucrase specificity considering the transfer of waste, donors and acceptors also polymerase and hydrolase activities. Fructose residues are usually transferred for all levansucrases. However, levansucrase from B. subtilis, produced as induced form and secreted in the extracellular environment, is capable of transferring L-sorbosil or D-xilulosil, a property not found in levansucrase from Aerobacter levanicum. Interestingly, only molecules endowed with a fructosyl group unsubstituted connected to an aldosil group by the same diglycosidic bond as sucrose may act as a donor. Sucrose and raffinose were the first donors known. Levan chains are initiated by compounds that have not reduced

fructose as terminal residue, with free hydroxyl at carbon 6. At the beginning of the levan polymerization trifructosyl series of primers are effective, and the efficiency of the primer increases with the polymerization degree. Polymerase activity rate is less than hydrolytic activity rate on presence of sucrose and always leads fructose formation in free form or oligosaccharide form plus levan. Levansucrase shows hydrolysis activity against small levan chains and this action is terminated when reaches the branch points. Levan synthesis begins with the transfructosilation reaction, where the sucrose molecule acts as a donor fructosyl and a second sucrose molecule acts as fructosyl residue initial acceptor. Sucrose attack may occur via oxygen 1, synthesizing 1-kestose, or via oxygen 6, synthesizing 6-kestose. 6-kestose is formed by bridges β(2→6) and levan can be polymerized [10]. Chambert and co-workers [12], [13] studied the mechanism of action of levansucrase. They confirmed the hypothesis of Ping-Pong mechanism and the involvement of an intermediate complex formed between the enzyme and fructosyl residue. The study of temperature dependence for the fructosylation and desfructosylation enzyme constant showed that the activation entropy for the two desfructosylation steps is almost the same, but the activation enthalpy to transfructosilation step to water is greater than for glucose. Studies of Martinez-Fleites et al. [10] on the crystal structure of levansucrase from B. subtilis showed the importance of amino acid residues located in active site for substrate recognition. Enzyme chain is composed of five parts in β conformation that is essential for enzymes catalytic activity of the hydrolase family members 68. The complex sucrose-levansucrase structure suggests that Glu342 and Asp86 are a essential pair to the active site characteristic of most glycosyl hydrolases, while Asp 247, although necessary for catalysis, does not appear to be directly involved in the chemical reaction mechanism. Cleavage chemical reaction analysis of the intermediate indicates that the aspartyl residue forms a covalent bond with fructosyl residue linked with the enzyme. The reaction mechanism shows that the canonical Asp86 and Glu242 must be at different protonation stages for substrate binding ([14]. Vigants et al. [15] studies confirmed that NaCl and KCl addition to the culture medium containing sucrose stimulates levan biosynthesis due to direct influence of salt in gene activation, with an increase in levansucrase synthesis. Vigantes and co-workers [16] observed that the stimulus occurred in the levan synthesis by NaCl on the Z. mobilis sucrose fermentation. Reaction catalyzed by levansucrase was inversaly proportional to the substrate concentration when salt was present, concluding sodium and potassium were heterotropics activators at lower sucrose concentrations.

3 Levansucrase production

The effect of different fermentation conditions on levansucrase production has been studied by several authors, considering the applications importance. The first study found on B. subtilis levansucrase production and purification was published in 1966 by Dedonder [11]. In the 1880s, Lyness and Doelle [17], [18], [19] conducted a series of studies related to levansucrase from Z. mobilis and the results showed the sucrose hydrolysis rate are dependente on temperature, sucrose concentration and pH. Cote [20] investigated levansucrase from Erwinia herbicola NRRL B-1678 production and tested the effect of various carbon sources (sucrose, glucose, fructose, sorbitol and mannitol) and nutrients sources (dried nutrient broth and corn steep liquor) in the enzyme secretion. The results indicated reduced of microorganism grown when fructose was used as carbon source. The reduction of levansucrase production was also observed. When glucose was used, the levansucrase activity reached a peak after the stationary phase, and at the end of fermentation the activity reached zero. The other carbon sources showed similar behavior, but the decline in activity was not as accentuated as for glucose. The highest levansucrase yield were produced in culture medium consisting of corn steep liquor and sorbitol, with activity of approximately 0.7 U ml -1, followed by medium composed of fructose and nutrient broth or corn steep liquor, both of wich yielding approximately 0.30 and 0.35 U mL-1 respectively. Microorganism growth and levansucrase production were identical using mannitol and sorbitol as carbon source [20]. Abdel-Fattah, Mahmoud e Esawy [21] studied constitutive and inducible extracellular production of levansucrase from B. subtilis NRC33a using sucrose and glucose as carbon source obtaining activity of 14.5 and 14.1 U mL-1 respectively. Among different nitrogen sources tested (soy, corn mash, baker's yeast, wheat, peptone, casein, urea and ammonium sulfate), culture medium containing baker's yeast had the highest extracellular levansucrase production. The highest activity (17.5 U mL-1) was found when used baker's yeast. Addition of 0.02% N2, increased the activity to 18 U mL-1 and addition of 0.15 g L-1 MgSO4 plus 5.0 g K2HPO4, increased to 19.5 U mL-1. Levan molecular weight varied between 49.91 and 56.8 kDa with increasing enzyme concentration from 5 to 1000 µg mL-1. Polysaccharide molecular weight was also influenced by sucrose concentration and temperature. A molecular weight reduction was observed with increasing sucrose concentration to 40%, and levan chains with higher molecular weight were synthesized at 15°C (58.52 kDa). Levan molecular weight decreased with temperature increasing. The reaction time did not influence in the levan molecular weight ([21]. Agricultural residues were also evaluated for the enzyme production. Ahmed [22] studied Bacillus megaterium levansucrase production by solid state fermentation, adopting orange and limes peels, sawing, banana and wheat bran waste as substrates. The maximum enzyme yield was 140.54 U g-1 of dry solids substrate, obtained from 5 g of sawdust moistened with 55 mL of culture medium with initial pH 6.0, after 72 h static incubation at 30°C. El-Refai, Abdel-Fattah e Mostafa [23] produced levansucrase from Bacillus circulans in medium composed of baker's yeast, sucrose and magnesium and phosphate salts, pH 5.2, incubated in orbital shaker for 72 h at 30°C. The partially purified enzyme showed specific activity of 16.82 U mg-1 protein after wool immobilization by physical adsorption. An increase of levan molecular weight produced with variation between 35.5 and 38.5 kDa was observed. At higher sucrose concentration (32%), the levan produced had low molecular weight (5 kDa). In opposite way, using the lower sucrose concentration (5%), the levan molecular weight obtained was greater than 35 kDa. The temperature and period of incubation were considered important factors to the levan polymerization. Levan higher molecular weight (38 kDa) was obtained at 30°C, with a gradual decrease with temperature increasing and a molecular weight increase between 6 and 24 h from 10 to 38 kDa was observed. Esawy, et al. [24] isolated six strains of B. subtilis from honey samples and evaluated the levansucrase production varying sucrose concentration (8-12%), temperature (37-40°C), time (24-28 h),

agitation (50-100 rpm), pH (6.0 to 7.0) and NaCl (0-4%). Levan produced molecular mass was determined and tested for antiviral activity. The highest activities and levan production observed varying between 62 and 59 U mL-1 and 11 and 16.25 g L-1, respectively. The presence of salt increased enzyme production by all strains up to 3 times, but reduced in 40-50% the levan production. The levan molecular weight varied between 14,200 and 154,638 kDa, and showed antiviral activity [24]. One of the first studies on levansucrase production by Bacillus subtilis Natto CCT 7712 using response surface Methodology was published by Gonçalves et al. [25]. In this study the substrate concentration, pH and agitation were variables evaluated using Box-Behnken 23 design. According to the optimized conditions the levan production and levansucrase activity was 205 g/L and 8.53 UA/mL, respectively. In addition, the levansucrase showed to be a thermo-stable and thermo-active enzyme.

4 Levansucrase characterization and purification

Levan was first identified by the researchers in 1901 through observations of solutions containing sucrose, as sugar cane juice, where one microorganism of the genus Bacillus produced a gum, leaving a viscous medium. This gum was named levan by diverting the plane of polarized light to left, in analogy dextran, a gum already known at the time. Levan is a polifructans linked by β fructofuranosidics-(2→6) straight chain. This exopolyssacharide may have varying degrees of branching with linked β(2→1) and different molecular weights, regulated by medium strength ion. Levan can be produced by certain bacteria that grows on sucrose medium, such as B. subtilis, Aerobacter levanicum, Erwinia herbicole, Streptococcus salivarius and Z. mobilis, by levansucrase transfructosilation reaction [6], [26], [27], [28]. Levan rheological properties as water solubility, low intrinsic viscosity (η = 0.17 dl g-1) and high tensile strength of 991 psi make it a very compact polymer that may be responsible for cellular aggregation, osmotic stress tolerance and desiccation [29]. According to Bekers et al. [30] characteristics such as viscosity, solubility in water, oil, suspension or rheological properties, compatibility with salts and surfactants, stability to heat, acids and bases, film forming capacity, water and chemicals retention, biological properties make it able to be used as stabilizing agent, emulsifier, thickener, encapsulating, cryoprotectant and osmoregulator making the levan attractive for application in many fields. Molecular weight of levan obtained by synthesis or microbial enzyme is estimated at about 2.5 x 106 Da, varying according to the producing microorganism and the culture conditions. In the therapeutic area, the levan has been used due to its antiviral [24], antidiabetic [31], and antitumor activity [32], [33], and these activities are related to its molecular chain [34]. For levan preparation for medical use, it is necessary to purify the sediment of levan and investigate its stability against different pH values and temperatures. Tanaka et al. [26] investigated the effect of different sucrose concentrations (1, 5, 10 and 20%) in the levan synthesis by levanasacarase from B. subtilis at 4°C for 10 days. The levan synthesis was more efficient at higher concentrations of sucrose. Lower temperatures favor levan synthesis and higher temperatures favor the sucrose hydrolysis. Ananthalakshmy and Guanasekaran [28] have isolated two Z. mobilis B4286 mutants overproducing levan, ZML1 and ZML2 and compared with the parental strain. Sucrose hydrolysis rate was high for all three strains (about 13 g L-1 h-1) and production levan rate was low (0.58 , 0.40 and 0.18 g L-1 h-1 respectively) on first 8 h of fermentation. Between 8 and 16 hours of fermentation, the hydrolysis sucrose rate decreased to 2.38 g L-1 h-1 and mutants levan production speed reached values of 1.72 and 1.68 g L-1 h-1, whereas the parental strain produced only 0.69 g L-1 h-1. Maximum levan production by the mutants was achieved with 24 h of fermentation, corresponding to 21.6 and 20.0 g L-1, with levansucrase activity: 30.6 and 36.0 U mg-1 and hydrolysis activity: 68.0 and 71.0 U mg-1, while the parental strain produced 16 g L-1 with 12.6 and 55.7 U mg-1 activity. Abdel-Fattah Mahmoud and Esawy [21] analyzed the enzyme concentration effect on the levan or oligosaccharides production. Higher enzyme concentrations produced higher levan amounts. Fructose conversion in levan was increased by 84% when used 1000 g mL-1 enzyme. Bekers et al. [30] studied levan stability produced by Z. mobilis 113 "S" during the liquid cultures stock at different pH and temperature. Levan was stable in the liquid culture during 120 hours, between 25 and 30°C and pH greater than 3.7. About 90% of levan was degraded at temperatures between 55 and 60°C, at pH 3.95 for 120 hours of storage, and 70% was degraded at pH 4.5. The authors concluded that levan degradation in liquid culture at high temperatures results from acid hydrolysis from organic acids produced by Z. mobilis which reduce the pH. Santos and co-workers [35] studying the production of levan from B. subtilis Natto CCT 7712 related that the conditions of sucrose (400 g/L) and 16 h of period of incubation resulted in 111.6 g/L of levan, which presented two molecular weights 568.000 Da and <50.000 Da.

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