

Enzymatic synthesis of fructo-oligosaccharides by recombinant levansucrase from *Leuconostoc mesenteroides* Lm17

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Dedicated to Acad. Ivan Juchnovski on the occasion of his 80th birthday

The levansucrase [EC2.4.1.10] can transfer fructosyl units from sucrose to different acceptors (maltose, lactose) to give a good yields of oligosaccharides that could have prebiotic potential. Selectivity of the reaction of synthesis of fructooligosaccharides (FOSs) using levansucrase was strongly dependent on the enzyme source, type of acceptors and donor/acceptor ratio (D/A). The acceptor specificity studies determined the ability of recombinant levansucrase Lm17 to synthesize different types of (FOSs) using different acceptors. In the present study we first describe that when maltose was the acceptor, the major products was a FOS with degree of polymerization (DP3) (75,5% of total FOS) identified as an erlose. Levansucrase was highly transfructosylating than hydrolytic activity. In carrying out the enzymatic reaction in the presence of 10% sucrose and 5% maltose, K_m of recombinant levansucrase Lm17 was 43.99 ± 7.01 mM sucrose, and V_{max} value was 2.1 ± 7.7 U/ml. This investigation of the properties of recombinant levansucrase Lm17 reveals the potential use of the enzyme as tools for synthesis of short chain FOSs (erlose) with prebiotic potential.

Key words: *Levansucrase; Leuconostoc mesenteroides; fructooligosaccharides; prebiotics*

INTRODUCTION

Lactic acid bacteria (LAB) belonging to the genera *Lactobacillus*, *Streptococcus*, *Leuconostoc* and *Weissella* have been reported as producers of fructosyltransferases (FTFs) [1,2,3,4]. FTFs are classified into a family 68 of glycoside hydrolase enzymes (GH68) (CAZY, <http://cazy.org>) [5]. According to the type of the glycosidic linkages between fructosyl units in the synthesized fructans these enzymes are divided into levansucrases (EC 2.4.1.10) synthesizing levan with β -(2 \rightarrow 6) linkages and inulosucrases (EC 2.4.1.9) synthesizing inulin with β -(2 \rightarrow 1) linkages in the main chain [6]. Microbial levansucrases have a molecular weight in a range of 46-120 kDa [7,8,9,10]. These enzymes catalyze hydrolysis of sucrose in the presence of water as an acceptor (hydrolase activity) and also a transfer of fructosyl residues to different acceptors (transferase activity). When a growing levan chain is used as an acceptor of β -D-fructosyl residues, levan with β -(2 \rightarrow 6) linkages is synthesized. FTF can catalyze fructose transfer from both sucrose and raffinose to variable acceptors. In presence of different monosaccharides, disaccharides and oligosaccharides as acceptors the transferase activity of levansucrase leads to synthesis of fructo-oligosaccharides [11]. The nature of the enzyme,

the sucrose concentration, the type and concentration of acceptors, the temperature and pH of the reaction influence the amount and molecular masses of the synthesized oligosaccharides [8,12,13]. In the case of transfructosylation reaction levansucrases enzyme can use different sugar acceptor molecules such as maltose, lactose, cellobiose, melibiose, isomaltose, D-galactose, D-fructose, and D-xylose, which leads to the synthesis of fructo-oligosaccharides [11]. Currently commercial available FOSs for human consumption are exclusively inulin-type prebiotics with α -(2-1)-linkages. However, the levan-type-FOSs, with α -(2-6)-linked fructose and some α -(2-1)-linked branching, have demonstrated prebiotic effects, in vitro, that surpass inulin-type FOSs [14]. The functional health attributes of prebiotic FOSs are dependent on their chemical structures, in particular the type of hexose moieties, the extent of polymerization and the glycosidic linkages. That's why it is important to characterize new enzymes, for production of fructo-oligosaccharides with different structure and biological activity. The nature of the enzyme, the sucrose concentration, the type and concentration of acceptors, the temperature and pH of the reaction are important characteristic that affect the amount and the structure of the synthesized oligosaccharides [8,12,13].

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In a previous study, we showed that *L. mesenteroides* Lm 17 strain, isolated from fermented cabbage, produces a glucosyltransferase of about 180 kDa and two FTFs with molecular masses about 120 kDa and 86 kDa [15].

The aim of this study was to optimize the transferase reaction of levansucrase from *L. mesenteroides* Lm 17 strain expressed in *E. coli*. The selected enzyme was characterized in terms of the effect of different substrates on its transferase activity, determining the Michaelis constant for different substrates and possible inhibition constant by glucose and fructose. These results will give information for industrial application of levansucrase for the production of novel prebiotic fructo-oligosaccharides.

EXPERIMENTAL

Preparation of a cell free extract and purification of the recombinant levansucrase L17

E. coli BL21(DE3) cells were collected by centrifugation (9,000 rpm, 15 min, 4°C) and washed two times with 20 mM sodium acetate buffer (pH 7.5). The cells were resuspended in a cold sonication buffer containing 20 mM sodium acetate buffer (pH 7.5), 300 mM NaCl, 5 mM imidazole and 5 mM β -mercaptoethanol and then were sonicated (Hielscher Ultrasound Technology UP50H, Germany). The cell debris was separated by centrifugation (9,000 rpm, 15 min, 4°C) and the supernatant was used as a cell free extract. Recombinant enzyme was purified using His Trap FF 5 ml Ni Sepharose Fast Flow column (GE Healthcare). The column was equilibrated with binding buffer (20 mM sodium acetate buffer (pH 7.5), 0.5 M NaCl, 30 mM imidazole) and then the cell free extract was added to the column. To remove the unbound proteins, the column was washed with three volumes of the same buffer. Then the bound His-tagged protein was separated from Ni Sepharose column by elution buffer (binding buffer with 500 mM imidazole). The eluted fraction was concentrated 10-fold using 10,000 MW cut off concentrators (Sartorius) and dialyzed against 20 mM sodium acetate buffer (pH 5.5).

Levansucrase assays and protein determination

One unit of levansucrase activity is defined as the amount of enzyme that catalyzes the formation of 1 μ mol glucose per minute at 30°C in 20 mM sodium acetate buffer (pH 5.5); 0.05 g/l CaCl and

100 g/l of sucrose. Levansucrase activity was determined by measuring the amount of reducing sugars derived from sucrose by the 3,5-dinitrosalicylic acid method (DNS) [16]. For determination of the specific enzyme activity, proteins were assayed by the method of Bradford [17], using bovine serum albumin as a standard. The transferase activity of the purified levansucrase was determined as a difference between the amount of released glucose from sucrose (total activity) and the amount of measured fructose (hydrolytic activity) in the presence of 100 g/l of sucrose. D-glucose and D-fructose were measured enzymatically with hexokinase, glucose-6P dehydrogenase and phospho-glucose isomerase (K-FRUGL kit from Megazyme). All measurements were performed at least in triplicate and average values are given.

Kinetic studies of recombinant levansucrase L17

The kinetics parameters were determined at 35°C in the presence of 20 mM sodium acetate buffer, pH 5.5, 0.05 g/l CaCl₂ and sucrose concentrations ranging from 20 to 1000 mM. All measurements were performed in triplicate and the received data were processed using SigmaPlot 12.0 (Systat Software Inc.). Km and Vmax were determined by nonlinear regression approach according and Lineweaver-Burk equation.

Determination of acceptor specificity of recombinant levansucrase and optimization the reaction conditions for fructooligosaccharides synthesis

To study the acceptor specificity of purified levansucrase various sugar acceptors (fructose, arabinose, maltose, lactose, lactulose, raffinose, fructooligosaccharides and galactooligosaccharides) were tested. The enzymatic synthesis in the presence of sucrose as fructosyl donor and various sugar acceptor molecules was carried out at 35 °C in 20 mM sodium acetate buffer, pH 5.5, containing 0.05 g/l CaCl₂ and 0.5 U/ml purified levansucrase. The reaction volume was 50 ml. Samples were taken at different time points (6 h, 12 h, 24 h, 48 h). In order to optimize the reaction conditions for synthesis of fructooligosaccharides different concentrations of sucrose and acceptors-maltose, lactose, raffinose were tested. To evaluate the influence of enzyme concentration on the reaction conditions and the amount of the synthesized products, synthesis reactions were carried out using 0.5 U/ml, 1 U/ml and 2 U/ml purified levansucrase.

HPLC-analysis of fructooligosaccharides

Separation of oligosaccharides was achieved by using a CarboPac PA1 anion-exchange column (250 mmx64 mm; Dionex) coupled to a CarboPac1 Guard column (Dionex). The following gradient was used: eluent A at 100% (0 min), 70% (10 min), 60% (25 min), 10%.

(80 min), 0% (83 min), 100% (91 min). Eluent A was 0,1 M sodium hydroxide, and eluent B was 0,1 M sodium hydroxide in 0,6 M sodium acetate. Detection was performed with an ED40 electrochemical detector (Dionex), with an Au working electrode, and an Ag.

AgCl reference electrode. The amount of sucrose utilized during the reaction reflected the total enzyme activity. The amount of fructose synthesized reflected the hydrolytic enzyme activity. The total activity minus the hydrolytic activity reflected the transglycosylation enzyme activity (polymer and fructooligosaccharide formation). Based on these data, the hydrolysis versus transglycosylation ratio (end-point conversion) was calculated.

RESULTS AND DISCUSSION

Effect of sucrose/enzyme ratio on the levan and fructooligosaccharides production

In a previous study, we showed that the synthesized polymer from recombinant levansucrase from *L. mesenteroides* Lm 17 is levan [18]. Using different concentrations of levansucrase, 0,5 U/ml, 1 U/ml and 2 U/ml, a study on the influence of enzyme concentration on polysaccharide and oligosaccharide synthesis from 10 % sucrose was carried out at 35°C in 20 mM sodium acetate buffer, pH 5.5, containing 0.05 g/l CaCl₂ (Table 1). The highest amount of the reaction products were established when the enzyme activity in the reaction mixture is 1 U/ml. Interestingly, increasing levansucrase activity to 2 U/ml didn't led to higher concentration of the products. At the 6th hour of the start of the enzyme reaction, maximum sucrose is hydrolyzed using 2U/ml levansucrase (86.2%), which is three times the hydrolysed sucrose using 0.5 U/ml levansucrase. From previous studies is known that glucose has inhibitory effect on hydrolysis and transfructosylation reactions of levansucrase [19]. Table 1 indicates that accumulation of higher amounts of glucose at the early stages of the enzyme reaction inhibits processes for polysaccharide and FOS synthesis. In all reactions

with varying enzyme activities the whole amount of sucrose is hydrolyzed at 12th hour, and hydrolysis of the substrate proceed faster with increasing the levansucrase activity in the reaction mixtures.

Table 1. Profile of the products synthesized by levansucrase L17 at different in presence of 10% sucrose.

Time (h)	Levansucrase activity (U/ml)	Consumed sucrose (%)	F _{POL}	F _{FOS}
3	0.5	2.5	14.9	0.0
	1.0	24.7	60.1	6.2
	2.0	62.4	47.8	1.4
6	0.5	22.7	22.7	0.0
	1.0	61.4	63.1	4.2
	2.0	86.2	54.9	2.0
12	0.5	100	62.3	3.2
	1.0	100	92.8	2.3
	2.0	100	65.8	1.7
24	0.5	100	69.6	3.3
	1.0	100	97.2	2.8
	2.0	100	73.6	2.5

Synthesis of oligosaccharides was observed at the early hours (3th hour) from the beginning of the reaction, when 1 U/ml and 2 U/ml enzyme was used. The results on Table 1 indicate that transfructosylation reaction for production of levan was favored after 6th hour of the beginning, and the hydrolysis reaction was not significant over the same period of the enzyme reaction. These results represent a very important characteristic of the studied recombinant levansucrase with regards to its high transferase to hydrolase ratio. It was reported that production of FOS by FTFs is competitively inhibited by glucose which is formed as a by-product in transfer reaction [20]. We tested the influence of glucose on (277,5 mM) on levansucrase activity. In presence of 277,5 mM and 292 mM sucrose recombinant levansucrase Lm17 shows 23 % of inhibition when 0,5 U/ml enzyme is used and 16 % - when 1 U/ml enzyme is used (Fig. 1). The received results confirmed that the lower amount of levan and FOS formed by levansucrase at 2 U/ml enzyme activity is due to the product inhibition. The higher enzyme activity leads to accumulation of higher amounts of glucose at the earlier stages of the reaction, and cause partial enzyme inhibition. Steinberg et al., 2004 demonstrated inhibitory dose-dependent effect of glucose on FTF activity from *S. mutans* V-1995, as the authors observed 75% inhibition and less fructan production at 160 mM concentration of glucose in the reaction mixture [21]. Product inhibition by fructose has also been reported for glucansucrases synthesizing glucans from sucrose [22, 23].

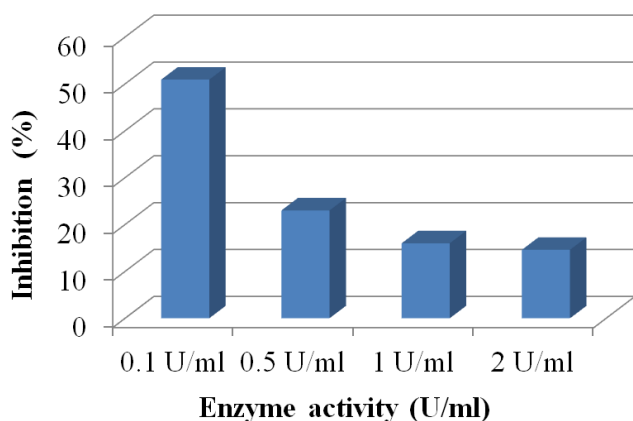


Fig. 1. Inhibition of levansucrase activity (%) by the released glucose.

Acceptor specificity of recombinant levansucrase Lm17

To investigate the donor/acceptor specificity (D/A) of levansucrase from *L. mesenteroides* Lm17 expressed in *E. coli* BL21(DE3), transfructosylation reactions were conducted by using sucrose as a donor of fructose units and fructose, arabinose, maltose, lactose, lactulose, raffinose, fructo-oligosaccharides or galacto-oligosaccharides as acceptors. Synthesis of oligosaccharides was detected only with maltose, lactose, fructo-oligosaccharides and galacto-oligosaccharides as acceptors (Table 2).

Table 2. Acceptor specificity of levansucrase L17.

Sugar acceptors	Synthesis of FOSs
Arabinose	-
Fructose	-
Maltose	+
Lactose	+
Lactulose	-
Raffinose	-
FOSs	+
Galacto-oligosaccharides	+

In the presence of fructose, arabinose, lactulose and raffinose, oligosaccharides were not detected. As a best acceptor for oligosaccharide production from recombinant levansucrase Lm17 was determined to be maltose, which is known to be the best acceptor for the action of dextransucrase enzymes [23]. The highest total amount of synthesized FOS – 20.4% was determined at 12th hour.

Table 2 indicate that the catalytic efficiency of the transfructosylation activity of the studied recombinant levansucrase is dominant after 6 hours of reaction.

Ratio between hydrolysatation and transfructosylation activity of levansucrase Lm17

Equivalent amounts of glucose and fructose from sucrose is released from the hydrolytic activity (Ht) of the levansucrases. The ratio between glucose and fructose, could therefore be used to determine the amount of fructose used for transfructosylation to other sugars, i.e., the transferase activity (Ft). Based on the concentration of released glucose and fructose during the reaction catalyzed by levansucrase L17, was determined the ratio between transferase and hydrolase activity of the enzyme. As is shown on a Fig. 2, during enzyme reaction the transferase activity of of levansucrase L17 was predominant as in the presence only of substrate sucrose (10%), thus in the presence of acceptor maltose (5%). In the presence of 10% sucrose the highest transferase activity was observed at 12th hour, and after that decreases until 48th h.

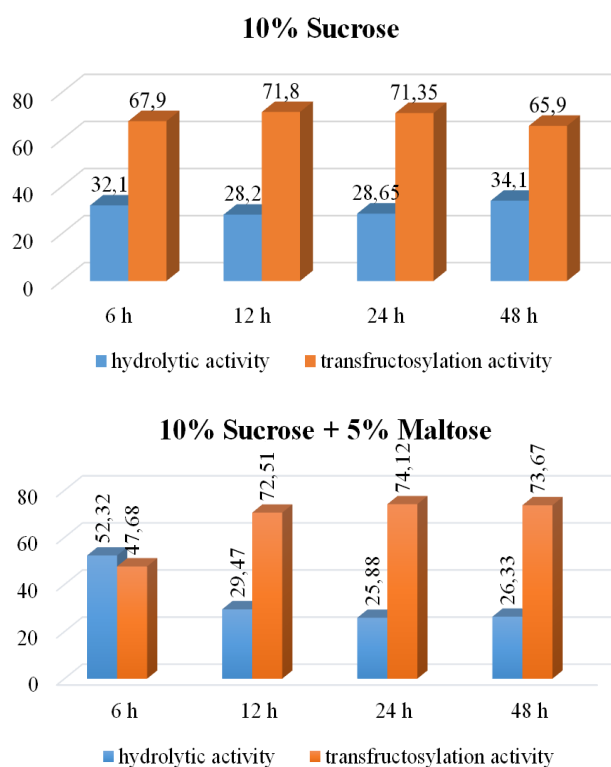


Fig. 2. Profile of hydrolase and transferase activity of levansucrase L17 in the presence of sucrose and maltose.

In the presence of acceptor maltose in the reaction mixture the transferase activity of the enzyme increases. The highest transferase activity in presence of 5 % maltose was observed at 24th hour. Different factors affect the ratio between transferase and hydrolase activity of levansucrase -

sucrose concentration, pH and temperature [10,11]. It is known that the formation of low-molecular-mass fructans at the early stages of the reaction affects the ratio between transferase and hydrolase levansucrase activity by directing the fructose units mostly to elongation of the fructan chains instead to water [24].

Determination of kinetic parameters in presence of 10 % sucrose and 5 % maltose

K_m and V_{max} values were determined at concentrations of sucrose ranging from 20 to 1000mM, and calculated from Lineweaver-Burk plots. K_m of the recombinant levan-sucrase Lm17 was 62.96±6.27mM sucrose, and V_{max} value was 6.34±0.14 U/ml (Fig.3).

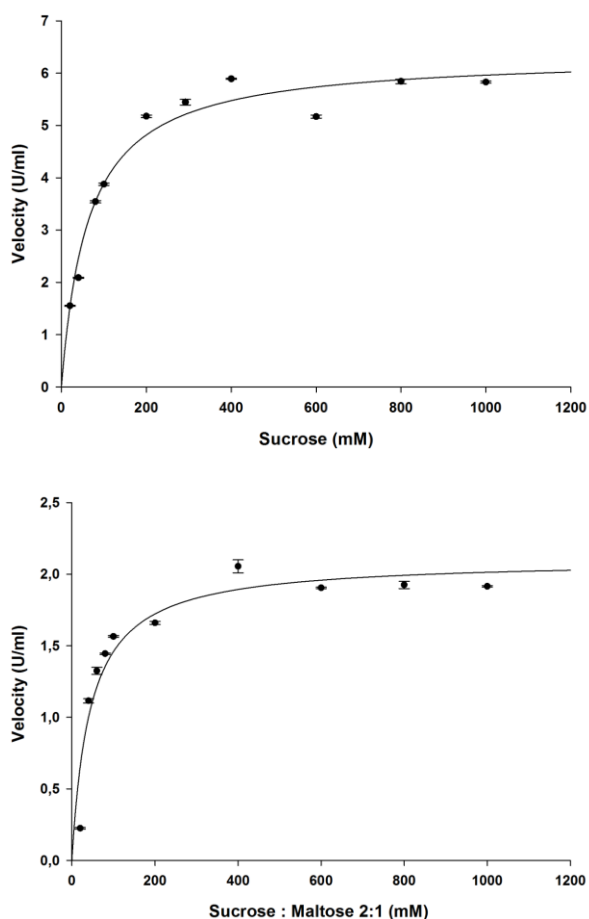


Fig. 3. Kinetics parameters of levansucrase L17 determined in the presence of sucrose and at sucrose/maltose ratio 2:1.

The value of K_m was higher than this one of other recombinant levansucrases: K_m = 36.7±5.4 mM for LevS from *L. mesenteroides* NRRL B-512F [28]; K_m = 27.3 mM for LevC from *L. mesenteroides* ATCC 8293 [25]; K_m = 26.6 mM

for levansucrase M1FT from *L. mesenteroides* B-512FMC [7], and K_m = 14.5±4.2 mM for levansucrase from *Lb. sanfranciscensis* TMW 1.392 [26].

K_m and V_{max} values were determined also in presence of acceptor maltose (D/A = 2 :1). In carrying out the enzymatic reaction in the presence of 10% sucrose and 5% maltose, K_m of recombinant levansucrase Lm17 was 43.99±7.01 mM sucrose, and V_{max} value was 2.1±7.7 U/ml.

CONCLUSION

Selectivity of the reaction of synthesis of FOSs using levansucrase was strongly dependent on the enzyme source, type of acceptors and donor/acceptor ratio. In the present study we first describe that when maltose was the acceptor, the major products was a fructooligosaccharide with DP3 (75,5% of total fructooligosaccharides) identified as an erlose. This investigation of the properties of recombinant levansucrase Lm17 reveals the potential use of the enzyme as tools for synthesis of short chain fructooligosaccharides (erlose) with prebiotic potential.

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ЕНЗИМЕН СИНТЕЗ НА ФРУКТО-ОЛИГОЗАХАРИДИ С ПОМОЩТА НА РЕКОМБИНАНТНА ЛЕВАНЗАХАРАЗА ОТ *LEUCONOSTOC MESENEROIDES* Lm17

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(Резюме)

Ензимът леванзахараза [EC2.4.1.10] трансферира фруктозилни остатъци от молекулата на захарозата към различни акцептори (малтоза, лактоза) и се получава добър добив на олигозахариди, които биха имали пребиотичен потенциал. Селективният характер на реакцията за синтез на фруктоолигозахариди (ФОЗ) с помощта на леванзахараза зависи строго от произхода на ензима, вида на акцепторите и съотношението донор/акцептор (Д/А). При изследване специфичността на акцепторите се определи възможността на леванзахараза Lm 17 да синтезира ФОЗ с различни структури при използване на различни акцептори. В настоящото изследване за първи път описахме, че при използване на малтозата като акцептор, основният фруктоолигозахарид, който се получава е със степен на полимеризация 3 (СПЗ) (75,5% от totalното количество синтезирани ФОЗ), идентифициран като ерлоза. Леванзахаразата беше с висока трансфруктозилазна отколкото хидролазна активност. При провеждане на ензимната реакция в присъствие на 10% захароза и 5% малтоза, Km на рекомбинантната леванзахараза Lm17 е 43.99 ± 7.01 mM захароза, и Vmax е 2.1 ± 7.7 U/ml. Това проучване на свойствата на леванзахараза Lm17 разкри потенциала на използване на ензима за синтез на късоверижни ФОЗ (ерлоза) с пребиотичен потенциал.