Food Sci. Biotechnol. Vol. 16, No. 3, pp. 482 ~ 484 (2007)

RESEARCH NOTE

Food Science and Biotechnology

Characterization of Levan Hydrolysis Activity of Levansucrase from *Zymomonas mobilis* ATCC 10988 and *Rahnella aquatilis* ATCC 33071

Ki-Hyo Jang*, Soon Ah Kang[1], Chul Ho Kim[2], Jae-Cheol Lee, Mi-Hyun Kim, Eunwha Son[3], and Sang-Ki Rhee[2]

Department of Food and Nutrition, Kangwon National University, Samcheok, Gangwon 245-711, Korea
[1]Department of Fermented Food Science, Seoul University of Venture and Information, Seoul 137-070, Korea
[2]Biotechnology Research Division, Korea Research Institute of Bioscience and Biotechnology, Yusong, Daejeon 305-333, Korea
[3]Department of Pharmacognosy Material Development, Kangwon National University, Samcheok, Gangwon 245-711, Korea

Abstract To investigate production and hydrolysis of levan, the levansucrase enzymes from *Zymomonas mobilis* ATCC 10988 and *Rahnella aquatilis* ATCC 33071 were used. The optimum temperature of *R. aquatilis* levansucrase for levan formation was 37°C, whereas that of *Z. mobilis* was 4°C, under the experimental conditions. Both levansucrases also catalyzed the reverse levan hydrolysis reaction. Levan hydrolysis reactions from both levansucrases were temperature dependent; high temperature (20°C) was more favorable than low temperature (4°C) by 4 times. Fructose was the only product from hydrolysis reaction by both levansucrases, showing that both levansucrases mediated the hydrolysis reaction of exo-enzyme acting. In both enzymes, initial levan hydrolysis activity was almost accounted to 1% of initial levan formation activity. The results allow the estimation of the fructose release rate in enzyme processing conditions.

Keywords: levan, levansucrase, transfructosylation, hydrolysis of levan

Introduction

In nature, there are two types of fructose polymers (fructan). One is inulin and the other is levan. Levan is composed of β-(2,6)-fructosyl linkages, whilst fructosyl residues of inulin are linked mainly by β-(2,1)-fructosyl linkages (1). Levan has an average degree of polymerization (DP) of up to 10^5, with a number of structural variants. Levan is present in a variety of living systems, including bacteria and higher plants (1). Levan has been also reported to have certain biological functions such as anti-cariogenicity, a growth factor of lactic acid bacteria, antitumor, and immunomodulatory activities (2-5). Utilization of levan by fisheries as cryoprotectans was also reported that the rate of surviving cells with levan and dimethylsulfoxide was the highest among other protectans (4). As a dietary fiber, levan is soluble in water and can be used as a prebiotic in the food industry (1). Levan is useful as a colloid-stabilizing agent in the food industry, as well as beverages, cosmetics, and pharmaceuticals. Levan polymers are valuable in stabilizing protein emulsions, in milk and other dairy products including ice cream, yogurt, custards, cheese, and cheese products (6).

Levansucrase (EC 2.4.1.10) is capable of producing levan from sucrose as a substrate (7). The enzyme is known to not only catalyze the transfer reaction producing levan, but also to hydrolyze levan. The overall reaction equations are as follows:

$$\text{Sucrose} + H_2O \rightarrow \text{Glucose} + \text{Fructose} \quad (1)$$

$$\text{Sucrose} + (\text{Fructose})_n \rightarrow \text{Glucose-(Fructose)}_{n+1} \quad (2)$$

$$\text{Glucose-(Fructose)}_{n+1} \rightarrow \text{Glucose-(Fructose)}_n + \text{Fructose} \quad (3)$$

In the view of industrial application of levan, hydrolysis reaction is undesirable reaction. In the current work, the activities of levan hydrolysis by levansucrases from *Z. mobilis* ATCC 10988 and *R. aquatilis* ATCC 33071 are determined at various temperatures.

Materials and Methods

Materials Levan was obtained from laboratory-scale production using *Z. mobilis* levansucrase (1). The estimated molecular weight of bacterial levan was about 6×10^6 Da (8). Purity of levan was above 98%, judging by HPLC analysis (8). Sucrose, glucose, and fructose were purchased from Sigma (St. Louis, MO, USA) and 1-kestose from Wako Pure Chemical Ind. (Osaka, Japan).

Levansucrase preparation To characterize the enzymatic properties of *Z. mobilis*- and *R. aquatilis* levansucrase, which contained His-affinity tag, were constructed and expressed in *Escherichia coli* DH5α (7). For the production of *Z. mobilis* levansucrase, the plasmid (pELCHis24) carrying a levansucrase gene (*levU*) of *Z. mobilis* was used. For the production of *R. aquatilis* levansucrase, the plasmid used was pRL1CP, a pBluescript II KS(+) (Stratagene, La Jolla, CA, USA) derivative carrying the promoter region and structural gene of levansucrase from *R. aquatilis* (9, 10). *E. coli* was grown aerobically at 37°C for 12 hr. Cells were harvested, sonicated, and centrifuged. The purification of levansucrase from *E. coli* lysate was done using Ni-NTA metal affinity chromatography (Qiagen, Valencia, CA, USA). The purification procedure was replicated and the resulting purified levansucrase formed a single band on the PAGE gel. Protein

*Corresponding author: Tel: 82-33-570-6882; Fax: 82-33-570-6889
E-mail: kihyojang@kangwon.ac.kr
Received September 8, 2006; accepted March 16, 2007

concentration of levansucrase was estimated using the method described by Bradford (11) in which a BioRad Lab. (Hercules, CA, USA) protein assay kit was used.

Measurement of enzyme activity Levansucrase activity was determined by either sucrose hydrolysis- or levan formation activity. For sucrose hydrolysis- and levan formation activity determination, reaction mixtures (1 mL) contained 10% sucrose (w/v) (2%, w/v, levan, for levan hydrolysis activity determination) and 50 μg of *Z. mobilis* levansucrase (or 6.6 μg of *R. aquatilis* levansucrase). One unit (U) of sucrose hydrolysis activity was defined as the amount of enzyme releasing one μmole of glucose per min. Levan hydrolysis activity was determined by measurement of fructose released from levan. Quantitative determination of glucose, fructose, oligosaccharides, and levan was conducted by HPLC with a refractive index and a Shodex Ionpack KS-802 column (Showa Denko Company, Tokyo, Japan). Deionized water was used as a mobile phase at a flow rate of 0.4 mL/min.

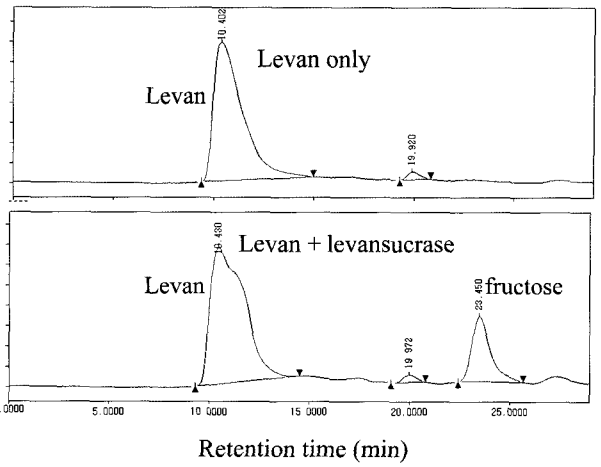

Fig. 1. Typical HPLC chromatograms of levan (top panel), and enzymatic products using levan and levansucrase (bottom panel). For reaction, 50 mg of *Z. mobilis* levansucrase was incubated with 2% levan, for 4 weeks at 4°C.

Results and Discussion

Purification and characterization of his-tagged levansucrase To characterize the enzymatic properties of *Z. mobilis*- and *R. aquatilis* levansucrase, levansucrases were constructed and expressed in *E. coli*, as reported previously (7, 12). Under the control of T7 promoter, the levansucrases were tagged with 6 histidine residues and produced up to 20% of the total cell protein in *E. coli*. After disruption of cells, the His-tagged levansucrases were purified by Ni-NTA affinity column chromatography. The purified levansucrases from *E. coli* lysate showed more than 95% homogeneity in SDS-PAGE analysis. Protein concentrations of *Z. mobilis*- and *R. aquatilis* levansucrases were 5 and 0.2 mg/mL, respectively, whilst sucrose hydrolysis activity of 2 enzymes were 100 U/mL in *Z. mobilis* levansucrase and 32 U/mL in *R. aquatilis* levansucrase. We are not sure why the activities of 2 levansucrases were different. One possible explanation is that the proportion of insoluble proteins in *E. coli* (pELCHis24) is higher than that of *E. coli* (pRL1CP). Sunita *et al.* (13) reported, when the gene encoding *Z. mobilis* levansucrase was cloned and expressed in *E. coli*, a high level of levansucrase was produced as inclusion bodies in the bacterial cytoplasm.

Effect of temperature on the enzyme activity The optimal temperature for sucrose hydrolysis activity was 37°C whilst the optimal temperature for levan formation activity was dissimilar (Table 1). *R. aquatilis* levansucrase showed high velocity for levan formation at 37°C. Compared to other bacterial levansucrases, the optimal temperature for levan formation activity of *Z. mobilis* levansucrase was very low, at 4°C (14, 15). However, the levan content formed was slightly decreased with the increase of incubation time. This phenomenon could be explained by the disproportionate reaction of this enzyme.

In order to determine the hydrolysis pattern of levansucrase, levan was reacted with 2 levansucrases at various temperatures. In the absence of levansucrase in the reaction mixture, no or little fructose were released from levan in the extended incubation for 28 days at 4 and 20 °C, suggesting that the levan solutions was stable in the analysis conditions used. When 2% levan were incubated with 1 U of levansucrase at pH 6.0 (50 mM sodium acetate buffer), the hydrolysis reaction was progressed. Both levansucrases were active toward levan, and the hydrolysis of the purified product generated fructose only in HPLC analysis, indicating that the purified levansucrase

Table 1. Characterization of 3 different enzyme activities of *Z. mobilis*- and *R. aquatilis* levansucrase

	Sucrose hydrolysis activity		Levan formation activity		Levan hydrolysis activity	
Temperature (°C)	Initial rate[1)]	Specific initial rate[2)]	Initial rate	Specific initial rate	Initial rate	Specific initial rate
Z. mobilis						
4	9.8	197	2.8	56	0.4	8
20	31.8	636	6.4	128	1.6	32
37	33.6	672	1.0	19	-[3)]	-
R. aquatils						
4	2.9	439	0.9	135	0.2	30
20	12.2	1848	2.7	409	0.9	132
37	29.6	4485	5.2	788	-	-

[1)]Initial rate (mg of sugar/time) was obtained from the slope of the curve obtained by plotting the concentration of the sugar against time.
[2)]Specific initial rate (mg of sugar/mg of protein/time) was determined by dividing the initial rate by the concentration of enzyme used.
[3)]No experiments were carried out.

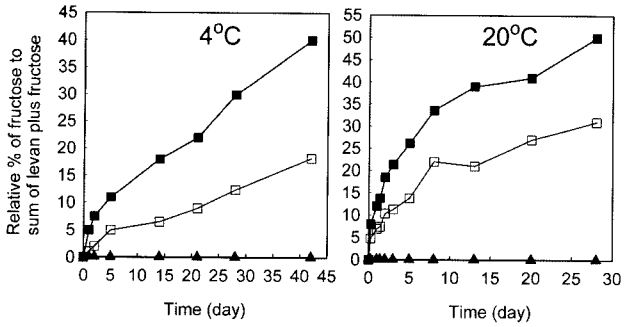

Fig. 2. Time course of levan hydrolysis reactions at various temperatures by *Z. mobilis*- and *R. aquatilis* levansucrase. ■, levan treated with *Z. mobilis* levansucrase; □, levan treated with *R. aquatilis* levansucrase; ▲, levan only.

was exo-type levan-degrading enzyme in the presence of levan (Fig. 1 and 2). In case of levan-degrading enzyme, levan is degraded to D-fructose, levanbiose, sucrose, levan oligomers, or low molecular-weight levan by the hydrolytic activity of levanase, levansucrase, or levan fructotransferase (LFTase) from some plants and microorganisms (16). The latter enzyme, LFTase, hydrolyzes levan to DFA IV (α-D-fructofuranose-β-D-fructofuranose 2',6:6,2' dianhydride). The levan hydrolysis activity of levansucrase was well studied with Gram-positive bacterial levansucrase, mainly with *Bacillus* species. The current study shows that the Gram-negative bacterial levansucrases also mediate levan hydrolysis reaction. In Fig. 2 and Table 1, the hydrolysis activities of levansucrases of *Z. mobilis* and *R. aquatilis* were changed according to temperature. Since *Z. mobilis* levansucrase was completely inactivated in extended incubation at 37°C, no data were available. Levan hydrolysis activities of *Z. mobilis* levansucrase were more favorable at high temperature. The same result was observed with that of *R. aquatilis* levansucrase. For instance, amounts of fructose released from levan by *Z. mobilis* levansucrase were 8 mg of fructose/mg protein/day at 4°C and 32 mg of fructose/mg of protein/day at 20°C. At 4°C, the formation rates of fructose generated from levan were 8 mg of fructose/mg of protein/day in *Z. mobilis* levanansucrase and 30 mg of fructose/mg of protein/day in *R. aquatilis* levansucrase. Therefore, levan hydrolysis activity from *Z. mobilis* was almost four times lower than that of *R. aquatilis*. It is interesting to note that levan hydrolysis acitivity of *Z. mobilis* is minimal at 4°C, which is optimal temperature for levan formation. In case of *R. aquatilis* levansucrase, both levan hydrolysis activity and levan formation activity are maximal at high temperature. In both enzymes, levan hydrolysis activity was almost accounted to 1% of levan formation activity.

The degree of polymerization of levan formed by *Z. mobilis*- (8) and *R. aquatilis* levansucrase (10) were greatly affected by the reaction temperature. The degree of polymerization and yield of levan at 37°C were lower than that at 4°C (10). Similar phenomenon (8) was observed in *Z. mobilis* levansucrase. Since such a hydrolysis activity is unfavorable in industrial application of levansucrase to produce levan, levan production at low temperature is an attractive character of *Z. mobilis* levansucrase since such a low temperature could help not only to increase levan synthesis activity during the extended enzyme reaction but also to avoid levan hydrolysis activity. In conclusion, our finding will help to estimate the fructose release rate in many foodstuff processing conditions.

References

1. Kim YY, Jang KH, Kang SA, Cho YC, Kim JS, Kim CH, Choue RW. Effect of types of levan with or without phytic acid on intestinal environment and mineral absorption in rats. Food Sci. Biotechnol. 13: 450-454 (2004)
2. Sung HY, Choi YS, Cho SH, Yun JW. Fructooligosaccharides alter profiles of fecal short-chain fatty acids and bile acids in rats. Food Sci. Biotechnol. 15: 51-56 (2006)
3. Choi NY, Shin HS. Effect of oligosaccharides and inulin on the growth and viability of bifidobacteria in skim milk. Food Sci. Biotechnol. 15: 543-548 (2006)
4. Iizuka M, Minamiura N, Ogura T. Glycoenzymes. Japan Scientific Societies Press, Tokyo, Japan. pp. 130-135 (2000)
5. Sung HY, Lim YJ, Choi YS. Soy isoflavones do not alter the effects of fructooligosaccharide on the intestinal ecosystem of colon-cancer model rats. Food Sci. Biotechnol. 15: 931-936 (2006)
6. Bekers M, Upite D, Kaminska E, Grebe M, Laukevics J, Vina I, Vigants A, Zikmanis P. Fructan biosynthesis by intra- and extracellular *Zymomonas mobilis* levansucrase after simultaneous production of ethanol and levan. Acta Biotechnol. 1: 85-93 (2003).
7. Rhee SK, Song KB, Kim CH, Park BS, Jang EK, Jang KH. Levan. Vol. 5, pp. 351-377. In: Biopolymers. De Baets S, Vandamme EJ, Steinbuchel A (eds). Wiley VCH Verlag Gmbh, Weinheim, Germany (2002)
8. Jang KH, Song KB, Kim CH, Chung BH, Kang SA, Chun UH, Choue R, Rhee SK. Comparison of characteristics of levan produced by different preparations of levansucrase from *Zymomonas mobilis*. Biotechnol. Lett. 23: 339-344 (2001)
9. Seo JW, Jang KH, Kang SA, Song KB, Jang EK, Park BS, Kim CH, Rhee SK. Molecular characterization of the growth phase-dependent expression of the *lsra* gene, encoding levansucrase of *Rahnella aquatilis*. J. Bacteriol. 184: 5862-5870 (2002)
10. Kang SA, Lee JC, Park YM, Lee C, Kim SH, Chang BI, Kim CH, Seo JW, Rhee SK, Jung SJ, Kim SM, Jang KH. Secretory production of *Rahnella aquatilis* ATCC 33071 levansucrase expressed in *Escherichia coli*. J. Microbiol. Biotechn. 14: 1232-1238 (2004)
11. Bradford MM. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein of protein-dye binding. Anal. Biochem. 72: 248-254 (1976)
12. Seo JW, Song KB, Jang KH, Kim CH, Jung BH, Rhee SK. Molecular cloning of a gene encoding the thermoactive levansucrase from *Rahnella aquatilis* and its growth phase-dependent expression in *Escherchia coli*. J. Biotechnol. 81: 63-72 (2000)
13. Sunita K, Chung BH, Jang KH, Song KB, Kim CH, Rhee SK. Refolding and purification of *Zymomonas mobilis* levansucrase produced as inclusion bodies in fed-batch culture of recombinant *Escherchia coli*. Protein Expres. Purif. 18: 388-393 (2000)
14. Elisashvili VI. Levan synthesis by *Bacillus* sp. Appl. Biochem. Micro+. 20: 82-87 (1984)
15. Hettwer U, Gross M, Rudolph K. Purification and characterization of an extracellular levansucrase from *Pseudomonas syringae pv. phaseolicola*. J. Bacteriol. 177: 2834-2839 (1995)
16. Saito K, Tomita F. Difructose anhydrides: their mass-production and physiological functions. Biosci. Biotech. Bioch. 64: 1321-1327 (2000)