

CLONING AND EXPRESSION OF LEVANASE GENE FROM *Bacillus licheniformis* RN-01 AND *Bacillus amyloliquefaciens* 1B

Noppadol Smak-karn,^{1,2} Kamontip Kuttiyawong,³ Rath Pichyangkura^{2,*}

¹Program in Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

²Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

³Faculty of Liberal Arts and Science, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand

*e-mail: prath@chula.ac.th

Abstract: Levanase (EC 3.2.1.65) is a hydrolytic enzyme which cleaves beta-(2, 6)-glycosidic linkage of levan, producing oligofructan. We have cloned levanase gene from *Bacillus licheniformis* RN-01 (*lnRN*) and *Bacillus amyloliquefaciens* 1B (*ln1B*) by PCR cloning technique with the size of 1,548 bp and 1,545 bp, respectively. When translated into protein the genes yield proteins of 515 and 514 amino acid residues in length, respectively. The deduced amino acid sequence of levanase genes from *B. licheniformis* RN-01 (*lnRN*), *B. amyloliquefaciens* 1B (*ln1B*), *Bacillus* sp. L7 (*SacC*) (GenBank accession number Y12619), and *Gluconacetobacter diazotrophicus* SRT4 (*lsdB*) (accession number L41732) were analyzed by CLUSTALW 2.1 multiple sequence alignment. The deduced amino acid sequence of levanase *lnRN* and *ln1B* showed 22% and 26% homology with *SacC*, and 21% and 27% homology with *lsdB*, respectively, while *lnRN* and *ln1B* shared 64% homology with each other. The genetic distances of levanases are *lsdB* : 0.37558, *lnRN* : 0.17631, *ln1B* : 0.17583 : 0.18304, *SacC* : 0.42030. *lnRN* was subcloned into pET19b and transformed into *Escherichia coli* BL21(DE3) for expression, while *ln1B* in pGEM ®-T Easy was expressed under its own promoter (338 bp up stream of the start codon) in *E. coli* Top10. Levanase secreted into the culture medium was assayed by DNS method. No activity was found for *lnRN*, however 94 mU/mL was detected for *ln1B*. TLC analysis showed that fructose was a major product when levan was hydrolyzed by *ln1B* in 50 mM citrate buffer pH 6.0 at 37° C overnight.

Introduction: Fructan is a homopolysaccharide composed of D-fructofuranosyl residues joined together by β -(2, 6) and β -(2, 1) glycosidic linkages. Two types of fructan can be found, inulin and levan. Levan is a fructan which composed of D-fructofuranosyl residues linked together by β -(2, 6) linkage in the main chain with β -(2, 1) at the branching points. Levanase is an enzyme that hydrolyzes levan at β -(2, 6)-D fructofuranosidic linkages, producing oligofructan and/or fructose. Levanase has two modes of catalysis, an endo-type and exo-type levanase. Endolevanase is an enzyme that hydrolyzes within the chain of levan, giving various sizes of oligofructan and/or fructose as a product. Exolevanase is an enzyme that hydrolyzes at end of levan chains, giving only single size product. Currently, levanase genes have been cloned from *Bacillus* sp. L7¹, *B. subtilis*², *G. diazotrophicus* SRT4³ and *Streptococcus* sp.^{4,5} Levanase from *Bacillus* sp. L7 is an endolevanase, in contrast, levanase from *G. diazotrophicus* SRT4 and *Streptococcus* sp. are exolevanases. Levanase gene from *B. licheniformis* RN-01 and *B. amyloliquefaciens* 1B isolated by our laboratory was cloned. The genes were compared, expressed, and digestion product analyzed. The potential used in oligofructan production will be further assessed.

Methodology:

Culture conditions: The bacterial cells were routinely grown at 37 °C in LB medium (1% Bacto Tryptone, 0.5% Bacto Yeast Extract, and 0.5% NaCl). For pET expression system, LB-

ampicillin (100 mg/liter) was used. *E. coli* cultures were induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at mid-log phase. For induction of *ln1B* under the regulation of its own promoter, 3X LB supplement with 1% levan was used.

Cloning and expression of levanase gene: Chromosomal DNA from *B.licheniformis* RN-01 and *B.amyloliquefaciens* 1B, was used as template for PCR amplification using primer sets; 5'CCCATGGAGAAGGCAGTATATAAGCG-3'/5'-CCTCGAGTTAATCGCGGATTGAACG-3' and 5'-CGCGGATCCTTACAAGCCGCTGAACAAAAC-3'/5'-GCGCTCGAGTATACATATTTGCATTTTACCACAGTC-3', respectively. The PCR product was cloned into pGEM®-T Easy. *lnRN* was subcloned into pET19b and transformed into *E. coli* BL21(DE3) for expression, while *ln1B* in pGEM®-T Easy was expressed under its own promoter (338 bp up stream of the start codon) in *E. coli* Top10.

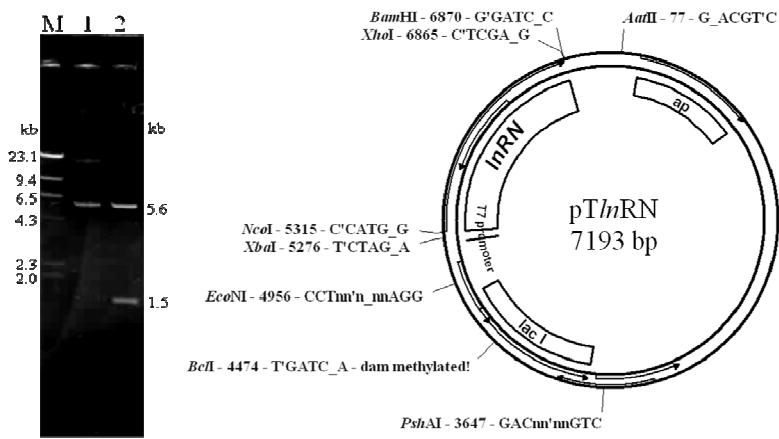
Determination of levanase activity: Levan synthesized by *B. licheniformis* RN-01 levansucrase was used as substrate. Enzymatic hydrolysis was performed in a reaction buffer (50 mM citrate buffer, pH 6.0) supplemented with levan to a final concentration of 100 mg/mL. The mixture was incubated at 37°C for 1 h. Total reducing sugar produced from reaction was quantified by using the dinitrosalicylic acid (DNS method)⁶. Fructose was used to construct a standard calibration curve. One unit of the enzymatic activity is defined as the amount of enzyme, generating one μ mole of reducing end equivalents per minute under the above conditions.

Product size analysis: Thin-layer chromatography (TLC) technique was used to analyze the hydrolysis product of levan by levanase. The products were separated on silica gel plates using acetic acid:butanol:water, 3:3:2 (v/v) as mobile phase. After three repetitive runs, the product was visualized by dipping the TLC plate in 10% sulfuric in methanol (v/v) followed by heating at 110°C for 15 min.

Results, Discussion and Conclusion:

PCR and cloning result: We have successfully cloned levanase gene from *B. licheniformis* RN-01 (*lnRN*) and *B. amyloliquefaciens* 1B (*ln1B*) (including its endogenous promoter) by PCR cloning technique. *lnRN* was then subcloned into pET19b yielding pT*lnRN*, and transformed into *E. coli* BL21 (DE3) for expression, while *ln1B* in pGEM®-T Easy, pG*ln1B*, was expressed under its own promoter in *E. coli* Top10. Plasmids pT*lnRN* and pG*ln1B* and restriction mapping are shown in Figure 1.

A.



B.

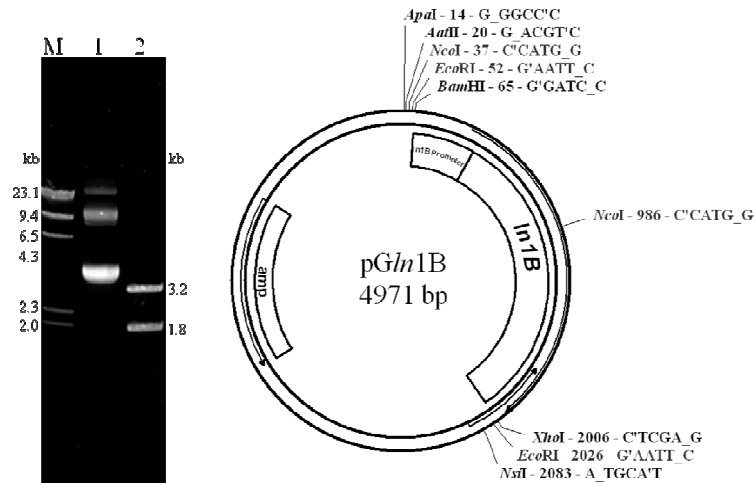


Figure 1. Restriction map and plasmid map of pTlnRN and pGln1B

Agarose gel electrophoresis analysis of **A**; pTlnRN and **B**; pGln1B double digested with NcoI and XhoI was performed. Lane M is λ /HindIII marker, lane 1 is undigested plasmid, lane 2 plasmid digested with NcoI and XhoI

Sequence analysis: Levanase gene from *B. licheniformis* RN-01 (*lnRN*) and *B. amyloliquefaciens* 1B (*ln1B*) was sequenced and found to have the size of 1,545 bp and 1,548 bp, respectively. When translated, *lnRN* and *ln1B* yielded a protein of 514 and 515 amino acid residues in length, respectively. Amino acid sequence comparison of levanase genes was performed. The deduced amino acid sequences of levanase gene *lnRN* and *ln1B* were compared with the deduced amino acid sequence of levanase gene from *Bacillus* sp. L7, *SacC* (accession number Y12619, GenBank) and *G. diazotrophicus* SRT4, *lsdB* (accession number L41732, GenBank). The deduced amino acid sequence of levanase *lnRN* and *ln1B* showed 22% and 26% homology with *SacC*, and 21% and 27% homology with *lsdB*, respectively, while *lnRN* and *ln1B* shared 64% homology with each other. The genetic distance of levanases are *lsdB* : 0.37558, *lnRN* : 0.17631, *ln1B* : 0.17583 : 0.18304, L7 : 0.42030 shown in Figure 2.

LnRN	-----MEKAVYKRISILTVCLGIFIMIFLSVRETEKKGESPDTPPEYR	42
Ln1B	-----MARKWPIAILIILGAF-LFVGLLPDKSNSSKTNKTKNPDYR	41
lsdB	-----MTMTGTVPRASGWRRLSRRCLPAAFALLFPLG-APHAADTPQWR	43
SacC	MMKWFAKLILSLAVVMAASSAAISFGASNSSLDTHASLVTLQDLSAASEAAEGKSAMIN	60
	
LnRN	AAFHLTTPDKWKNDPQK-----PVYFNGEY	67
Ln1B	AAYHFSTTPDKWKNDPQK-----PIFFDGKY	66
lsdB	PVLHFSPAAYWMNDPNG-----PILLDGVY	68
SacC	ESAINSNVTGWKLHGKGRMEVTGEGRLRLTSDPQENVMAISETVADDFIYEADVMTDPQA	120
	: * . :	: :
LnRN	HYYYLYNRDYPDNGTEWRHAVSDDLVHWQDKG-----	100
Ln1B	HYYYLYNRDYPNGTEWRHAVSEDLVHWTDG-----	99
lsdB	HLFYQYAPGSMTWGHPSWGATSTDDLHWTEHG-----	101
SacC	DATLLFRSGEDGWSSYMLQLALGAGVIRLKDASGGEGVLNVERKVEAKPGDIYHLRVKAE	180
	. : . . * . . : : .	
LnRN	-----VAIPKYTNKNGDPWSGSVVVDSKNTAGFGK-----	131
Ln1B	-----TAIPKYTNPNNGDIWTGSVVIDKHNTAGFGKN-----	130
lsdB	-----VAIAATPGE--EIFSGSLVDPDLNRSGLGSTDAPPLLAF--	138
SacC	GTRLQVYWGQYEPVIDTEAAAHRTGRLGLHVWNGSALFQNIQVSDMSGNTLEPISSOGL	240
	. * . : . : * : : . : .	

LnRN	-----TIVAVMTQPSANDGKEEQFLWYSQNGGKTFKPYGE-----	166
Ln1B	-----ALVAITQPTAKTEAQEQYLWYSTDKGKTFTSYSD-----	165
lsdB	-----HTSVFHDNPAHPDGTQAQSVSVSHDGGFTWRPYAH-----	173
SacC	WQPDLKGLKGTGEDGLEAKKVFVRNHEADVVLVLEGLDILNGQGSAGLLFRSNAQGTGYAAV	300
	.. : : : . . * : . . .	
LnRN	-----EPVLP-NPGTVDFRDPKVIWDEQDDKWMALAEGTK--VGFYES-----	210
Ln1B	-----QPVMK-NPGTKDFRDPKVIWDEQDNKWMAMAEGEK--IGFYES-----	209
lsdB	-----NPVLTLPDSRQFRDPSVFWYQDGGCWIMTTVVGDAQLVKLYRS-----	220
SacC	LQEGGERVVRVYLKADGTILHESRVTYPSQRESRHHLEVKAIGERIQIFVDGYEPAIDM	360
	* :.. ::: * : : : . . : : : . : :	
LnRN	KEWRYTGSFQTENIG----IIECPDLYKMRADDG--TYKWVLGASANGKGTGKPNITYAYW	264
Ln1B	KNWHYTGGFIAQQIG----LIECPDLYMMRADDG--TTKWILGASANGKPOGKPNITYAYW	263
lsdB	LHWSFLSDFQPSGYRKPMLWEMPTLVPLKLDGNPRATRWVMIVSVNPNWSIAGGSGVQYF	280
SacC	VDTAFPSGYHGMMASSGIAYFQDVYITPYASYT-EKYRPOYHYSPIRGSASDPNGLVYF	419
	. : . : . : : : : * : . . . * :	
LnRN	TGSFNGNEFTADEAEP-----QWLDYGFWDYAGVTFEDGETNDPYEKRY	308
Ln1B	TGNFDGKEFTADQAE-----QWLDYGFWDYGGVTFEDGNSKDLPTKRY	307
lsdB	VGRFDGVTFTPDALPPGS-----DPSRYDWLDHGADQYATTLFANTGSGAP-----	328
SacC	EGEYHLFHQDGGQWAHAVSRDLIHWKRLPIALPWNDLGHVWVSGSAVADTTNAGLFGSSG	479
	* : . . . * * * . . . :	
LnRN	ALAWMNNWDYANRTPTWKDFNGTDSIVRQIRLKHKGGDRYSLASHPIDQDELTESS---	365
Ln1B	ALAWMNNWDYPNETPTLKNFNGTDSIVRETRLQQDGGTYSLMSEPVEALNQLTSS---	364
lsdB	LIGWMDNWDYATDLPTAP--WRGQMTLPVDIALKTVDG-HPTVIQTPTRAYEDMVRKKGV	385
SacC	GKGLIAYYTSYNPDRHNGNQKIGLAYSTDRGRTWKYSEHPVVIENPGKTGEDPGGWDFR	539
	. : : . . * : . . . * : :	
LnRN	-ADELDRIEVNGSKTLQIKG---NTYQLEADISWDDLKNAGFRLRESADRKRHRIDVIGISA	421
Ln1B	-TDGTEDKQVNGSETLPKIG---DTYQLDMDISWSDIKNAGVRLRESADHKRHRIDVIGISA	420
lsdB	VTYGDRNLPQDGRVTIPSRG---EVLDIRLVLRGGARRAGIVVRETPDGRGTGTSVSYDF	442
SacC	DPKVVRDEANNRWMVVSAGDHIRLFTSTNLLNWLTDQFGYGAYIRGGVWECDFLQFLP	599
	. : : * : . . . * . . . :	
LnRN	EGGYSYVNRGFTGQPDSTRTYLESKAPFDPEKK-RVHFTIIVDQNTVEAFIDDGETTHSN	480
Ln1B	EDGCAYVNRSFTDQPKTGTYTESKAPFDGNGK-RVHLKILVDKTSIEVFADDGKTVLTN	479
lsdB	VDGTLTVDRGRSGLVGFSPRFSTRHIAAYLAAPGGEVALHLVVDRAVELFANDGVLKMTD	502
SacC	VEGSKKRKWLMISTGANPNTQGSDAEYFIGDLTPEGKFINDNPAGTVLTKTDWGKEYYAS	659
	* : : : * : . :	
LnRN	LAFPDMDNTG-----ITLFSNGTAVFENLKIKHL	510
Ln1B	EVFPKPEDQG-----ITLYSEGGSAVFQHIKIKHL	509
lsdB	LIFPPAGSDR-----ISLFAEGGATIHGLRVAVL	532
SacC	MSFSMDMPDGRIRMLAWMTNWDYPPSFPTTGWKQLSIPRQVSLKETEEGIRMHQTPIEEL	719
	* . . : : * : . : *	
LnRN	RSIRD-----	515
Ln1B	GSIH-----	514
lsdB	AR-----	534
SacC	AQLRSPVLHITNREVGDFRRKSAQRDHIRRL	750

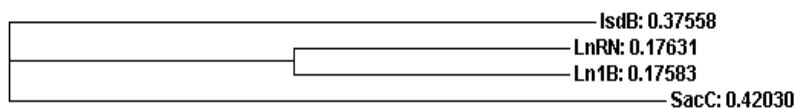


Figure 2. Amino acid sequence alignment and phylogram of levanase genes. Deduced amino acid sequence of levanase genes from *B. licheniformis* RN-01 (LnRN), *B. amyloliquefaciens* 1B (Ln1B), *Bacillus* sp. L7 (SacC), and *G. diazotrophicus* SRT4 (lsdB) were analyzed by CLUSTALW 2.1 multiple sequence alignment⁷.

Activity and Product analysis: LnRN and Ln1B was expressed in *E. coli*. Levanase activity was not detected for LnRN, however 94 mU/mL was detected for Ln1B. TLC analysis showed that fructose was a major product when levan was hydrolyzed by Ln1B in a reaction buffer containing 50 mM citrate buffer pH 6.0 at 37 °C overnight as shown in Figure 3. Suggesting that recombinant levanase from *B. amyloliquefaciens* 1B is either an exo-type which cleaves

fructose from the end of levan chains or endo-type enzyme which can hydrolyze tri- and di-saccharide into fructose.

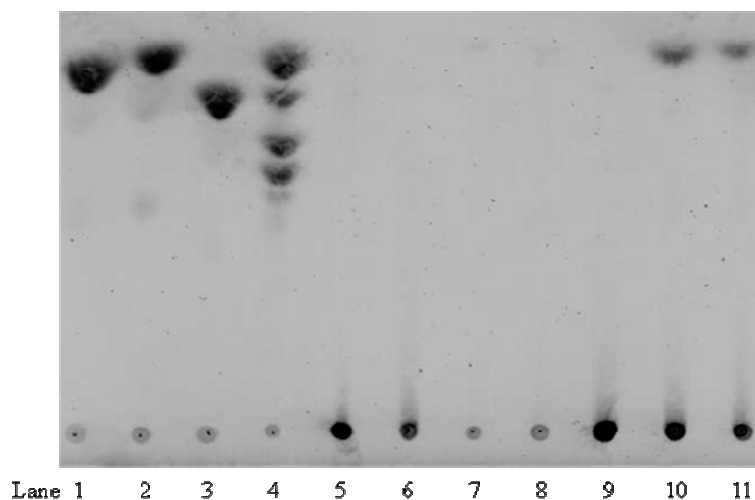


Figure 3. TLC analysis of hydrolytic product of Ln1B

Levan was digested with crude Ln1B. The reaction was carried out in 50 mM citrate buffer pH 6.0 at 37 °C overnight. Lanes 1 to 5 are glucose, fructose, sucrose, standards GF-GF₄, and levan, respectively. Lanes 6 to 8 are culture medium of *E. coli* top10, *B.amyloliquefaciens*1B, and *E. coli* top10 harboring pGln1B, respectively. Lanes 9 to 11 are hydrolytic product when levan was incubated with culture medium of *E. coli* top10, *B.amyloliquefaciens*1B, and *E. coli* top10 harboring pGln1B, respectively.

We have successfully cloned *lnRN* and *ln1B*. The deduced amino acid sequence of levanase *lnRN* and *ln1B* were compared with levanase from *Bacillus* sp. L7 (*SacC*), and *G. diazotrophicus* SRT4 (*lsdB*) and was found to have approximately 21-27% homology with the previously reported levanases. We have successfully expressed *ln1B* in *E. coli* and the recombinant enzyme can hydrolyze levan, producing fructose.

References:

1. Miasnikov, A. 1997. Characterization of a novel endo-levanase and its gene from *Bacillus* sp. L7. *FEMS Microbiology Letters*, 154: 23–28.
2. Martin, I., D'barbouill', M., Ferrari, E., Klier, A., and Rapoport, G. 1987. Characterization of the levanase gene of *Bacillus subtilis* which shows homology to yeast invertase. *Molecular Genetics and Genomics*. 208:177-184
3. Menéndez, C., Hernández, L., Selman, G., Mendoza, M., Hevia, P., Sotolongo, M., Arrieta, J. 2002. Molecular cloning and expression in *Escherichia coli* of an exo-levanase gene from the endophytic bacterium *Gluconacetobacter diazotrophicus* SRT4. *Current Microbiology*. 45:5-12
4. Burne, R., and Penders E, J. 1992. Characterization of the *Streptococcus mutans* GS-5 *fruA* Gene Encoding Exo-,1-D-Fructosidase. *American Society for Microbiology*. 60(11): 4621-4632
5. Burne, R., Schilling, K., Bowen, W., and Yasbin, R. 1987. Expression, Purification, and Characterization of an Exo-1-D-Fructosidase of *Streptococcus mutans*. *Journal of Bacteriology*. 169(10): 4507-4517
6. Miller, L., (1959). Use of dinitrosalicylic acid reagent for determination of reducing



- sugar. *Anal. Chem.* 31(3): 426–428
7. Larkin, A., Blackshields, G., Brown, P., Chenna, R., McGettigan, A., McWilliam, H., Valentin, F., Wallace, M., Wilm, A., Lopez, R., Thompson, D., Gibson, J., and Higgins, G. 2007. ClustalW and ClustalX version 2. *Bioinformatics* 2007. 23(21): 2947-2948

Acknowledgements: This research work was partially supported by the “CU.GRADUATE SCHOOL THESIS GRANT” Chulalongkorn University, Bangkok, Thailand.

Keywords: Oligofructan, Levan, Levanase