Identification of catalytic acidic residues of levan fructotransferase from *Microbacterium* sp. AL-210

Hee-Kyung Sung, Keum-Ok Moon, Ki-Won Choi, Kyung-Hwa Choi, Kyung-Ju Hwang, Myo-Jung Kim[1] and Jaeho Cha*

Department of Microbiology, College of Natural Sciences, Pusan National University, Busan 609-735, Korea
[1]Food Research Institute and School of Food and Life Science, and Biohealth Products Research Center, Inje University, Gimhae 621-749, Korea

Received September 25, 2006 / Accepted November 22, 2006

β-Fructofuranosidases, a family 32 of glycoside hydrolases (GH32), share three conserved domains including the W(L/M)(C/N)DP(Q/N), FRDPK, and ECP(D/G) motifs. The functional role of the conserved acidic residues within three domains of levan fructotransferase, one of the β-fructofuranosidases, from *Microbacterium* sp. AL-210 was studied by site-directed mutagenesis. Each mutant was overexpressed in *E. coli* BL21(DE3) and purified by using Hi-Trap chelating affinity chromatography and fast performance liquid chromatography. Substitution of Asp-63 by Ala, Asp-195 by Asn, and Glu-245 by Ala and Asp decreased the enzyme activity by approximately 100-fold compared to the wild-type enzyme. This result indicates that three acidic residues Asp-63, Asp-195, and Glu-245 play a major role in catalysis. Since the three acidic residues are present in a conserved position in inulinase, levanase, levanfructotransferase, and invertase, they are likely to have a common functional role as nucleophile, transition state stabilizer, and general acid in β-fructofuranosidases.

Key words – levan fructotransferase, site-directed mutagenesis, β-fructofuranosidases, glycosyl hydrolase family 32

Introduction

A new coryneform actinobacteria subfamily *Microbacterium* sp. AL-210 produces an extracellular levan fructotransferase (LFTase), which is specifically induced by levan as the sole carbon source [3]. The enzyme synthesizes a di-d-fructose-2,6′:6,2′-dianhydride (DFA IV), a cyclic disaccharide consisting of two fructose units linked at their reducing carbons, from levan by an intramolecular levan fructosyl transfer reaction [13]. The physiological and physical functionalities of four kinds of DFA (DFA I, III, IV and V) are known to be due to their unique structures and properties [12]. DFAs have been known to be a promising sweetener for people with diabetes because of their half sweetness of sucrose and structural stability at extreme pH. It also enhances calcium absorption in the small intestine of rats suggesting their potential applications to prevent osteoporosis [12].

The studies of LFTases have mainly focused on the mass production of DFA IV by optimization of reaction conditions or overexpression of LFTase in *E. coli* through cloning of the *lftase* genes [12,14,16]. However, the catalytic properties studies for the evaluation of the reaction mechanism of LFTase have little been studied. LFTases belong to β-fructofuranosidases of family 32 of the glycoside hydrolases (GH32) (http://afmb.cnrs-mrs.fr/CAZY) [5], which includes inulinase (EC 3.2.1.7), levanase (EC 3.2.1.65), transfructosidase, and invertase (EC 3.2.1.26). Comparison of the primary sequences of GH32 enzymes available in the literature shows three highly conserved domains including the W(L/M)(C/N)DP(Q/N), FRDPK, and ECP(D/G) motifs, which may be involved in enzyme activity. Glycoside hydrolases are a widespread group of enzymes displaying a great variety of protein folds and substrate specificities. They share common defining feature in two critically located acidic residues, which make up the catalytic machinery responsible for the cleavage of glycosidic bonds. These two invariant residues (Asp-23 and Glu-204) were identified in yeast invertase as a nucleophile and a general acid/base, respectively [9,10]. Saito et al. [13] also proposed that the Asp-52 (Asp-23 in invertase) and Glu-232 (Glu-204 in invertase) residues of *Arthrobacter nicotinovorans* GS-9 LFTase may play a role as a nucleophile and a proton donor in the catalytic mechanism of this enzyme. Attempts to identify those amino acid residues essential

*Corresponding author
Tel : +82-51-510-2196, Fax : +82-51-514-1778
E-mail : jhcha@pusan.ac.kr

for LFTase activity led to the implication of a carboxylate group. Recently, the crystal structures of three GH32 enzymes, *Thermotoga maritima* invertase [1], exo-inulinase from *Aspergillus awamori* [6], and a plant fructan 1-exohydrolase from *Cichorium intybus* [17] have been reported. The three-dimensional structures of three enzymes revealed that three carboxylate groups of two aspartates and one glutamate at the bottom of a deep, negatively charged funnel-shaped depression of the β-propeller structure are essential for catalysis and function as nucleophile, transition state stabilizer, and general acid.

We have previously reported the cloning, overexpression and purification of a recombinant LFTase (LftM) from *Microbacterium* sp. AL-210 and investigated the kinetic and pH dependence studies for DFA IV production [3,19]. The present paper extends this study by identifying the aspartate and glutamate residues involved in catalysis by means of site-directed mutagenesis.

Materials and Methods

Materials

Taq DNA polymerase was purchased from Bioneer (Korea). Wizard PCR Preps DNA Purification system, a DNA cleaning kit, and Wizard DNA Clean-Up System were purchased from Promega (USA). Kieselgel 60 F_{254} thin-layer chromatography plate was purchased from Merck (Germany). DFA IV was a gift from Realbiotech (Korea). Centriprep Centrifugal Filter was purchased from Millipore (USA). Olgonucleotides for cloning, nucleotide sequencing and site-directed mutagenesis of the target gene were synthesized by Bioneer Co. (Korea)

Bacterial strains and media

Escherichia coli cells harboring pELFT-NX [19] or one of its derivatives were grown with shaking (150 rpm) at 37°C in Luria-Bertani medium supplemented with kanamycin (30 μg/ml).

Site-directed mutagenesis

Site-directed mutagenesis was performed by two-step PCR method using mutagenesis primers. One pair of mutagenic oligonucleotides for each mutant was constructed. Both primers contain the desired base change but are complementary to different strands of the plasmid pELFT-NX. Two PCR fragments were made by first round PCR reaction by using two sets of primers. One is done by *lftM* forward primer (LFT*Nde*I-F; 5'-GATATACATATGACCGA ATCCTCC-3') and mutagenic reverse primer and the other is done by mutagenic forward primer and *lftM* reverse primer (LFT*Xho*I-R; 5'-GTGGTGCTCGAGGATCGGCGT-3'). Amplification of *lftM* was performed in a total volume 50 μl: 200 ng of template DNA; 1 μM of each primer; the four dNTPs (dATP, dGTP, dTTP, and dCTP), 0.25 mM each; 1.5 mM $MgCl_2$; 2.5 U Taq polymerase; 1×Taq polymerase buffer. Two-step PCR amplification was carried out on a thermal cycler (GeneAmp PCR System 2400, Perkin Elmer) with a program of a predenaturation for 1 min at 94°C, followed by 30 cycles (denaturation 98℃, 20 sec; annealing and extension 68°C, 3 min), and final incubation for 7 min at 72°C. The purified two DNA fragments were used as templates for second round PCR. The condition of PCR was the same as the first round PCR except for adding two fragments as templates and *lftM* forward and reverse primers instead of one pair of mutagenic primers. The purified mutant fragment digested with *Nde*I and *Xho*I was introduced to pET-29b(+) which digested with the same restriction enzymes to generate each mutant. Each mutant was confirmed by sequencing.

Expression of recombinant levan fructotransferase

E. coli BL21(DE3) cells harboring the recombinant plasmid were grown, and recombinant proteins were induced by adding IPTG as an inducer. *E. coli* cells were grown in 1 l of LB medium at 37°C to a density of 0.6 at 600 nm, and IPTG was added to a final concentration of 0.5 mM. Then, the culture was grown for an additional 4 hr. The induced cells were harvested and the pellet was resuspended in 20 mM phosphate buffer (pH 7.0), and sonicated. The cell lysate was centrifuged and the supernatant was recovered and purified by Hi-Trap chelating Ni-NTA column. The proteins bound to the resin were eluted by 0.2 M imidazole and dialyzed against 20 mM sodium phosphate buffer (pH 7.0).

Enzyme assay

The activity of LFTase and other mutants were assayed according to 3,5-dinitrosalicylic acid (DNS) method determining the amount of a reducing sugar released from the levan. The assay mixture for levan-hydrolyzing activity consisted of 1% levan in a 50 mM sodium phosphate buffer (pH 7.0) and the crude enzyme solution. The reaction

mixture was adjusted to 1.0 ml by adding 50 mM phosphate buffer (pH 7.0). The reaction was carried out at 37°C for 30 min, and then stopped by adding 3 ml of DNS reagent. Reaction without addition of enzyme was used as a blank. Development of color by the reaction was detected by heating at 100°C for 5 min, followed by cooling in running water to stabilize the color. The absorbance of the reaction mixture was measured at 575 nm using a spectrophotometer (UV-240, Shimadzu, Japan). One unit of levan-hydrolyzing activity was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to fructose per min under these assay conditions.

Sequence analysis

DNA sequencing was performed on a DNA auto sequencer (model ABI PRISM 377; Perkin Elmer) at Macrogen (Korea). Oligonucleotides for sequencing were synthesized by Bioneer Co. (Korea). Computer analyses of the DNA sequence data and the deduced amino acid sequence were done with programs available on the Expasy Molecular Biology server (http://www.expasy.ch/tool/) on the worldwide web. The GenBank and Swiss-Prot databases were searched for protein sequence homology by using BLAST search engines.

Thin-layer chromatography

A Thin-layer chromatography (TLC) analysis of the fructo-oligosaccharides produced was done as described previously [16]. Two to four μl aliquots of the reaction mixture were spotted onto a Silica gel 60 F_{245} plate with a capillary tube and developed with a solvent system of n-butanol/pyridine/water (6:4:1. v/v/v) in TLC developing tank. Ascending development was repeated twice at room temperature. The plate was allowed to air-dry in a hood and then developed by soaking rapidly into methanol solution containing 20% (v/v) sulfuric acid. The plate was dried and placed in 110°C oven for 10 min to visualize the reaction spots.

Results and Discussion

Acidic residues have been found to be responsible for catalysis in most glycoside hydrolases studied to date [4]. Multiple sequence alignment of bacterial fructosyl hydrolases available in the NCBI databases revealed the presence of three well-conserved domains containing aspartate or glutamate residues (Table 1). First targets for mutagenesis are Asp-63 in domain I [the W(L/M)(C/N)DP(Q/N) motif] and Glu-245 in domain III [ECP(D/G) motif]. Both residues were proposed to be ionization groups responsible for pH dependence of LFTase (LftM) from *Microbacterium* sp. AL-210 [19]. The importance of aspartate residue in the conserved RDP motif for sucrose hydrolysis has also been reported in invertases and sucrases [2]. Therefore, second target is designated to aspartate in RDP motif. Substitution of three acidic residues of LftM was carried out and a single base change was generated in each mutated *lftM* gene.

The expression vector of each mutant was constructed by subcloning of mutant PCR product into plasmid pELFT-NX and expressed in *E. coli* BL21(DE3) by IPTG induction. All mutant enzymes were located in cytoplasmic fraction with a level of about 70% of total protein. TLC analysis of the reaction products of four active site mutant enzymes (D63A, D195N, E245A, E245D) did not show any detectable product spot while the native enzyme showed DFAIV as a major product and some of the fructo-oligo-

Table 1. Sequence homology comparison of the catalytic residues of LftM with other fructosylhydrolases

Enzyme	Source	Sequence			Sequence Identity (%)	GenBank accession number
LFTase	*Microbacterium sp.*	**W**LC**D**PQR	**FRDPKIH**	**E**CPDLF	100	AAG01554
LFTase	*A. nicotinovarans*	**W**LC**D**PQR	**FRDPKIH**	**E**CPDLF	80	BAA24915
LFTase	*A. ureafaciens*	**W**LC**D**PQR	**FRDPKIH**	**E**CPDLF	79	AAF73829
Levanase	*M. laevaniformans*	**W**MN**D**PQR	**FRDPKVV**	**E**CPDLF	44	BAB59060
Levanase	*B. polymixa*	**W**MN**D**PNG	**FRDPKVI**	**E**CPDLF	29	A55206
Levanase	*B. subtilis*	**W**MN**D**PNG	**FRDPKVF**	**E**CPDLF	32	CAB14645
Inulinase	*P. mucidolens*	**W**MN**D**PNG	**FRDPKVF**	**E**CPDLF	30	AAF44125
FTase	*B. polymixa*	**W**MN**D**PNG	**FRDPKVI**	**E**CPDLF	29	CAA81392
Invertase	*S. cerevisiae*	**W**MN**D**PNG	**FRDPKVF**	**E**CPGLI	28	CAA87030

Conserved residues are highlighted in bold. For the LFTase from *Microbacterium sp.*, the underlined D represents Asp-63 and Asp-195, and the underlined E represents Glu-245. FTase, fructosyltransferase.

saccharides as a minor products (Fig. 1). All mutant enzymes were purified to examine the enzyme activity more clearly. The native and mutant enzymes resulted in a protein with the molecular mass of 54 kDa, as deduced by SDS/PAGE (Fig. 2) and their enzymatic activities were examined by TLC and DNS method. As shown in Table 2, the enzyme activities of all four mutants were at least 100 times less than that of native enzyme, indicating that three acidic residues we targeted (Asp-63, Asp-195, and Glu-245) play essential roles in the catalytic mechanism of LftM. E245D mutant showed two-fold higher activity than that of E245A. It indicates that the negatively charged residues

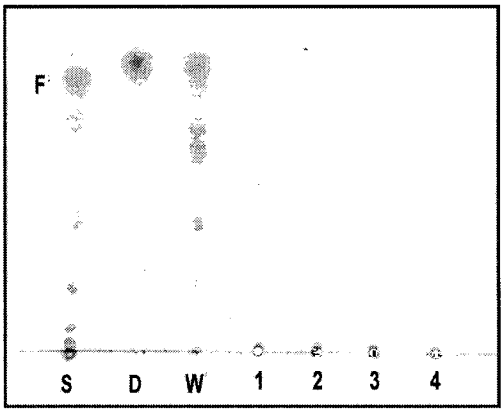

Fig. 1. TLC analysis of the reaction products by LftM mutants. The reaction was carried out with the crude enzyme and 1 mg levan as a substrate at 40°C for 3 hr. lane S, levan hydrolysate (fructose standard); lane D, 5 mg/ml DFA IV standard; lane W, wild-type LFTase, lane 1, D63A mutant; lane 2, D195N mutant; lane 3, E245A mutant; lane 4, E245D mutant; F, fructose.

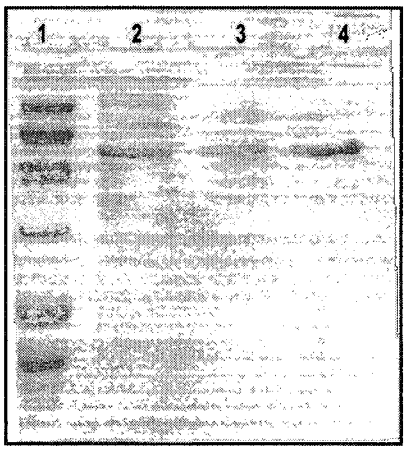

Fig. 2. SDS-PAGE analysis of the purified LftM mutants. lane 1, size marker; lane 2, cell free extract of D63A mutant; lane 3, purified native enzyme; lane 4, purified D63A mutant.

Table 2. Comparison of the enzyme activities of LftM mutants

Enzyme	Activity (Units/ml)	Relative activity (%)
wild-type	7.4×10^{-1}	100
D63A	7.4×10^{-3}	1
D195N	7.7×10^{-3}	1.04
E245A	4.6×10^{-3}	0.62
E245D	9.3×10^{-3}	1.26

One unit of activity was expressed by the amount of enzyme that required to liberate 1 μmol of fructose per min.

somehow important for activity in Glu-245 position. However, the extremely low activity compared to the wild-type enzyme was very interesting. Generally the alteration of Asp to Glu or vice versa (Glu to Asp) did not significantly change the enzyme activities in case of mutation of the acidic residue in the active site of the enzyme because both residues contain the same carboxylate group.

From the three-dimensional structure of *Thermotoga maritima* yeast invertase complexed with glycerol, which helped define the precise position of the modeled sucrose molecule, Asp-17 (Asp-63 in LFTase) and Glu-190 (Glu-245 in LFTase) point to the space to the incoming substrate and forms a hydrogen bond to the O1 of fructose and O1 and O2 of fructose. This model also shows that the strictly conserved Asp-138 (Asp-195 in LFTase) forms hydrogen bonds to O3 and O4 of the fructose unit and thus most likely plays a crucial role in substrate binding and recognition (Fig. 3) [1]. Unexpected low activity observed in E245D means that the carboxylate side chain of Asp-245 has a distance which cannot hydrogen bond to the O1 and O2 of

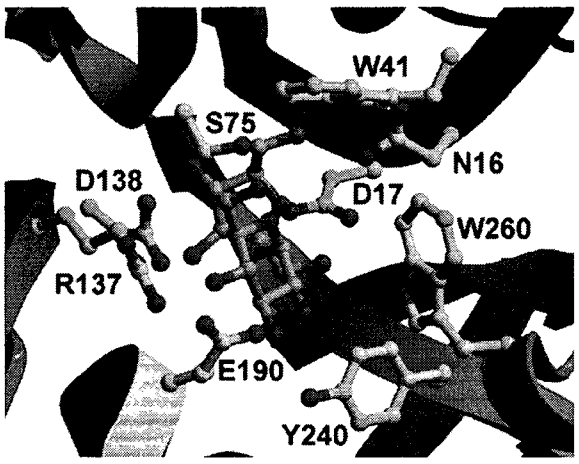

Fig. 3. Close-up view of the catalytic site of *T. maritima* invertase. The residues surrounding the modeled sucrose molecule are involved in binding and recognition. The figure was quoted from ref. 1.

Table 3. Comparison of the activities for three catalytic acidic residues of fructosylhydrolases

	Enzyme	Residue	Mutant	Activity decreased (fold)	References
Nucleophile	LFTase	Asp-63	D63A	100	This study
	Inulinase	Glu-20	E20A	>100	[8]
			E20D	>91	[8]
	Levansucrase	Asp-249	D249N	394,000	[7]
	Inulosurase	Asp-272	D272N	146,000	[7]
Transition state stabilizer	LFTase	Asp-195	D195N	96	This study
	Levansucrase	Asp-309	D309N	75	[2]
	Levansucrase	Asp-404	D404N	308,000	[7]
	Inulosucrase	Asp-424	D424N	71,200	[7]
	Levansucrase	Asp-194	D194N	3,400	[18]
	FTase	Asp-397	D397S	ND	[15]
Acid/base catalyst	LFTase	Glu-245	E245A	160	This study
			E245D	80	This study
	Invertase	Glu-204	E204A	3,000	[10]
	Levansucrase	Glu-503	E503Q	32,300	[7]
	Inulosucrase	Glu-523	E523Q	16,700	[7]

fructose. Earlier mutagenesis studies of yeast invertase showed that the mutation of Glu-204 to Ala in EC(P/G) motif resulted in a 3,000-fold reduction in the k_{cat} of invertase, indicating that Glu-204 plays a major role in catalysis [10]. Batista et al. [2] also reported that the mutant D309N in RDP motif of *Acetobacter diazotrophicus* levansucrase reduced the k_{cat} by 75-fold, but its K_m was similar to that of the wild-type enzyme, indicating that Asp-309 involves sucrose hydrolysis. The comparison of our results with the other mutant studies of three catalytic residues of GH32 enzymes was summarized in Table 3.

In order to understand the detailed catalytic mechanism of LFTase, the kinetic parameters and pH dependence studies of three carboxylate mutants are prerequisite. First of all, the enzymatic characterization of various Asp-63 mutants is under progress for the elucidation of the catalytic role of this residue in catalysis.

Acknowledgements

This work was supported for two years by Pusan National University Research Grant.

References

1. Alberto, F., C. Bignon, G. Sulzenbacher, B. Henrissat, and M. Czjzek. 2004. The three-dimensional structure of invertase (beta-fructosidase) from *Thermotoga maritima* reveals a bimodular arrangement and an evolutionary relationship between retaining and inverting glycosidases. *J. Biol. Chem.* **279**, 18903-18910.
2. Batista, F. R., L. Hernandez, J. R. Fernandez, J. Arrieta, C. Menendez, R. Gomez, Y. Tanbara, and T. Pons. 1999. Substitution of Asp-309 by Asn in the Arg-Asp-Pro (RDP) motif of *Acetobacter diazotrophicus* levansucrase affects sucrose hydrolysis, but not enzyme specificity. *Biochem. J.* **337**, 503-506.
3. Cha, J., N. H. Park, S. J. Yang, and T. H. Lee. 2001. Molecular and enzymatic characterization of a levan fructotransferase from *Micobacterium* sp. AL-210. *J. Biotechnol.* **91**, 49-61.
4. Davies, G. and B. Henrissat. 1995. Structures and mechanisms of glycosyl hydrolases. *Structure* **15**, 853-859.
5. Henrissat, B. 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **280**, 309-316.
6. Nagem, R. A. P., A. L. Rojas, A. M. Golubev, O. S. Korneeva, E. V. Eneyskaya, A. A. Kulminskaya, K. N. Neustrov, and I. Polikarpov. 2004. Crystal structure of exo-inulinase from *Aspergillus awamori*: the enzyme fold and structural determinants of substrate recognition. *J. Mol. Biol.* **344**, 471-480.
7. Ozimek, L. K., S. A. F. T. van Hijum, G. A. Van Koningsveld, M. J. E. C. Van der Maarel, G. H. van Geel-Schutten, and L. Dijkhuizen. 2004. Site-directed mutagenesis study of the three catalytic residues of the fructosyltransferases of *Lactobacillus reuteri* 121. FEBS Lett. **560**, 131-133.
8. Park, S., Y. Han, H. Kim, S. Song, T. B. Uhm, and K. S. Chae. 2003. Trp17 and Glu20 residues in conserved WMN(D/E)PN motif are essential for *Aspergillus ficuum* endoinulinase (EC 3.2.1.7) activity. *Biochemistry* **68**, 658-661.
9. Reddy, A. and F. Maley. 1990. Identification of an active-site residue in yeast invertase by affinity labeling and site-directed mutagenesis. *J. Biol. Chem.* **265**, 10817-10820.

10. Reddy, A. and F. Maley. 1996. Studies on identifying the catalytic role of Glu-204 in the active site of yeast invertase. *J. Biol. Chem.* **271**, 13953-13957.
11. Saito, K., H. Goto, A. Yokota, and F. Tomita. 1997. Purification of levan fructotransferase from *Arthrobacter nicotinovorans* GS-9 and production of DFAIV from levan by the enzyme. *Biosci. Biotechnol. Biochem.* **61**, 1705-1709.
12. Saito, K. and F. Tomita. 2000. Difructose anhydrides: their mass-production and physiological functions. *Biosci. Biotechnol. Biochem.* **64**, 1321-1327.
13. Saito, K., A. Yokota, and F. Tomita. 1997. Molecular cloning of levan fructotransferase gene from *Arthrobacter nicotinovorans* GS-9 and its expression in *Escherichia coli*. *Biosci. Biotechnol. Biochem.* **61**, 2076-2079.
14. Song, K. B., K. S. Bae, T. B. Lee, K. Y. Lee, and S. K. Lee. 2000. Characteristics of levan fructotransferase from *Arthrobacter urefaciens* K2032 and difructose anhydride IV formation from levan. *Enz. Microb. Technol.* **27**, 212-218.
15. Song, D. D. and N. A. Jacques. 1999. Mutation of aspartic acid residues in the fructosyltransferase of *Streptococcus salivarius* ATCC 25975. *Biochem. J.* **344**, 259-264.
16. Tanaka, K., T. Uchiyama, K. Yamauchi, U. Suzuki, and S. Hashiguchi. 1982. Formation of a di-d-fructose dianhydride from levan by *Arthrobacter ureafaciens*. *Carbohydr. Res.* **99**, 197-204.
17. Verhaest, M., W. V. D. Ende, K. L. Roy, C. J. D. Ranter, A. V. Laere, and A. Rabijns. 2005. X-ray diffraction structure of a plant glycosyl hydrolase family 32 protein: fructan 1-exohydrolase IIa of *Cichorium intybus*. *Plant J.* **41**, 400-411.
18. Yanase, H., M. Maeda, E. Hagiwara, H. Yagi, K. Taniguchi, and K. Okamoto. 2002. Identification of functionally important amino acid residues in *Zymomonas mobilis* levansucrase. *J. Biochem.* **132**, 565-572.
19. Yang, S. J., N. H. Park, T. H. Lee, and J. Cha. 2002. Expression, purification and characterization of a recombinant levan fructotransferase. *Biotechnol. Appl. Biochem.* **35**, 199-203.

초록 : *Microbacterium* sp. AL-2100이 생산하는 levan fructotransferase의 효소활성에 중요한 아미노산의 동정

성희경 · 문금옥 · 최기원 · 최경화 · 황경주 · 김묘정[1] · 차재호*
(부산대학교 미생물학과, [1]인제대학교 식품생명과학부)

당 분해효소의 family 32 (GH32)에 속하는 β-fructofuranosidase는 3차구조를 근거로 볼 때 W(L/M)(C/N)DP(Q/N), FRDPK, 그리고 ECP(D/G) 부위를 포함하는 세 군데의 보전적인 영역을 가지고 있다. 이러한 β-fructofuranosidase family에 속하는 *Microbacterium sp.* AL-210 유래 levan fructotransferase (LFTase)의 보전적인 산성 아미노산들의 역할이 특정위치 돌연변이법으로 검사되었다. 각각의 돌연변이체는 대장균인 *E. coli* BL21 (DE3)균주에서 발현되어 대량 생산되었고, 금속 친화 크로마토그래피법과 FPLC법으로 순수 정제되었다. wild-type LFTase의 효소의 활성은 0.74 unit 인 반면 네 개의 돌연변이체인 D63A, D195N, E245A, E245D 각각은 specific activity를 측정해 본 결과 원 균주와 비교해서 약 100배 정도 감소한 효소활성을 보여 주었다. 이로써 아미노산 변형의 target이 되었던 Asp-63, Aps-195, 그리고 Glu-245가 모두 효소 활성 및 기질과의 결합에 상당히 중요한 역할을 하고 있음이 판명되었다. 이러한 세 부위의 산성 아미노산들은 inulinase, levan fructotransferase와 invertase에 모두 보전적으로 위치하므로 이들은 β-fructofuranosidase family내에서 공통된 역할을 할 것으로 사료된다.