J. Microbiol. Biotechnol. (2008), **18**(6), 1064–1069

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Probing the Critical Residues for Intramolecular Fructosyl Transfer Reaction of a Levan Fructotransferase

Moon, Keum-Ok[1], Kyoung-Hwa Choi[1], Ho-Young Kang[1], Jeong-Il Oh[1], Se Bok Jang[1], Cheon-Seok Park[2], Jong-Hoon Lee[3], and Jaeho Cha[1]*

[1]*Department of Biological Sciences, Pusan National University, Busan 609-735, Korea*
[2]*Department of Food Science and Biotechnology, Graduate School of Biotechnology and Institute of Life Sciences and Resources, KyungHee University, Yongin 446-701, Korea*
[3]*Department of Foods and Biotechnology, Kyonggi University, Suwon 442-760, Korea*

Received: October 2, 2007 / Accepted: January 9, 2008

Levan fructotransferase (LFTase) preferentially catalyzes the transfructosylation reaction in addition to levan hydrolysis, whereas other levan-degrading enzymes hydrolyze levan into a levan-oligosaccharide and fructose. Based on sequence comparisons and enzymatic properties, the fructosyl transfer activity of LFTase is proposed to have evolved from levanase. In order to probe the residues that are critical to the intramolecular fructosyl transfer reaction of the *Microbacterium* sp. AL-210 LFTase, an error-prone PCR mutagenesis process was carried out, and the mutants that led to a shift in activity from transfructosylation towards hydrolysis of levan were screened by the DNS method. After two rounds of mutagenesis, TLC and HPLC analyses of the reaction products by the selected mutants revealed two major products; one is a di-D-fructose-2,6':6,2'-dianhydride (DFAIV) and the other is a levanbiose. The newly detected levanbiose corresponds to the reaction product from LFTase lacking transferring activity. Two mutants (2-F8 and 2-G9) showed a high yield of levanbiose (38–40%) compared with the wild-type enzyme, and thus behaved as levanases. Sequence analysis of the individual mutants responsible for the enhanced hydrolytic activity indicated that Asn-85 was highly involved in the transfructosylation activity of LFTase.

Keywords: DFAIV, error-prone PCR mutagenesis, levan fructotransferase, *Microbacterium* sp., transfructosylation

Levan is a highly branched fructose homopolysaccharide that is composed of β-2,6-linked fructosyl residues and is found in monocotyledons as a reserve carbohydrate or synthesized by microbial levansucrase (E.C. 2.4.1.10) from sucrose [6]. A microbial levan has many branching points at the C-1 position, depending on the sources of the enzyme. Levan-degrading enzymes can be classified into several types based on their degradation products: exo-β-fructosidase (E.C. 3.2.1.80), which hydrolyzes levan into fructose [2]; levanase (E.C. 3.2.1.65), which hydrolyzes levan into fructooligosaccharide; 2,6-β-D-fructan 6-levanbiohydrolase (E.C. 3.2.1.64), which hydrolyzes levan into levanbiose [3]; and levan fructotransferase (LFTase), which produces di-D-fructose-2,6':6,2'-dianhydride (DFAIV) from levan, a cyclic disaccharide consisting of two fructose units linked by their reducing carbons [14, 17].

Among them, 2,6-β-D-fructan 6-levanbiohydrolase and LFTase hydrolyze levan to difructose units. The former produces a linear difructose levanbiose, whereas the latter produces a cyclic difructose DFAIV through an intramolecular levan fructosyl transfer reaction [16]. We previously cloned and sequenced LFTase from *Microbacterium* sp. AL-210 and levanbiohydrolase from *Microbacterium laevaniformans*, respectively [5, 15]. The amino acid sequence of the *Microbacterium* sp. AL-210 LFTase showed 44% identity and 55% similarity with that of the *M. laevaniformans* levanbiohydrolase. Both enzymes belong to the β-fructofranosidases of family 32 of the glycosyl hydrolases (GH32), which includes inulinase (E.C. 3.2.1.7), levanase (E.C. 3.2.1.65), transfructosidase, and invertase (E.C. 3.2.1.26). The catalytic regions in the enzymes that hydrolyze the fructose polymers are highly conserved. Therefore, it is predicted that the difference in the reaction products of the levanbiohydrolase (levanbiose: linear form) and the LFTase (DFAIV: cyclic form) could result from the intramolecular transfructosylation activities in LFTase. The production of fructosyl derivatives by the transfructosylation reactions of the fructosyltransferase enzymes has recently become a topic of interest for its industrial application [9, 10].

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

In this paper, we used an error-prone PCR mutant library of LFTase from *Microbacterium* sp. AL-210 in a screening process for LFTase mutants displaying high hydrolytic activity over transfructosylation activity in order to identify the residues that are critical to the fructosyl transfer reaction. The results for this investigation suggest that Asn-85 near the substrate binding pocket found in the GH32 enzymes is highly involved in transfructosylation activity in LFTase.

MATERIALS AND METHODS

Materials

Escherichia coli BL21(DE3) harboring plasmid pELFT-NX (encoding the *Microbacterium* sp. LFTase [20]) or one of its derivatives was grown at 37°C on Luria-Bertani (LB) agar plates or in 200 μl of LB medium in 96-well microtiter plates (polystyrene) supplemented with kanamycin (30 μg/ml), with continuous shaking at 750 rpm. Microbial levan was prepared by laboratory-scale production using a levansucrase, and was isolated by ethanol precipitation [21]. DFAIV was a gift from Realbiotech (Chungnam, Republic of Korea). *Taq* DNA polymerase and dNTPs for polymerase chain reaction (PCR) amplification were purchased from Bioneer, Inc. (Cheongwon, Republic of Korea). Oligonucleotide primers used in an error-prone PCR, nucleotide sequencing, and expression of target genes were purchased from Bioneer. A QIAquick gel extraction kit, for PCR products, and DNA restriction fragments were purchased from Qiagen (Hilden, Germany). For the purification of recombinant proteins, Hi-Trap chelating affinity and gel filtration columns were purchased from Amersham Pharmacia Biotech (Cleveland, U.S.A.). For thin-layer chromatography (TLC) analysis, silica gel 60 F_{254} TLC plates were purchased from Merck (Darmstadt, Germany). For the concentration of the protein, Centriprep centrifugal filter units with Ultracel YM-30 membrane were purchased from Millipore (Billerica, U.S.A.). All other chemicals were of analytical grade.

Error-Prone PCR Mutagenesis

Error-prone PCR mutagenesis was performed using an unbalanced dNTP concentration in the presence of $MnCl_2$ (Genofocus, Daejeon, Korea). The LFTase gene was amplified from pELFT-NX with a T7 promoter primer and a T7 terminator primer. The reaction mixture contained 5 ng of plasmid DNA, 40 mM KCl, 10 mM Tris-HCl (pH 9.0), 5.5 mM $MgCl_2$, 0–0.64 mM $MnCl_2$, 0.25 mM each of dATP and dGTP, 1 mM each of dCTP and dTTP, 10 pmol each of primers, and 1 U *Taq* DNA polymerase (Bioneer, Daejeon, Korea) in a 20-μl reaction volume. PCR amplification was carried out on a thermal cycler (GeneAmp PCR System 2400, Perkin Elmer) with a program of predenaturation for 2 min at 95°C; followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min; and a final incubation for 7 min at 72°C. One-tenth of each PCR mixture was analyzed on a 0.8% (w/v) agarose gel containing ethidium bromide (0.25 mg/ml). Agarose gel electrophoresis was carried out in Tris-acetate buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) at 100 volts, and the size marker used was a 1-kb ladder (Elpis Biotech, Daejeon, Korea). The purified DNA fragment was used as a template for a second round of PCR with the LFTNdeI-F primer and LFTXhoI-R primer [20]. The reaction mixture contained 20 ng of an amplified DNA fragment, 40 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.25 mM each of dNTP, 10 pmol each of primers, and 2.5 U *Taq* DNA polymerase. The PCR-amplified DNA fragment digested with NdeI and XhoI was subcloned into the expression vector pET-29b. Each mutant was confirmed by sequencing. The mutated plasmid containing the LFTase gene was transformed into *E. coli* BL21(DE3) cells.

High-Throughput Screening

Screening of the mutants showing high hydrolytic activity was carried out by the modification of a previously described method [11]. *E. coli* clones carrying LFTase mutants were transferred to 96-well microtiter plates containing 200 μl of LB per well and supplemented with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 30 μg/ml kanamycin, and the microtiter plates were then incubated for 24 h at 37°C. Next, 100 μl of each culture was transferred to a second microtiter plate, and the cells were frozen at −20°C and thawed for 30 min at room temperature. This procedure lyses cells to release the proteins, which allows for measurement of the activities of enzymes located within cells. After the addition of 50 μl of 1% (w/v) levan in 20 mM sodium phosphate buffer (pH 7.0), the microtiter plates were incubated for 2 h at 37°C. Fifty μl of each reaction was added to 50 μl of dinitrosalicylic acid (DNS) reagent in polypropylene microtiter plates (Costar, U.S.A.). After sealing with a sealing mat, the plates were heated at 100°C for 5 min, and were then cooled in an ice bath to stabilize the color. DNS reagent was prepared by mixing 10.6 g of 3,5-dinitrosalicylic acid, 19.8 g of NaOH, 306 g of sodium potassium tartrate, 8.3 g of sodium metabisulfite, and 7.6 ml of phenol with 1,300 ml of distilled water. The total volume was then brought up to 1,500 ml with distilled water. The development of color was measured at 570 nm. Otherwise, the microtiter plates were screened visually, as increasing amounts of reducing sugars give darker colors.

Expression and Purification of the Enzyme

E. coli BL21(DE3) cells harboring the pELFT-NX were subcultured in 50 ml of LB medium supplemented with kanamycin (30 μg/ml) for 12 h at 37°C. Five ml of the subculture was inoculated into 500 ml of LB medium containing kanamycin (30 μg/ml) in a 2-l flask. The cells were incubated with shaking at 37°C to a density of 0.6 at 600 nm, and IPTG was added to a final concentration of 1 mM. The culture was then grown for an additional 4 h. The induced cells were harvested by centrifugation at 8,000 ×*g* for 15 min at 4°C, and the pellet was resuspended in ice-cold 20 mM sodium phosphate buffer (pH 7.0) and disrupted by a French pressure cell press (Thermo IEC, Needham Heights, MA, U.S.A.). The cell lysate was centrifuged at 15,000 ×*g* for 30 min at 4°C, and the supernatant was filtered and purified on a Hi-Trap chelating Ni-NTA column (Qiagen). The column was washed twice with 20 mM sodium phosphate buffer (pH 7.0) containing 500 mM NaCl and 50 mM imidazole. The proteins bound to the resin were eluted with 250 mM imidazole and dialyzed against 20 mM sodium phosphate buffer (pH 7.0). After Hi-Trap chelating affinity chromatography, the enzyme solution was further purified on a Hi-Prep 16/60 Sephacryl S-200 high resolution column (Pharmacia Biotech) using fast-performance liquid chromatography (FPLC; UPC-900 model; Pharmacia Biotech). The fractions showing enzyme activity were pooled and concentrated. The purity of the enzyme was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Thin-Layer Chromatography

The sugars produced by the enzyme reaction were analyzed by thin-layer chromatography (TLC) [8]. The reaction mixture was composed of 0.1 ml of the enzyme solution (0.01–0.02 mg/ml) and 0.2 ml of 1% levan in the presence of a 20 mM phosphate buffer (pH 7.0). The reaction mixture was incubated at 40°C for 30 min, and was then heated in boiling water for 5 min to stop the reaction. After centrifugation, an aliquot (1–3 μl) of the reaction mixture was spotted onto a Kieselgel 60 F_{254} TLC plate and developed with a solvent system of *n*-butanol/pyridine/water (6:4:1, v/v/v) in a developing tank. Ascending development was repeated twice at room temperature. The plate was allowed to air-dry in a hood, and was then developed by soaking it briefly in methanol solution containing 20% (v/v) sulfuric acid. The plate was dried and placed in an 110°C oven for 10 min to visualize the reaction spots.

HPLC Analysis of the Reaction Products

A quantitative assay for the production of DFAIV and levanbiose was performed by high-performance liquid chromatography (HPLC) as described previously [20]. The reaction mixture was composed of 0.1 ml of the enzyme solution (0.01–0.02 mg/ml) and 0.2 ml of levan (1 mg/ml) in the presence of a 20 mM phosphate buffer (pH 7.0). The reaction mixture was centrifuged, and the supernatant was filtered and analyzed on a Waters-associated HPLC system with a SugarPac column (6.5 mm×300 mm; Waters, Milford, U.S.A.). The elution was conducted using degassed deionized water at a flow rate of 0.3 ml/min. Buffers were degassed on the same day of use. The detection of fructose, DFAIV, and levanbiose was monitored with a 410 differential refractometer (Waters, U.S.A.) at 80°C and the quantitative amount of each sugar was calculated using a known standard sugar concentration. The amount of each sugar of the wild-type and mutant enzymes was converted to the relative amounts to compare the changes of the products by mutation.

Analytical Methods

Denatured proteins were separated on a 12% SDS-PAGE gel and stained with Coomassie blue R-250. The molecular weight was estimated using molecular weight standards. The protein concentration during purification was measured using a Bio-Rad protein microassay. The purified enzyme was determined by amino acid analysis. An extinction coefficient of 150,000 M^{-1} cm^{-1} at 280 nm was determined for the purified enzyme. Subsequent protein concentrations were based on the absorbance at 280 nm using a spectrophotometer (Ultrospec-2100 Pro; Amersham Bioscience).

Results

Screening of the LFTase Mutants Showing Increased Hydrolytic Activities

In order to probe the residues that are critical to the intramolecular fructosyl transfer reaction of the *Microbacterium* sp. AL-210 LFTase (LftM), we performed error-prone PCR mutagenesis on the *lftM* gene. The expression plasmid, pELFT-NX, which contains the *lftM* gene with a tag of six-histidine residues at the C-terminus, was used as a template in the first-round error-prone PCR. Before the screening of the mutants, the optimal condition for mutagenesis was examined at various $MnCl_2$ concentrations ranging from 0 to 0.64 mM. PCR-amplified DNA showed a tendency to decrease above a concentration of 0.32 mM $MnCl_2$ (data not shown). At 0.08 mM $MnCl_2$, a nucleotide mutation ratio is approximately 0.06%, which is an ideal mutagenesis ratio for directed evolution (one amino acid substitution per gene). The isolated mutants were screened for high hydrolytic activities, which produce more reducing sugars, as indicated by the appearance of a red color in the DNS assay. In contrast, the mutants producing no reducing sugars resulted in the appearance of a yellow color. The activities were measured at pH 7 and 40°C because there are no differences among the wild type and mutants in terms of optimum temperature and pH [5, 20]. The formation of DFAIV by the wild-type enzyme does not increase the number of reducing sugar ends, and thus reveals a yellow color. Afterwards, the activities of the best mutants were quantitatively analyzed by TLC and HPLC in order to identify reaction products.

Among approximately 7,000 recombinant clones screened on LB containing 1% levan, eight mutants (C2, C11, D10, E8, E12, F10, G7, and H2) that showed a slightly red color (high hydrolytic activity) were chosen for further analysis (Fig. 1A). Analysis of products by TLC revealed that all of the mutants catalyzed the formation of more hydrolyzed products than did the wild-type enzyme (Fig. 1B). As we expected, the mutant enzymes showed two major products; one was a DFAIV (no reducing sugar) and the other was a levanbiose. Moreover, with the C2 mutant, the hydrolytic

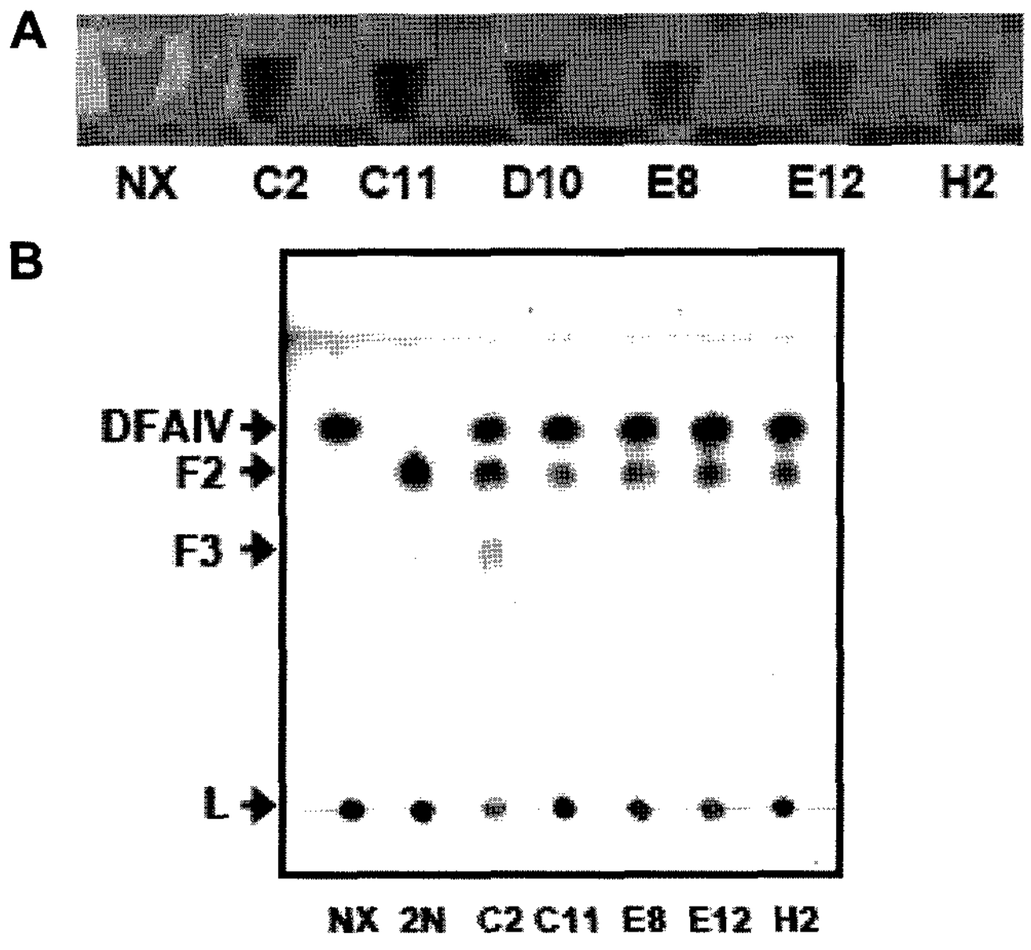

Fig. 1. Detection of levan hydrolytic activity in LFTase mutants using error-prone PCR mutagenesis.

A. Screening for LFTase mutants with increased hydrolytic activity in a microtiter plate. **B**. TLC profile of the LFTase mutants. The reaction was carried out with 1% levan at 40°C for 3 h. NX, wild-type LFTase; 2N, levanbiohydrolase; L, levan; F2, levanbiose; F3, levantriose.

Table 1. The amino acids changed by error-prone PCR mutagenesis.

Mutagenesis	Enzyme	Changed amino acids	Relative activity[a] (%)
	Wild type	None	10.7
First round	C2, D10, F10, G7	N85S	34.8
	C11	N85S/D268E	30.0
	E8	A264G	20.4
	E12	P313R/F518L	15.8
	H2	N238K	23.6
Second round	2-F8	N85S/A264G	49.9
	2-G9	N85S/N224K/Q229E/T253N	37.0

[a]Relative activity indicates the relative hydrolyzing activity compared with the levanbiohydrolase activity.

activity was relatively high because the presence of levantriose was detectable along with levanbiose. Sequences of mutant enzymes revealed the presence of one to two mutations in the different genes (Table 1). Interestingly, the Asn-85 residue was found to be substituted by a Ser in the four independent mutants, C2, D10, F10, and G7.

Enzymatic Properties of Selected Mutant Enzymes

The hydrolytic activities of mutant enzymes were determined spectrophotometrically by measuring the reducing sugar released after the enzyme reaction. The hydrolytic activities of the mutant enzymes were evaluated by comparing them with that of levanbiohydrolase from *M. laevaniformans*. The C2, D10, F10, G7 mutants (N85S), C11 (N85S/D268E), E8 (A264G), E12 (P313R/F518L), and H2 (N238K) showed 34.8%, 30%, 20.4%, 15.8%, and 23.6% hydrolytic activity, respectively, whereas the wild-type enzyme showed only 10.7% hydrolytic activity (Table 1). This observation indicates that the N85S substitution is responsible for the strong enhancement of the hydrolytic activity. Because the N85S mutant was very efficient, we carried out a second error-prone PCR mutagenesis procedure to acquire more effective mutants; through this process, 17 mutants were selected, and finally two mutants, 2-F8 and 2-G9, were obtained among about 4,000 clones. Double-mutant 2-F8 (N85S/A264G) and quadruple-mutant 2-G9 (N85S/N224K/Q229E/T253N) showed 49.9% and 37% hydrolytic activity, respectively.

Quantification of the Reaction Products

In order to further characterize the reaction products from selected mutant enzymes, we purified them using Hi-Trap chelating affinity and gel filtration chromatographies. The wild-type LFTase and levanbiohydrolase mainly produced DFAIV and levanbiose, respectively, as reported previously. However, the mutant enzymes we screened produced DFAIV and levanbiose in a different ratio, while also producing fructose and levantriose. The quantitative amounts of the fructose, levanbiose, levantriose, and DFAIV of each mutant enzyme were compared with those of wild-type LFTase and levanbiohydrolase (Fig. 2). Total reducing sugars, including fructose, levanbiose, and levantriose, were highly increased in all of the mutant enzymes. In particular, double-mutant 2-F8 and quadruple-mutant 2-G9 showed a high yield of levanbiose (38–40%), whereas the wild-type enzyme

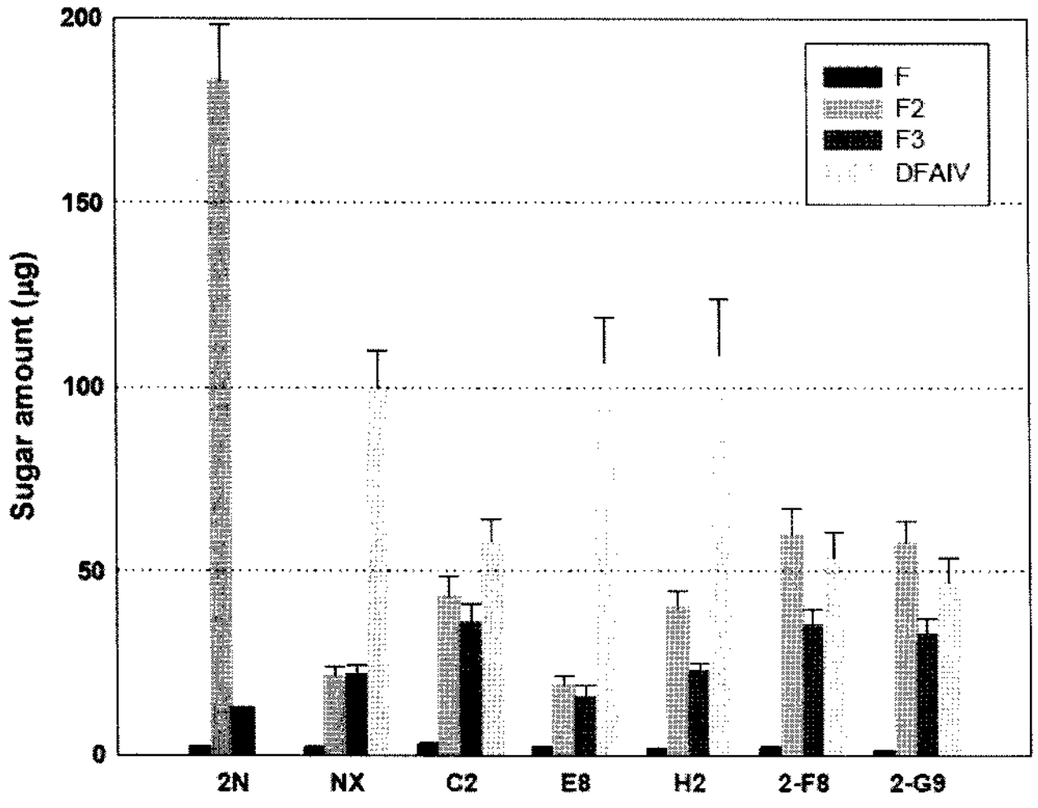

Fig. 2. The amounts of the sugars produced by the wild-type and mutant LFTases.

Sugar amounts based on the standard solution were determined using HPLC. NX, wild-type LFTase; 2N, levanbiohydrolase; F, fructose; F2, levanbiose; F3, levantriose.

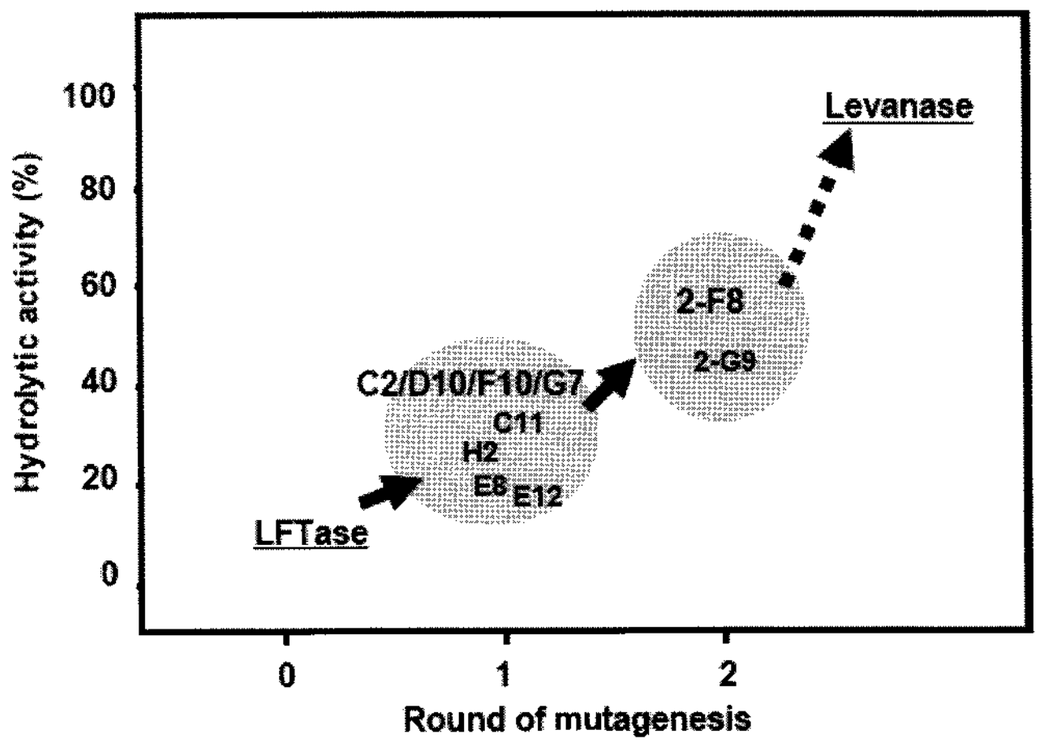

Fig. 3. Progression of LFTase mutants towards the levan-hydrolyzing enzyme, levanase.

could not produce more than 14% levanbiose. The slight decrease of levantriose for 2-F8 and 2-G9 compared with the C2 mutant is due to the relative increase of levanbiose for these two mutants. Moreover, both mutants behaved as somewhat levanase-type enzymes by increasing the concentration of levanbiose over that of DFAIV (Fig. 3).

Discussion

A variety of bacteria and approximately 12% of higher plants form carbohydrate reserves based on fructans [4, 7]. In plants, several fructosyltransferases with distinct fructosyl-donor and fructosyl-acceptor specificities are required for fructan biosynthesis, whereas in bacteria, levan biosynthesis is catalyzed by a single enzyme, levansucrase. Although it is recognized that fructosyl transfer activity is critical to fructan biosynthesis, little is known about the structural framework of its function in enzymes. Recently, the crystal structure of *Bacillus subtilis* levansucrase revealed that Arg-360 near the putative general acid was important for polymerization activity in this enzyme, because it served as a docking site for the transfer of the coming acceptor to an enzyme-donor intermediate [12].

In previous studies, we cloned LFTase and levanbiohydrolase from *Microbacterium* species [5, 15]. LFTase catalyzes intramolecular transfructosylation, in addition to its hydrolytic action on the contrary to levanbiohydrolase or levanase, although both enzymes belong to the same GH32 family and show high sequence similarity. It is known that the intramolecular fructosyl transfer reaction and the hydrolysis of levan to fructose and/or levanbiose are mediated by LFTase itself [18]. LFTase produces DFAIV as a main product, but it also produces a trace amount of fructose, levanbiose, and levantriose at the same time. The distribution of these products is constant regardless of the time course of the enzyme reaction. It will be interesting to know which factors are involved in the product specificity (hydrolysis versus intramolecular transfructosylation) differentiating between LFTase and levan-hydrolyzing enzyme, levanase or levanbiohydrolase. Therefore, we attempted to identify the residues that are highly involved in the intramolecular fructosyl transfer reaction in LFTase using random mutagenesis. A couple of mutants showed increased hydrolytic activities after first-round mutagenesis. A TLC chromatogram revealed that most of the mutants possess two reaction products instead of one major product, DFAIV, which is usually observed in the wild-type enzyme. A novel hydrolytic product was confirmed to be a levanbiose through TLC and HPLC analyses. These results imply that the intramolecular fructosyl transfer reaction following the hydrolysis of levan to levanbiose was partially inhibited by mutation. The result of the amino acid sequencing of the mutants indicates that the enhancement of the hydrolytic activity is attributed to the substitution of the asparagine with serine at the 85th position. Its substitution caused a corresponding decrease in the transfer of the intramolecular fructosyl group, which is indicative of the central role played by Asn-85 in the catalysis of intramolecular transfructosylation by LFTase. Further improvement of the hydrolytic activity of LFTase was carried out by second-round mutagenesis, and Ala-264 and Asn-238 were also confirmed to be involved in the fructosyl transfer reaction.

Recently, the crystal structures of three GH32 enzymes, *Thermotoga maritima* invertase [1], exoinulinase from *Aspergillus awamori* [13], and a plant fructan, 1-exohydrolase, from *Cichorium intybus* [19], have all been elucidated. The three-dimensional structures of these three enzymes revealed a similar bimodular arrangement, with catalytic-domain folding in a rather unusual five-bladed β-propeller shape linked to a C-terminal β-sandwich domain of a putative carbohydrate-recognition function. The similarities of the amino acid sequences and activities toward the fructose-containing poly- and oligosaccharides may reflect an evolutionary relationship among the members of the GH32 family. Based on the invertase active-site mutant structure with raffinose, Trp-41 (corresponds to Asn-85 in LFTase) forms a hydrogen bond to O5' of the fructose moiety of raffinose. The fructose moiety is located in subsite −1 at the deepest end of the active-site pocket, and Trp-41 is compatible with the spatial requirements of a fructofuranose group and could serve as a docking site to recognize the fructose moiety that is to be cleaved. Overlap of Trp-41 in invertase for the Asn-85 found in LFTase retains the position of Trp-41 in the active site (Fig. 4). Trp-65 forms a hydrogen bond to OH-5 of fructose. The same hydrogen bonding pattern has also been described for fructose bound with exoinulinase [13]. Therefore, it is certain that Asn-85 may play an important role in the fructosyl transfer reaction following the hydrolysis in LFTase. Ala-264 (corresponds to Ile-204 in invertase) and Asn-238 (corresponds to Asp-183 in invertase) mutated in E8 and H2 mutants do not appear to be related to substrate binding or catalytic activity, although they are located in the interior of the β-sheet and on a loop that connects adjacent β-sheets within the N-terminal five-bladed β-propeller. The rest of the amino acids (Pro-313, Phe-518, Asn-224, Gln-229, and Thr-253) changed by mutants E12 and 2-G9 showed only subtle changes in hydrolytic activity of LFTase. Therefore, it will be very difficult to understand the role of these amino acid residues for fructosyl transfer reaction unless the three-dimensional structure of LFTase has been solved. At this point, more detailed interpretation may not be possible because there are certain limitations in the structural similarity between LFTase and other GH32 enzymes. We are now in the process of solving the three-dimensional structure of LFTase to better understand the transfructosylation specificity of the enzyme.

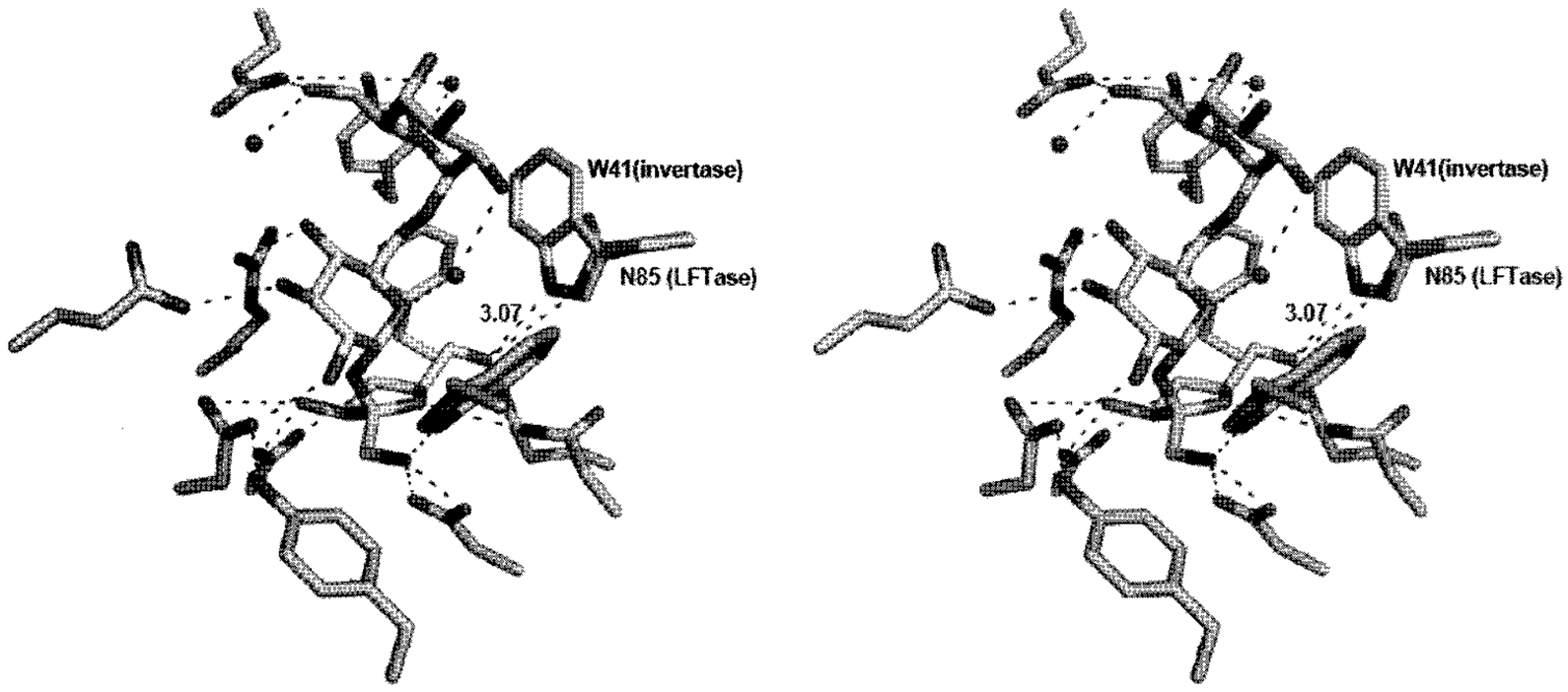

Fig. 4. Stereographic view of the catalytic site of *Thermotoga maritima* invertase in complex with raffinose (adapted from Ref. 1).
Trp-41 (corresponds to the Asn-85 in LFTase) forms a hydrogen bond to O5' of the fructose moiety of raffinose. The figure was prepared with PyMOL v1.0 (http://pymol.sourceforge.net/) using PDB Accession No. 1W2T.

Acknowledgment

This work was supported for two years by a Pusan National University research grant.

REFERENCES

1. Alberto, F., E. Jordi, B. Henrissat, and M. Czjzek. 2004. Crystal structure of inactivated *Thermotoga maritima* invertase in complex with the trisaccharide substrate raffinose. *Biochem. J.* **395:** 457–462.
2. Avigad, G. and S. Bauer. 1996. Fructan hydrolases. *Methods Enzymol.* **8:** 621–628.
3. Avigad, G. and R. Zelikson. 1963. Cleavage of fructans to levanbiose by a specific hydrolase. *Bull. Res. Counc. Isr.* **11A4:** 253–257.
4. Cairns, A. J. 2003. Fructan biosynthesis in transgenic plants. *J. Exp. Bot.* **54:** 549–567.
5. Cha, J., N. H. Park, S. J. Yang, and T. H. Lee. 2001. Molecular and enzymatic characterization of a levan fructotransferase from *Microbacterium* sp. AL-210. *J. Biotechnol.* **91:** 49–61.
6. Han, Y. W. 1990. Microbial levan. *Adv. Appl. Microbiol.* **35:** 171–194.
7. Hendry, G. 1987. The ecological significance of fructan in a contemporary flora. *New Phytol.* **106:** 201–216.
8. Jung, Y.-J., C. S. Park, H. G. Lee, and J. Cha. 2006. Isolation of a novel gellan-depolymerizing *Bacillus* sp. strain YJ-1. *J. Microbiol. Biotechnol.* **16:** 1868–1873.
9. Kim, M. J., H. E. Park, H. K. Sung, T. H. Park, and J. Cha. 2005. Action mechanism of transfructosylation catalyzed by *Microbacterium laevaniformans* levansucrase. *J. Microbiol. Biotechnol.* **15:** 99–104.
10. Lee, C. Y., K. H. Kim, S. Y. Hur, J.-H. Heo, M. H. Choi, S. K. Rhee, and C. H. Kim. 2006. Enzymatic synthesis of ascorbic acid fructoside by transfructosylation using levan fructotransferase. *J. Microbiol. Biotechnol.* **16:** 64–67.
11. Leemhuis, H., G. J. Euverink, and L. Dijkhuizen. 2003. High-throughput screening for gene libraries expressing carbohydrate hydrolase activity. *Biotechnol. Lett.* **25:** 1643–1645.
12. Meng, G. and K. Fütterer. 2003. Structural framework of fructosyl transfer in *Bacillus subtilis* levansucrase. *Nat. Struct. Biol.* **10:** 935–941.
13. 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.
14. 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. Biotech. Biochem.* **61:** 1705–1709.
15. Song, E. K., H. Kim, H. K. Sung, and J. Cha. 2002. Cloning and characterization of a levanbiohydrolase from *Microbacterium laevaniformans* ATCC 15953. *Gene* **291:** 45–55.
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. Tanaka, K., T. Karigane, F. Yamaguchi, S. Nishikawa, and N. Yoshida. 1983. Action of levan frutosyltransferase of *Arthrobacter ureafaciens* on levanoligosaccharide. *J. Biochem.* **94:** 1569–1578.
18. Tanaka, K., M. Shimonishi, M. Kitagaki, and M. Ikunaga. 1990. Action of levan fructotransferase of *Arthrobacter ureafaciens* on three oligosaccharides containing a bifurcose residue. *Agric. Biol. Chem.* **54:** 815–817.
19. Verhaest, M., W. V. Ende, K. L. Roy, C. J. De 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.
20. 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.
21. Yokota, A., K. Kondo, M. Nakagawa, I. Kojima, and F. Tomita. 1993. Production of levanbiose by a lvan-degrading enzyme from *Streptomyces exfoliatus*. *Biosci. Biotech. Biochem.* **57:** 745–749.