Molecular insight into regioselectivity of transfructosylation catalyzed by GH68 levansucrase and β-fructofuranosidase

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Glycoside hydrolase family 68 (GH68) enzymes catalyze β-fructosyltransfer from sucrose to another sucrose, the so-called transfructosylation. Although regioselectivity of transfructosylation is divergent in GH68 enzymes, there is insufficient information available on the structural factors involved in the selectivity. Here, we found two GH68 enzymes, β-fructofuranosidase (FF) and levansucrase (LS), encoded tandemly in the genome of Zymomonas mobilis, displayed different selectivity: FF catalyzed the β-(2→1)-transfructosylation (1-TF), whereas LS did both of 1-TF and β-(2→6)-transfructosylation (6-TF). We identified His79FF and Ala343FF and their corresponding Asn84LS and Ser345LS respectively as the structural factors for those regioselectivities. LS with the respective substitution of FF-type His and Ala for its Asn84LS and Ser345LS (N84H/S345A-LS) lost 6-TF and enhanced 1-TF. Conversely, the LS-type replacement of His79FF and Ala343FF in FFZm (H79N/A343S-FFZm) almost lost 1-TF and acquired 6-TF. H79N/A343S-FFZm exhibited the selectivity like LSZm but did not produce the β-(2→6)-fructosyl-linked levan and/or long levanoooligosaccharides that LSZm did. We assumed Phe189LS to be a responsible residue for the elongation of levan chain in LSZm and mutated the corresponding Leu187FF in FFZm to Phe. An H79N/L187F/A343S-FFZm produced a higher quantity of long levanoooligosaccharides than H79N/A343S-FFZm (or H79N-FFZm), although without levan formation, suggesting that LSZm has another structural factor for levan production. We also found that FFZm generated a sucrose analog, β-D-fructofuranosyl α-D-mannopyranoside, by β-fructosyltransfer to α-mannose and regarded His79FF and Ala343FF as key residues for this acceptor specificity. In summary, this study provides insight into the structural factors of regioselectivity and acceptor specificity in transfructosylation of GH68 enzymes.

The so-called fructooligosaccharides (FOSs) are structurally divided into two types: the reducing FOSs and the nonreducing FOSs. The former is composed of only fructose units with reducing power, whereas the latter has the former conformation blocked by a glucose unit at its reducing fructosyl residue, thereby meaning “glucosides” (or “fructoside”) for disaccharide). Natural nonreducing FOSs are mainly categorized into inulooligosaccharide (INO; whose long saccharide is an inulin with less than around sixty β-(2→1)-linked fructosyl units) and levanoooligosaccharide (LVO; whose polysaccharide is a levan linked by β-(2→6)-fructosyl units). Also, natural reducing FOSs are present by cleaving a terminal Glc-Fru unit (sucrose unit) of INO or LVO with α-glucosidase and acidic condition. These natural FOSs are originally regarded to be responsible for energy storage in plants. Other possible functions, including their contribution to the tolerance against drought and freezing, are also proposed in plants and microbes (1). Moreover, their associated health benefits also receive considerable attention, such as the selective stimulation of the growth and/or the activity of beneficial intestinal bacteria, contributing to the increase of the absorption of several minerals as well as the lowering of the cholesterol levels (2–4).

Figure 1 depicts the structures of INO (e.g., 1-kestose and nystose), LVO (e.g., 6-kestose), levan, and neokestose [e.g., β-fructosyl-(2→6)-glucosyl fructose]. These nonreducing FOSs and levan are produced from sucrose with the aid of fructosyl-transferring enzymes. Syntheses of INOs and inulin in plants are initiated by sucrose:sucrose 1-fructosyltransferase, resulting in 1-kestose. Subsequently, 1-kestose is further elongated by the action of fructan:fructan 1-fructosyltransferase. In microorganisms, the inulosucrase is responsible for the inulin synthesis. β-Fructofuranosidase (FF) is often used for the in vitro synthesis of INO, including commercially available INO, such as the 1-kestose and the nystose, which are produced by fungal FF (3). On the other hand, LVO and levan are well known to be produced by microbial levansucrase (LS). Plants, including timothy (Phleum pratense), orchard grass (Dactylis glomerata), and big bluegrass (Poa secunda), accumulate simple linear levan, of which timothy is identified to be synthesized with sucrose:fructan 6-fructosyltransferase (5, 6). 6-Kestose is also demonstrated to be efficiently formed by Microbial FF from Schwanniomyces occidentalis (7, 8). Furthermore, neokestose, neonytose, and the same type of long oligosaccharides are synthesized by FF in Xanthophyllomyces dendrorhous (9), a mutated FF of S. occidentalis (10), and a plant fructan:fructan 6G-fructosyltransferase (11, 12), in which the term for the produced saccharides (e.g., neokestose and neonytose) may become “neolevanoooligosaccharides.”

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Fructosyl-linkage hydrolyzing and transferring enzymes acting on sucrose are classified as members of the glycoside hydrolase family (GH) 32 or 68 (13). Among them, plant-derived enzymes and fungal FF generally belong to GH32, whereas the inulosucrase, LS, and bacterial FF are classified as GH68. GH32, and 68 proteins have a 5-bladed β-propeller fold as a catalytic domain, grouped into clan GH-J. The enzymes in this clan are known to show a catalytic double displacement mechanism with two carboxy groups, a catalytic nucleophile, and a general acid/base, for hydrolysis and/or transfructosylation. During the synthesis of inulin and levan, the transfructosylation occurs successively.

Bacterial LS, especially LS from Gram-positive Bacillus spp. (LSBspp), is one of the most characterized GH68 enzymes (14–16). The molecular weight of the levan produced by these LSBspp, reaching $3 \times 10^6$, can be controlled by the reaction conditions and the mutagenesis of the enzyme. The protein engineering works and other results are well reviewed by Ortiz-Soto et al. (17). There are different types of LSs derived from Gram-negative bacteria, a Gluconacetobacter diazotrophicus LS (LSGd) and an Erwinia amylovora LS (LSea), which synthesize a low amount of levan with the low molecular weight (18–20). Interestingly, a certain amount of INO (1-kestose and nystose) are also produced by these enzymes (18, 20), which is assumed to be associated with the low levan synthesis, although the detail mechanism is still obscure.

Gram-negative Zymomonas mobilis is known to grow on sucrose, glucose, and fructose as a carbon source. Therefore, it is possible that the enzymes associated with sucrose degradation of this bacterium have undergone its own molecular evolution under the limitation of having to use sucrose as a carbon source. Z. mobilis has two GH68 enzyme genes with ZZ6_0874 and ZZ6_0875 locus tags, which are positioned side by side in the chromosome, as if one of them was formed by a tandem gene duplication. Gene duplication has been of long-standing interest in the evolution as once a gene has duplicated, a novel function may be acquired by one of the copies (21). In fact, the functional evolution has occurred in these two GH68 proteins: ZZ6_0874 and ZZ6_0875 encode FF and LS (22–24), which hereafter will be referred to as FFZm and LSZm, respectively. The motivation for this study is to compare the structure and function of these enzymes that have undergone molecular evolution while relatively retaining sequence identity and to clarify the structural factors that distinguish the reaction specificity of two enzymes that act on sucrose. Relationship between the structure of LSZm and its levan synthesis has been well-studied (25–27), while limited enzymatic study has been done on FFZm, which might be due to the lack of clear difference in regard of the molecular mechanisms between the two GH68 enzymes in Z. mobilis. In this study, we found a distinction in regioselectivity of the FFZm- and LSZm-catalyzed transfructosylation as well as identified the structural factors responsible for the regioselectivity by mutation works. Until now, no studies have been performed to our knowledge on the regulation of the regioselectivity of GH68 enzymes. Our mutation works also enabled to learn one of important residues to elongate LVO chain. The FFZm was found to generate β-d-fructofuranosyl α-d-mannopyranoside (Fru-Man; a sucrose analog) with a good yield, which was firstly produced by Bacillus subtilis LS (LSBc) (28), although in not high-yield formation.

Results and discussion

Characterization and initial transfructosylation of the LSZm and FFZm

Wild-type LSZm and FFZm had the specific activities of 247 and 1620 U mg$^{-1}$, respectively (Table 1). The pH optimum for both enzymes was at pH 5.0, and the pH stability was between pH 4.2 and 5.5 after treatment at 4 °C for 24 h, as well as the thermal stability was up to 45 °C after a 15 min incubation. Although the initial velocity can be measured at 45 °C, the following transfructosylation experiments were carried out at 35 °C, where the activities were maintained even after prolonged reaction.

The LSZm- and FFZm-catalyzed initial 10 min reaction was analyzed using various sucrose concentrations ([sucrose]) (Fig. 2, A and B, respectively), in which the formed carbohydrates were measured with high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The initial products were glucose, fructose,
1-kestose, and 6-kestose, at which the hydrolysis generates glucose and fructose from sucrose and water as well as the transfructosylation forms trisaccharide (1-kestose or 6-kestose) and glucose from two sucrose molecules. Therefore, the initial reactions of both enzymes follow the scheme of Figure 2C, similarly to many glycosylases (28). Transfructosylation products were different in LSZm and FFZm, where 6-kestose and 1-kestose were synthesized by the former (Fig. 2A), while 1-kestose with a trace amount of 6-kestose was synthesized by the latter (Fig. 2B). 1-Kestose (or 6-kestose) is produced from two-substrate reaction using two sucrose molecules as noted above, at which a fructosyl residue of donor sucrose is transferred to an acceptor sucrose to form the β-(2→1)-fructoside linkage [or β-(2→6)-fructoside linkage]. The formation velocities of the transfructosylation products, 6-kestose (v_{6-kestose} [E]_{0}^{-1}; abbreviated as v_{6-Kes}) and 1-kestose (v_{1-kestose} [E]_{0}^{-1}; v_{1-Kes}), increased with an increase of [sucrose] (Fig. 2, A and B). In the range of [sucrose] up to 300 mM, the formation velocities of glucose (v_{glucose} [E]_{0}^{-1}; v_{Glc}) and fructose (v_{fructose} [E]_{0}^{-1}; v_{Fru}) increased with showing almost equal amounts (Fig. 2, A and B), meaning that the main reaction is a hydrolysis. Moreover, as a [sucrose] increased, v_{Glc} and v_{Fru} decreased (especially v_{Fru}), meaning that the hydrolysis reduces and the transfructosylation increases. According to the scheme that is followed by the LSZm and the FFZm (Fig. 2C), the velocity of hydrolysis is reflected by v_{Fru} and that of transfructosylation is indicated by v_{6-Kes} and/or v_{1-Kes}. The v_{Glc} represents a sucrose degradation velocity, meaning the whole reaction velocities, i.e., the sum of hydrolysis and transfructosylation. The decrease in v_{Fru} can be ascribed to the increase in transfructosylation at the high [sucrose]. On the other hand, the diminution in v_{Glc} indicates the substrate inhibition of enzyme reaction. There are two possibilities that can explain this inhibitory phenomenon. First, the accumulation of an E-F-F-G complex is assumed (Fig. 2C) (29, 30). The concentration of the E-F-F-G complex ([E-F-F-G]) increases with increasing [sucrose], eventually exceeding the limit of the ability to catalyze the fructosidic linkage formation, so that the accumulation of the E-F-F-G is expected to occur. Since the [E-F-G], [E-F], and [E-F-F-G] are in steady state, an increase in the [E-F-F-G] causes a decrease in the v_{Glc}. Secondly, a nonproductive binding that, for instance, the excess sucrose occupies the +1 and +2 subsites of the catalytic site, prevents the productive binding of the sucrose at −1 and +1 subsites (31). The occurrence of the nonproductive binding increases at a higher [sucrose]. We cannot identify the correct mechanism among the two possibilities [or more correct further candidate(s) might exist, if the above two possibilities are not accurate].

The proportion of the transfructosylation velocity in all reaction velocities, which can be estimated from the equation of \((v_{1,kestose} + v_{6,kestose})/v_{glucose}\) was 0.52 in LSZm and 0.34 in FFZm using 1 M sucrose as substrate (Table 2). Yanase et al. (24) reported that the FFZm, purified from the periplasmic fraction of Z. mobilis, exhibited no transfructosylation activity, in regard of which the detailed experimental conditions were missing. A certain reaction condition might be inadequate, suggesting that one factor is a less enough [sucrose].

Transfructosylation of LSZm and FFZm at late reaction stage

The LSZm- and FFZm-generating transfructosylation products from 1 M sucrose were analyzed at 1 h (Fig. 3). The wild-type LSZm synthesized 6-kestose, 1-kestose, and neokestose (Fig. 3A). Neokestose, produced by β-(2→6)-transfructosylation (6-TF) of a fructose unit to the glucosyl residue of sucrose, was not detected during the initial reaction, implying a slower formation than that of the 6-kestose and 1-kestose. At 24 h, LSZm formed several oligosaccharides (6-kestose, neokestose, and nystose) as well as some possible LVOs with high degree of polymerizations (DPs) (Fig. 3B). The long LVOs should have DPs at least 25 (inset of Fig. 3B), although their exact DPs could not be estimated due to unavailable standards. They might be the intermediates of low-molecular weight levan after the elongation with successive 6-TF. On the other hand, the FFZm generated a sole trisaccharide of 1-kestose at 1 h (Fig. 3C). At 24 h, further trisaccharides of the 6-kestose, neokestose, and nystose were observed along with a small amount of the products above DP4 (Fig. 3D). In cases of both initial and late reaction stages, FFZm and LSZm show different regioselectivity in the transfructosylation process (Figs. 2 and 3), at which the former mainly catalyzes β-(2→1)-transfructosylation (1-TF), whereas the latter does both of 1-TF and 6-TF.

### Table 1

Properties of FFZm, LSZm, and their variants

| Enzyme          | Specific activity (U mg⁻¹) | Optimum pH | pH-stability * | Thermal stability (°C)* |
|-----------------|---------------------------|------------|----------------|-------------------------|
| FFZm Wild type  | 1620                      | 5.0        | 4.2–6.0        | <45                     |
| HS9N-FFZm       | 502                       | 5.0        | 4.1–6.0        | <45                     |
| A343S-FFZm      | 380                       | 5.0        | 4.1–6.0        | <45                     |
| H79N/A343S-FFZm | 274                       | 5.0        | 4.1–6.0        | <45                     |
| H79N/L187F/A343S-FFZm | 350                 | 5.0        | 4.1–6.0        | <40                     |
| LSZm Wild type  | 247                       | 5.0        | 4.1–5.5        | <45                     |
| N84H-LSZm       | 480                       | 5.5        | 3.7–5.5        | <60                     |
| S345A-LSZm      | 800                       | 5.0        | 3.7–5.0        | <60                     |
| N84H/S345A-LSZm | 440                       | 5.5        | 3.7–5.5        | <60                     |

*pH stability or *thermal stability shows the range to maintain the residual activity of more than 90% after treating with various pH at 4 °C for 24 h or *after incubating at various temperatures for 15 min, respectively.

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Table 2

| Enzyme                               | Reaction velocities of FF<sub>Zm</sub>,<sub>Wild type</sub> | Reaction velocities of FF<sub>Zm</sub>,<sub>H79N</sub> | Reaction velocities of FF<sub>Zm</sub>,<sub>A343S</sub> | Reaction velocities of FF<sub>Zm</sub>,<sub>H79N/L187F/A343S</sub> | Transfructosylation ratio a |
|--------------------------------------|-------------------------------------------------------------|--------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|-----------------------------|
| FF<sub>Zm</sub>,<sub>Wild type</sub> | 2980 ± 360                                                 | 900 ± 30                                               | 870 ± 105                                                    | 1000 ± 55                                                      | 3.8                         |
| FF<sub>Zm</sub>,<sub>H79N</sub>     | 1500 ± 130                                                 | 500 ± 29                                               | 420 ± 13                                                     | 430 ± 65                                                       | 0.34                        |
| FF<sub>Zm</sub>,<sub>A343S</sub>    | 600 ± 29                                                   | 216 ± 13                                               | 120 ± 8                                                      | 120 ± 8                                                        | 0.20                        |
| FF<sub>Zm</sub>,<sub>H79N/L187F/A343S</sub> | 920 ± 23                                                   | 600 ± 35                                               | 120 ± 8                                                      | 120 ± 8                                                        | 0.35                        |
| LS<sub>Zm</sub>,<sub>Wild type</sub> | 1820 ± 216                                                 | 600 ± 35                                               | 420 ± 63                                                     | 420 ± 63                                                       | 1.0                         |
| LS<sub>Zm</sub>,<sub>N84H</sub>     | 790 ± 92                                                   | 216 ± 13                                               | 120 ± 8                                                      | 120 ± 8                                                        | 0.82                        |
| LS<sub>Zm</sub>,<sub>N84H/S345A</sub> | 1600 ± 120                                                 | 600 ± 35                                               | 420 ± 63                                                     | 420 ± 63                                                       | 1.0                         |

Transfructosylation ratio was calculated from the equation of \( \frac{v_{6-kestose}}{v_{6-kestose} + v_{1-kestose}} \). The transfructosylation ratio of 0.69 means 69% of transfructosylation to glucose, indicative of the proportion of transfructosylation to whole reactions.

Prediction of residues responsible for regioselectivity of transfructosylation

As mentioned above, the regioselectivity of transfructosylation diverges at GH68 FF<sub>Zm</sub> and LS<sub>Zm</sub>, despite that their amino-acid sequences share the significantly high 61.5% identity and 72.0% similarity with only 5.1% gaps, leading us to unravel the structural factor(s) in connection with the regulation of regioselectivity distinction. However, the three-dimensional structures (3Ds) of these enzymes remain unresolved currently, and it is hard to identify the amino acid residues that are key to the differences in specificity. To visualize the 3Ds of amino acid residues at the active-site pocket of FF<sub>Zm</sub> and LS<sub>Zm</sub>, both enzymes were modeled (Fig. 4) using the 3D of LSEa (32) as it had the most similarity to the amino-acid sequence of the 3D-known GH68 proteins. Pairwise alignments for the construction of model structures were created with Needleman–Wunsch algorithm using BLOSUM62 as the score matrix, and FF<sub>Zm</sub> and LS<sub>Zm</sub> showed 45.3% and 51.1% sequence identity with the template sequence, respectively. Comparative modeling was done in automodel mode of MODELLER. GA341 and zDOPE in the respective models were 1.0 and ~0.88 (FF<sub>Zm</sub>) and 1.0 and ~0.82 (LS<sub>Zm</sub>), indicating a reliable model. Besides, the 1-kestose and 6-kestose docked to FF<sub>Zm</sub> and LS<sub>Zm</sub> using AutoDock Vina (33), respectively. The docking scores were ~7.5 (1-kestose to FF<sub>Zm</sub>) and ~7.7 (6-kestose to LS<sub>Zm</sub>), respectively. The residues forming -1 and +1 subsites were well conserved, except for His79 FF<sub>Zm</sub> and Ala343 FF<sub>Zm</sub> (Fig. 4A), corresponding to Asn84 LS<sub>Zm</sub> and Ser345 LS<sub>Zm</sub>, respectively (Fig. 4B). His79 FF<sub>Zm</sub> and Asn84 LS<sub>Zm</sub> are at an intervening loop between β1B and βIC of the blade I in 5-bladed β-propeller to participate in the +1 subsite. In the model structure, His79 FF<sub>Zm</sub> possibly forms a hydrogen bond with C6-OH of fructosyl residue on +1 subsite (Fig. 4A). Asn84 LS<sub>Zm</sub> likely forms two hydrogen bonds with C1-OH of fructosyl residues on the -1 and +1 subsites (Fig. 4B). Furthermore, both of Ala343 FF<sub>Zm</sub> and Ser345 LS<sub>Zm</sub>, located on the βVA of the blade V, are in the inner part of active pocket, spatially situating close to Asp44 and Asp48 catalytic nucleophile residues, respectively (Fig. 4, A and B). We anticipated that these divergences were involved in regioselectivity difference, allowing to generate six mutated enzymes: H79N FF<sub>Zm</sub>, A343S-FF<sub>Zm</sub>, H79N/A343S-FF<sub>Zm</sub>, N84H-LS<sub>Zm</sub>, S345A-LS<sub>Zm</sub>, and N84H/S345A-LS<sub>Zm</sub>.

Characterization of mutated enzymes

Identical procedures used for wild-type FF<sub>Zm</sub> and LS<sub>Zm</sub> were performed for characterizing their mutants (Table 1). The specific activities of FF<sub>Zm</sub> variants, measured using 50 mM sucrose, decreased to 31%–17%, whereas those of LS<sub>Zm</sub> derivatives increased to 176%–320%. Among these findings, the latter phenomena are of interest since generally mutated enzyme reduces specific activity. The original specific activity of wild-type FF<sub>Zm</sub> is greater than that of wild-type LS<sub>Zm</sub>, so that the other five mutants display similar values of 270–500 U mg<sup>−1</sup>. S345A-LS<sub>Zm</sub> exhibits the highest value,
Reactions were performed for 10 min at 35°C. F-F-G is 1-kestose or 6-kestose, depending on LS-tertiary complex (E-F and fructose (F)) or to transfructosylation by accepting F-intermediate (E-F). The E-F either proceeds to hydrolysis to degrade into E and trisaccharide (F-F-G). Each experiment was repeated three times, and the mean was plotted (error bar, SD).

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Transfructosylation regioselectivity of LSZm and FFZm variants, S345A-LSZm and S453A-FFZm, which synthesized the same initial products as wild type, decreased in every initial velocity (v_{1-Kes}) and 6-Kes decreased to 43% of the parent enzyme. All variants of LSZm increased v_{1-Kes} but decreased v_{6-Kes}. In comparison with wild type, v_{1-Kes} increased to 170% (S345A-LSZm) of parent value, 190% (N84H-LSZm), and 510% (N84H/S345A-LSZm), whereas v_{6-Kes} did 14% (S345A-LSZm), 0.8% (N84H-LSZm), and 0.4% (N84H/S345A-LSZm). An increase in the transfructosylation ratio of each mutant can be explained by the enhancement of v_{1-Kes}. Among the mutants, N84H/S345A-LSZm, of which [v_{6-Kestose}/(v_{1-kestose} + v_{6-kestose})] value is substantially small (0.14%), is found to be quite similar to FFZm in regard of its trisaccharide production profile (Table 2).

Figure 3, A and B show the late reaction products of LSZm and FFZm variants at 1 and 24 h, respectively. At 1 h, three variants displayed the lack or a decreased production of 6-kestose due to the reduction of v_{6-Kes} at the initial reaction stage (Table 2), in which N84H-LSZm maintained the less formation even at 24 h. While S345A-LSZm still produced neokestose similarly to the wild type, N84H-LSZm and N84H/S345A-LSZm almost lost the formation of this trisaccharide. Besides, all mutations were observed to abolish the ability to generate large DP products (insets of Fig. 3B), probably coincident with the lowered capability to synthesize the β-(2→6)-fructosidic linkage. S345A-LSZm could produce LVOs up to DP10 in trace amounts by maintaining a limited ability to catalyze the successive 6-TF. These results indicate that Asn84LSZm and Ser345LSZm are involved in the 6-TF specificity of LSZm. The regioselectivity can be switched from LSZm-type to FFZm-type by the substitutions of Asn84LSZm and Ser345LSZm with FFZm-type His and Ala, respectively.

**Transfructosylation of FFZm mutants**

FFZm variants (H79N-FFZm, A343S-FFZm, H79N/A343S-FFZm), which synthesized the same initial products as wild type, decreased in every initial velocity (v_{1-Kes}, v_{5-Fru}, and v_{Gluc} (total reaction velocity)). The mutants showed a decreased transfructosylation ratio but an enhanced [v_{6-Kestose}/(v_{1-kestose} + v_{6-kestose})] value (Table 2). These results are attributed to the substantial reduction in v_{1-Kes}. Even H79N-FFZm, which showed the highest value in the mutants, decreased to 5.7% compared with that of the wild type. While v_{6-Kes} decreased to 11% in A343S-FFZm, it increased slightly in H79N-FFZm and H79N/A343S-FFZm. The H79N/A343S-FFZm, of which v_{1-Kes} decreased greatly, demonstrates the notable [v_{6-Kestose}/(v_{1-kestose} + v_{6-kestose})] value (97%), and its regioselectivity almost completely changes from 1-TF to 6-TF.

The late reaction of FFZm mutants at 1 and 24 h is shown in Figure 3, C and D, respectively. At 1 h, H79N-FFZm and A343S-FFZm synthesized both the 6-kestose and 1-kestose. H79N/A343S-FFZm did 6-kestose as the almost sole transfructosylation product similarly to initial reaction, although the synthesis of 6-kestose was not found in the wild type. At 24 h,

800 U mg⁻¹, alternating its catalytic efficiency. Moreover, all mutants were similar to their parent enzymes in regard of their optimum pH and pH-stability range. The thermal stability of LSZm variants is higher than that of their wild type, whereas FFZm derivatives maintain the heat stability of their parent enzyme.

**Transfructosylation of LSZm mutants**

The transfructosylation products of LSZm mutants were identical to those of the wild type. The enzymes yielded 1-kestose and 6-kestose in the initial reaction mixture using 1 M sucrose as substrate. The initial v_Glc, v_Fru, v_{1-Kes}, and v_{6-Kes} are summarized in Table 2. Two mutants, S345A-LSZm and N84H/S345A-LSZm, almost maintained the whole reaction velocities (v_Glc), whereas N84H-LSZm decreased to 43% of the parent enzyme. All variants of LSZm increased v_{1-Kes} but decreased v_{6-Kes}. In comparison with wild type, v_{1-Kes} increased to 170% (S345A-LSZm) of parent value, 190% (N84H-LSZm), and 510% (N84H/S345A-LSZm), whereas v_{6-Kes} did 14% (S345A-LSZm), 0.8% (N84H-LSZm), and 0.4% (N84H/S345A-LSZm). An increase in the transfructosylation ratio of each mutant can be explained by the enhancement of v_{1-Kes}. Among the mutants, N84H/S345A-LSZm, of which [v_{6-Kestose}/(v_{1-kestose} + v_{6-kestose})] value is substantially small (0.14%), is found to be quite similar to FFZm in regard of its trisaccharide production profile (Table 2).

Figure 2, A and B show the late reaction products of LSZm and FFZm from 1 M sucrose. A and B, plots of initial velocity versus [sucrose] for LSZm (A) and FFZm (B). Reactions were performed for 10 min at 35°C and pH 5.0. HPAEC-PAD measured concentrations of saccharides: fructose (open circle), glucose (closed circle), [glucose] (open diamond), and [1-kestose] (closed diamond). Each experiment was repeated three times, and the mean was plotted (error bar, SD). C, reaction scheme. Free enzyme (E) binds to sucrose (F-G) to form the Michaelis-complex (E-F-G), and release glucose (G) to form the second intermediate (E-F). The E-F either proceeds to hydrolysis to degrade into E and fructose (F) or to transfructosylation by accepting F-G to form the tertiary complex (E-F-F-G), which degrades into E and trisaccharide (F-F-G). F-F-G is 1-kestose or 6-kestose, depending on LSZm or FFZm.
Figure 3. Products at the late reaction stages of LSZm, FFZm, and their variants from 1 M sucrose. A and B, LSZm and its variants at 1 h (A) and 24 h (B). C and D, FFZm and its variants at 1 h (C) and 24 h (D). Insets of B and D are the magnified figures to clearly display the long saccharides with a small amount. F, fructose; G, glucose; S, sucrose; N, nystose; NK, neokestose; 1K, 1-kestose; 6K, 6-kestose.
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The relevant positions are conserved as a pair of His and Ala or that of Asn and Ser in the UniRef50_F8DT27 cluster, to which FFZm and LSZm belong. The enzymes having the former pair are predicted to be FF, whereas other enzymes having the latter pair are LS. On the other hand, the relationship between the residue pair and the regioselectivity of transfructosylation is found to be not necessarily conserved in GH68. Numerous GH68 enzymes derived from Gram-negative bacteria have a pair of His and Ser, although atypical for FFZm or LSZm. It has been demonstrated that LSGd and LSEa with this pair produce 1-kestose and nystose in addition to levan. Their reaction specificities are more similar to that of LSZm having a pair of Asn and Ser than that of A343S-FFZm and N79H-LSZm, which possess a pair of His and Ser. A further atypical example is the LS from Gram-positive bacteria, such as LSbc and Bacillus megaterium LS (LSbm), which have Ser at the second position of pair residue, corresponding to Ala343FFZm and Ser345LSZm; however, neither LSbc nor LSbm has a comparable residue, because the loop, carrying the relevant residue, is short. These LSs have been reported to possess another structural factor involved in the regioselectivity, at which a substitution of Arg370LSbm with Gln is observed to increase 1-kestose synthesis (34). These concrete examples suggest a diversity of the relationship between structure and specificity in GH68 enzymes.

the neokestose production was detected at H79N-FFZm and A343S-FFZm but was hardly observed in the H79N/A343S-FFZm. A little amount of tetrasaccharide nystose was produced by H79N-FFZm, but H79N/A343S-FFZm did not.

These results indicated that His79FFZm and Ala343FFZm are involved in the regioselectivity of 1-TF of FFZm. The double mutation using two LSZm-type residues enables FFZm to synthesize the 6-kestose. However, FFZm variants, gaining the 6-TF ability similarly to LSZm, do not efficiently generate long LVOs with high DPs and levan, as compared with the parent LSZm, which can do (inset of Fig. 3B). Only the H79N/A343S-FFZm can produce an exceedingly small amount of LVOs having DP7 or less (Fig. 6, described below).

Functions of mutated residues

Our mutational study reveals that the position at the His79FF or at its equivalent Asn84LS is involved in the regioselectivity for 1-TF of FFZm or 6-TF of LSZm, respectively. The model structures suggest that His79FFZm and Asn84LSZm form hydrogen bonds with the hydroxy groups of 1-kestose and 6-kestose, respectively (Fig. 4, A and B). Introduction of Asn at His79FFZm allows the formation of the hydrogen bond suitable to produce 6-kestose. Contrarily, the replacement of Asn84LSZm with His may enable to form the hydrogen bonds suitable for 1-kestose synthesis. Besides, Ala343FF and Ser345LS are also concerned in the regioselectivity of the transfructosylation, although their mutations affect modestly rather than those of His79FFZm and Asn84LSZm. It is hard to interpret how the Ala343FFZm and Ser345LSZm contribute to the selectivity because these residues cannot contact directly to the substrate or product from each position, that is, the inner part of catalytic pocket (Fig. 4, A and B). However, the mutations of Ala343FFZm and Ser345LSZm are able to alter the orientation of the side chains of adjacent amino acid residues, such as Tyr342FFZm and Tyr344LSZm, both of which lie at the +1 subsite. The alteration could modify the architecture of the +1 subsite, influencing the transfructosylation process containing regioselectivity.

Figure 4. Structural models of FFZm and LSZm with ligands of 1-kestose (A, C), 6-kestose (B, D), and Fru-Man (E). A and B, catalytic pocket structures of FFZm (A) and LSZm (B) with 1-kestose and 6-kestose, respectively. Predicted hydrogen bonds are represented by orange dashed line between the mutation-candidate residues and ligands. C and D, structures around Leu187FF and 2-OH of mannosyl residue. For all panels, Modeller constructed model structures of FFZm and LSZm. Autodock Vina docked 1-kestose or Fru-Man to FFZm as well as 6-kestose, 1-kestose, or Fru-Man to LSZm.
Levan synthesis using FFzm mutants

While H79N/A343S-FFzm can catalyze the 6-TF, this mutant is devoid of synthesizing levan-type polysaccharides (Fig. 3D). Goldman et al. demonstrated that the levan production of Z. mobilis LS from ATCC10988 was related to its protein form depending on the pH (27). Below pH 6.0, the enzyme formed the well-ordered microfibril-shaped structure and synthesized levan, allowing us to conduct the levan-generation at pH 3.7–7.5 using H79N/A343S-FFzm together with LSzm and FFzm as controls (LSzm as a positive control and FFzm as a negative control). The analysis using thin-layer chromatography (TLC) showed that LSzm produced levan and/or long LVOs at any pH, although such saccharides were not generated by FFzm and H79N/A343S-FFzm (Fig. 5). As shown by Goldman et al. (27), the levan synthesis of LSzm is more efficient at a lower pH. The pH is unrelated to the H79N/A343S-FFzm-associated levan formation, suggesting that this mutant possesses further essential factor(s).

The structural factors of LSbspp for levan synthesis are well-studied (16, 34–37), mentioning that Tyr247LSBm, Asn252LSBm, Asp257LSBm, Arg370LSBm, and Lys373LSBm (the residue numbers are of LSBm) were responsible for levan generation. Among them, the position of Asn252LSBm is found to be divergent at FFzm and LSzm. Other residues are conserved in FFzm (and also LSzm), probably without being involved in the elongation by 6-TF. Asn252LSBm, locating at a surface loop to connect blades II and III of 5-bladed β-propeller, is conserved in LSbspp, although it varies in other GH68 enzymes. In FFzm and LSzm, Leu187FFzm and Phe189LSzm are equivalent to Asn252LSBm (Fig. 4, C and D). In addition, Phe189LSzm seems situated at a possible subsite far from −1 and +1 subsite of LSzm, based on its model structure. We thus anticipated that the divergence of Leu187FFzm and Phe189LSzm is concerned with the ability (LSzm) or inability (FFzm) of levan synthesis.

H79N/L187F/A343S-FFzm was generated using H79N/A343S-FFzm as a parent enzyme to examine its ability in association with the levan synthesis by the substitution of Leu187FFzm with Phe. The specific activity of H79N/L187F/A343S-FFzm (C) was incubated with various [sucrose] (50, 100, 200, 400, 600, 800, and 1000 mM) in 30 mM sodium acetate buffer (pH 5.0) containing 0.01% Triton X-100 at 37 °C for 24 h, followed by analysis using HPAEC-PAD. Numbers on peaks are DP of their saccharides.

Figure 5. TLC analysis of levan- and long LVO-formation by LSzm, FFzm and H79N/A343S-FFzm. LSzm, FFzm and H79N/A343S-FFzm were incubated with 1 M sucrose at pH 3.7, 5.0, 7.0, and 7.5. Reaction was performed at 37 °C for 24 h. A spot of levan and quite long LVO appears at original position due to their large DP.

Figure 6. Long LVO formation of H79N/L187F/A343S-FFzm. A, LSzm; (B) H79N/A343S-FFzm; (C) H79N/L187F/A343S-FFzm. LSzm (A), H79N/A343S-FFzm (B), and H79N/L187F/A343S-FFzm (C) were incubated with various [sucrose] (50, 100, 200, 400, 600, 800, and 1000 mM) in 30 mM sodium acetate buffer (pH 5.0) containing 0.01% Triton X-100 at 37 °C for 24 h, followed by analysis using HPAEC-PAD. Numbers on peaks are DP of their saccharides.
showed similarity in optimum pH and pH-stability, while heat stability was slightly decreased. The initial reaction velocities (Table 2) indicated that the \( v_{\text{Glc}} \) (meaning a total reaction velocity), \( v_{\text{Fru-Man}} \) as well as fructosylation ratio increased compared with those of H79N/A343S-FFZm. The \( [v_{\text{Fru-Man}}/(v_{\text{Fru-Man}} + v_{\text{Fru-Man}})] \) value was almost the same as that of H79N/A343S-FFZm, meaning that triple mutant maintains 6-GT regioselectivity. 

**Table 3**
| Enzyme                | \( v_{\text{Fru-Man}} \) (s\(^{-1}\)) | Yield (mM) |
|-----------------------|----------------------------|------------|
| FFZm                  |                            |            |
| Wild type             | 105                        | 60         |
| H79N-FFZm             | 14                         | 18         |
| A343S-FFZm            | 7.2                       | 29         |
| H79N/A343S-FFZm       | 2.7                       | 16         |
| LSZm                  |                            |            |
| Wild type             | 3.6                       | 20         |
| N84H-LSZm             | 8.8                       | 24         |
| S345A-LSZm            | 6.1                       | 19         |
| N84H/S345A-LSZm       | 26                        | 30         |

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Below. Oligosaccharides that were further synthesized included nystose, 1-kestose, and two kinds of unknown disaccharides, the structures of which were almost identified with associated results appearing elsewhere. Using 0.5 M sucrose as a donor substrate and 1.0 M mannose as an acceptor substrate, we measured the initial velocity of Fru-Man synthesis (\( v_{\text{Fru-Man}} \) (s\(^{-1}\); abbreviated as \( v_{\text{FM}} \)) and the yield of Fru-Man at 24 h of late reaction (Table 3). FFZm shows by far the highest \( v_{\text{FM}} \), 105 s\(^{-1}\), whereas the \( v_{\text{FM}} \) values of FFZm mutants are 2.6–13% of FFZm \( v_{\text{FM}} \); and the \( v_{\text{FM}} \) values of LSZm and its mutants (except for N84H/S345A-LSZm having 25%) are 3.4–8.4% of FFZm \( v_{\text{FM}} \). While LSZm variants carry low \( v_{\text{FM}} \), values, these are 1.7–7.2-times larger than wild-type LSZm does. Besides, the concentration of Fru-Man produced at 24 h became 60 mM (Table 3), which means that 12% of sucrose is converted to be Fru-Man. This is the greatest yield among the enzymes tested, as other enzymes generate 27–50% compared with that of FFZm.

Seibel *et al.* (28) proposed that the LSBc-associated small yield of Fru-Man was in association with the reduction of catalytic efficiency due to the deprivation of the hydrogen bond between general acid/base catalyst (Glu342\(^{\text{LSBc}}\) and axial 2-OH of mannose. Since the spatial position of the general acid/base would be conserved in the GH68 members, the Fru-Man-generating FFZm also forms this hydrogen bond, suggesting that LSZs has unknown factor(s) to reduce Fru-Man yield. It is assumed that a reason why FFZm can synthesize Fru-Man is due to the His79\(^{\text{FFZm}}\) to stabilize the Fru-Man synthetic reaction. The model structure of FFZm docking Fru-Man appears to depict the interaction between His79\(^{\text{FFZm}}\) and the axial 2-OH of mannose (Fig. 4E), indicative of the possible significance of His79\(^{\text{FFZm}}\). The fact that Fru-Man syntheses are decreased by both H79N-FFZm and H79N/A343S-FFZm, in which His79\(^{\text{FFZm}}\) is replaced, whereas the yields are inversely increased by N84H-LSZm and N84H/S345A-LSZm, which newly acquire His79\(^{\text{FFZm}}\), supporting the proposed interpretation. As previously mentioned, LSBSpp does not have a residue corresponding to His79\(^{\text{FFZm}}\). This absence might be one reason for LSBC to accept a small amount of mannose into its catalytic site. On the other hand, it is difficult to explain that A343S-FFZm and S345A-LSZm alternate the Fru-Man production (\( v_{\text{FM}} \) and yield) in comparison with their wild types, because, as mentioned earlier, Ala343\(^{\text{FFZm}}\) and Ser345\(^{\text{LSZm}}\) do not directly contact with the substrate sucrose. Similarly to the changes in regioselectivity, the orientation of the adjacent Tyr residue, Tyr342\(^{\text{FFZm}}\) and Tyr344\(^{\text{LSZm}}\), may be responsible for the changes in Fru-Man synthesis.

**Conclusion**

We have truly realized how important the analysis of the paralogous relationship in the field of protein engineering through the present study. Generally, one of the two paralogous proteins is thought to evolve independently and uniquely because its gene has undergone...
relatively less restricted indels and substitutions. Therefore, the study of paralogous proteins is equivalent to the analysis of the results of protein engineering in nature and often provides important insights into the structure–function relationship of a protein. Based on this background, we have identified His79FFZm and Ala343LSZm and their respectively corresponding Asn84LS and Ser345LSZm as the amino acid residues associated with the regioselectivity in transfructosylation. We also demonstrated that Phe189LSZm is fairly involved in the elongation of the fructosyl residues in levan synthesis by analyzing an active fraction. For the expression of LSZm, both enzymes were present with sufficient specific activity. This may be because Z. mobilis is only able to utilize sucrose as a disaccharide, and the enzymes engaged in sucrose degradation may be able to have evolved without losing their function.

**Experimental procedures**

**Gene cloning, production, and purification of FFZm and LSZm**

Genes of ZZ6_0874 and ZZ6_0875 locus_tags, respectively encoding FFZm and LSZm, were amplified from the chromosomal DNA of Z. mobilis NBRC 13756 (ATCC 29191) by PCR using primers: for ZZ6_0874, [5’-GGCGAGCTCTT-3’ (sense); SacI-site underlined] and 5’-GAGTGTAGTTTTGCAGCAGAT-3’ (antisense; XbaI-site underlined); and for ZZ6_0875, [5’-CTACACATGTGATTTATAAGCGGACTG-3’ (sense); PciI-site underlined] and 5’-AAACTCGAGTCACAGCCAGGCCTG-3’ (antisense; XhoI-site underlined). The primers were designed based on the published whole genome sequence of Z. mobilis ATCC 29191 (accession number, CP003704.1). After amplification using PrimeSTAR MAX DNA polymerase (Takara Bio), a PCR product for FFZm was restricted with SacI and XbaI and cloned into appropriately digested pColdI DNA (Takara Bio). The completed plasmid DNA was named pColdFFZm. A PCR product for LSZm was digested with PciI and XhoI and ligated to pET23 days (+) DNA (Novagen-Merck Millipore) digested with Ncol and XhoI. The resulting plasmid DNA was called pET23-LSZm.

For the expression of FFZm, *Escherichia coli* BL21(DE3) cells harboring pCold-FFZm were cultured in Luria–Bertani medium (10 g L\(^{-1}\) Bacto tryptone, 5 g L\(^{-1}\) Bacto yeast extract, and 5 g L\(^{-1}\) NaCl) supplemented with 0.1 g L\(^{-1}\) ampicillin at 37 °C until reaching mid-exponential phase (OD600 \(\sim\) 0.4), followed by cooling on ice. To culture the 2500 \(\mu\)L volume of 0.5 M isopropyl β-thiogalactoside was added, and cells were grown in a shaking incubator at 15 °C for 24 h. For the expression of LSZm, *E. coli* BL21(DE3) cells harboring pET23-LSZm were cultured by shaking in a auto-inducing medium (40) [10 g L\(^{-1}\) Bacto tryptone, 5 g L\(^{-1}\) Bacto yeast extract, 50 mM Na\(_2\)HPO\(_4\), 50 mM KH\(_2\)PO\(_4\), 25 mM (NH\(_4\))\(_2\)SO\(_4\), 2 mM MgSO\(_4\), 5 g L\(^{-1}\) glycerol, 5 g L\(^{-1}\) glucose, and 2 g L\(^{-1}\) lactose] supplemented with 0.1 g L\(^{-1}\) ampicillin at 30 °C for 24 h. The cells bearing FFZm or LSZm were harvested by centrifugation (5000g, 5 min, 4 °C), resuspended in 20 mM sodium phosphate, 300 mM NaCl, pH 8.0 (buffer A), and disrupted by sonication. The supernatant was obtained by centrifugation (20,000g, 20 min, 4 °C).

Recombinant proteins were purified from the above supernatant, loaded onto a Ni\(^{2+}\)-chelating Sepharose Fast Flow column (GE healthcare), which was prepared according to the instructions of manufacturer and pre-equilibrated with buffer A. After washing the column with buffer A containing 20 mM imidazole, the absorbed proteins were quickly eluted with 300 mM imidazole in buffer A, and then the pH of effluent immediately changed to pH 6.0 for FFZm or to pH 5.0 for LSZm. Active fractions were passed through a Bio-Gel P-6 (Bio-Rad) column pre-equilibrated with 20 mM sodium phosphate (pH 6.0) for FFZm or with 20 mM sodium acetate (pH 5.0) for LSZm. The obtained fractions were pooled and concentrated using a 30 kDa MWCO Vivasin 20 (Sartorius AG). The concentration of purified enzyme ([enzyme]) was estimated by amino acid analysis of protein hydrolysate (6 M HCl, 110 °C, 24 h) using an Amino Acid Analyzer L-8900 (Hitachi High-Tech) equipped with a ninhydrin-detection system.

**Construction of model 3D protein structures**

Model 3D structures were prepared using the Modeller (41), Dock prep (31), and Autodock Vina (33) functions of UCSF Chimera version 1.14 (http://www.rbvi.ucsf.edu/chimera) (42). A template structure (PDB identifier, 4D47) was selected by Sequence Navigator server (https://pdbs.org/seqNavi). All model building was done with the default settings of the software. The GHECOM 1.0 server (https://pdbj.org/ghecom/) (43, 44) was used to detect the cavity in the docking simulation. Figures showing 3D structures were prepared using open-source PyMol (The PyMOL Molecular Graphic System version 2.4, Schrödinger, LLC). Sequence alignments were generated with T-coffee Expresso (http://tcoffee.crg.cat/apps/tcoffee/do: expresso) (45) or PROMALS3D (http://prodata.swmed.edu/promals3d/promals3d.php) (46).

**Site-directed mutagenesis**

Point mutations were introduced using the PrimeSTAR mutagenesis basal kit (Takara Bio). The oligonucleotides used for each mutation were as follows: for H79N-FFZm, [5’-GATGGCAATTTCTCATGCGATCGGC-3’ (sense); substituted codon in boldface] and 5’-ATGAGATTGGCATATCTCCAGGAAT-3’ (antisense); for A343LSZm, [5’-GGCTATCCCATACATGGAAT-3’ (sense) and 5’-GTAATGGAATAGGCTCTGCGGCTG-3’ (antisense)]; for N84H-LSZm, [5’-ATCTGCAATGTTGGCAGCGCCAGTGTC-3’ (sense) and 5’-GCCATCTCCAGGAATCTCGCTG-3’ (antisense)]; for S345A-LSZm, [5’-GCTTATGCATTTTATATATGCTAAAT-3’ (sense) and 5’-ATATATGGCTTAAGGCTGAGCTG-3’ (antisense)]; for L187F-FFZm, [5’-ATAAATTTCCGGAATTTCCGATCTCCT-3’ (sense) and 5’-ATCTTATATATATGCTCAAT-3’ (antisense)].
TTCTGCATAATT-3’ (antisense)). The mutated enzymes were purified by the aforementioned procedures.

**Enzyme assay and characterization**

Enzyme activity was measured at 35 °C in a standard reaction mixture consisting of 50 mM sucrose (Nacalai Tesque), 30 mM sodium acetate buffer (pH 5.0), 0.01% Triton X-100, and appropriately diluted enzyme solution. Reaction was terminated by heating at 100 °C for 3 min. After adjusting pH to 7.0 by adding the double amount of 2 M Tris-HCl (pH 7.0), the concentration of liberated glucose in the reaction mixture was measured by a Glucose CII Test Wako (FUJIFILM Wako Pure Chemical). One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzed the consumption of 1 μmol sucrose per minute under the above standard conditions.

pH-Dependent reaction velocities for identifying optimal pHs of FFZm, LSZm, and their variants were measured at 50 mM sucrose in the McIlvaine buffer (pH 3.9–7.0) containing 0.01% Triton X-100 at 35 °C. The [enzyme] used was specified for FFZm (2.1 nM), LSZm (2.0 nM), H79N–FFZm (4.8 nM), A343S–FFZm (4.0 nM), H79N/A343S–FFZm (19 nM), H79N/L187F/A343S–FFZm (4.2 nM), N84H–LSZm (5.3 nM), S345A–LSZm (0.34 nM), and N84H/S345A–LSZm (2.6 nM). pH-Stable region was evaluated as follows: the enzyme (FFZm [11 nM], LSZm [10 nM], H79N–FFZm [29 nM], A343S–FFZm [25 nM], H79N/A343S–FFZm [46 nM], H79N/L187F/A343S–FFZm [52 nM], N84H–LSZm [27 nM], S345A–LSZm [9.0 nM], or N84H/S345A–LSZm [13 nM]) was incubated with McIlvaine buffer (pH 2.7–7.0) for 24 h at 4 °C, and then the residual activity was determined at pH 5.0 according to the standard procedure described previously. To estimate the thermal stability of the enzymes, we incubated the enzyme (FFZm [11 nM], LSZm [10 nM], H79N–FFZm [36 nM], A343S–FFZm [12 nM], H79N/A343S–FFZm [28 nM], H79N/L187F/A343S–FFZm [32 nM], N84H–LSZm [16 nM], S345A–LSZm [5.1 nM], or N84H/S345A–LSZm [7.8 nM]) containing 33 mM sodium acetate buffer (pH 5.0) and 0.01% Triton X-100 at 30–70 °C for 15 min, followed by measurement of the residual activity under the standard conditions described above.

**HPAEC-PAD analysis**

HPAEC-PAD was performed with a Dionex ICS-3000 system (Dionex/Thermo Fisher Scientific) using an analytical column (CarboPac PA1, 4 mm I.D. x 250 mm; Dionex/Thermo Fisher Scientific) and a flow rate of 0.8 ml min⁻¹. The eluent system comprised MilliQ water (a), 0.8 M NaOH prepared from super special grade 50% NaOH solution (FUJIFILM Wako Pure Chemical Co) (b), and 1.0 M sodium acetate (c), in which two procedures were used, namely method A and method B, as described below. In the method A, the products were separated by the elution system comprising the first linear gradient from 97.2:2.8:0 (% a:b:c) to 83.8:16.2:0 (% a:b:c) for 10 min and the second linear gradient from 83.8:16.2:0 (% a:b:c) to 65.9:24.1:10 (% a:b:c) for 6 min, followed by isocratic elution at 65.9:24.1:10 (% a:b:c) over 7 min. In the method B, the products were separated by a linear gradient from 87.5:12.5:0 (% a:b:c) to 51.5:12.5:36 (% a:b:c) for 25 min, followed by isocratic elution at 51.5:12.5:36 (% a:b:c) over 5 min. Chromatograms were evaluated with Chromeleon software (Dionex/Thermo Fisher Scientific). The concentration of glucose, fructose, 1-kestose, 6-kestose, or neokestose was calculated using a calibration curve prepared based on the ration between chromatographic peak area of sorbitol as an internal standard and that of concentration-known saccharide standard. Neokestose was the kind gift from Prof Onodera S (Rakuno Gakuen University, Ebetsu, Japan).

**Initial transfructosylation**

For the measurement of $v_{\text{Glc}}$, $v_{\text{Fru}}$, $v_{1-\text{Kest}}$, and $v_{6-\text{Kest}}$, the reaction was done by incubating FFZm (4.6 nM) or LSZm (7.3 nM) with sucrose (0.025, 0.05, 0.1, 0.2, 0.3, 0.4, and 1.0 M) and 30 mM sodium acetate buffer (pH 5.0) containing 0.01% Triton X-100 at 35 °C for 10 min, followed by terminating reaction using heat treatment at 100 °C for 3 min. We also measured $v_{\text{Glc}}$, $v_{\text{Fru}}$, $v_{1-\text{Kest}}$, and $v_{6-\text{Kest}}$ against 1.0 M sucrose using FFZm, LSZm, and their variants ([enzyme], 30 to 800 nM) in the same way. The concentration of carbohydrates was determined by HPAEC-PAD using method A. For the measurement of $v_{\text{Fru-Man}}$, 0.5 M sucrose and 1.0 M mannose were used as substrates, and the used [enzyme] was 2.2 μM (FFZm), 16 μM (LSZm), 2.4 μM (H79N–FFZm), 5.8 μM (A343S–FFZm), 9.3 μM (H79N/A343S–FFZm), 2.7 μM (N84H–LSZm), 6.8 μM (S345A–LSZm), or 2.6 μM (N84H/S345A–LSZm). Other experimental conditions were the same as those described above.

**Late transfructosylation at 1 h and 24 h**

For the qualitative analysis of the products of the transfructosylation of FFZm, LSZm, and their variants using 1.0 M sucrose, the reaction was performed at 35 °C and pH 5.0 for up to 24 h in 30 mM sodium acetate buffer (pH 5.0). The [enzyme] was 0.03 μM (FFZm), 0.34 μM (LSZm), 0.13 μM (H79N–FFZm), 1.3 μM (A343S–FFZm), 0.64 μM (H79N/A343S–FFZm), 0.44 μM (H79N/L187F/A343S–FFZm), 0.38 μM (N84H–LSZm), 0.24 μM (S345A–LSZm), or 0.26 μM (N84H/S345A–LSZm). At 1 and 24 h, a part of the reaction mixture was recovered, heated at 100 °C for 3 min to terminate the reaction, and diluted to 100⁻¹ with 10 mM sorbitol of internal standard. The reaction products were analyzed by HPAEC-PAD. Resultant reaction samples at 1 h and 24 h were analyzed by methods A and B, respectively.

For investigating the changes in the long LVO production of LSZm, H79N/A343S–FFZm and H79N/L187F/A343S–FFZm, we incubated each enzyme with different [sucrose] bearing 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, or 1.0 M at 35 °C and pH 5.0 for 24 h in 40 mM sodium acetate buffer (pH 5.0), followed by heating at 100 °C for 3 min. The reaction mixture was diluted with sorbitol solution and analyzed by HPAEC-PAD using method B.
Transfructosylation regioselectivity of GH68 enzymes

For the Fru-Man synthesis, the reaction of FFZm (2.2 μM), LSZm (16 μM), H79N-FFZm (2.4 μM), A3433S-FFZm (5.8 μM), H79N/A3435S-FFZm (9.3 μM), N84H-LSZm (2.7 μM), S345A-LSZm (6.8 μM), or N84H/S345A-LSZm (2.6 μM) was performed with 0.5 M sucrose and 1.0 M mannose at 35 °C and pH 5.0 for 24 h, and was subsequently terminated by heating at 100 °C for 3 min. A sample for HPAEC-PAD analysis using method A was prepared by 100⁻¹-dilution of reaction mixture with 10 mM sorbitol.

pH-influence on production of long LVO and levan

For analyzing the synthesis of long LVO and levan at the different pHs, we incubated H79N/A3433S-FFZm, FFZm, or LSZm (5 U mL⁻¹ for each enzyme) with 1.0 M sucrose in McIlvaine buffer (pH 3.7, 5.0, 7.0, and 7.5) for 24 h at 35 °C. After heat treatment at 100 °C for 3 min, the reaction mixture was appropriately diluted in deionized water and analyzed by TLC using a TLC-plate (0.25-mm layers of silica gel 60 F254 (Merck Millipore)). The separation of carbohydrates was done with 10 mM sorbitol. After heat treatment at 100 °C for 3 min, the reaction mixture was subjected to gel filtration chromatography and purified.

Production of 6-kestose and Fru-Man

This research required the preparation of an appropriate amount of standard 6-kestose. For this, we did the 10-mllvel reaction of 37 U mL⁻¹ H79N/A3433S-FFZm with 1.0 M sucrose at 35 °C and pH 5.0 for 2 h. Reaction solution was desalted by Amberlite MB-4 resin (Organo), and then concentrated to 2 ml under reduced pressure. The reaction products were separated by an HPLC system (Jasco) equipped with a refrective index monitor (Jasco RI-2031) with a Cosmosil Sugar-D column (10 mm I.D. x 250 mm; Nacalai Tesque). Separation was achieved using a mobile phase (acetonitrile:water, 80:20, v/v) with a flow rate of 3 ml min⁻¹. For Fru-Man synthesis, 1.25 M sucrose and 2.5 M mannose with 25 U mL⁻¹ FFZm in 10 ml of 40 mM sodium acetate buffer were incubated at 35 °C for 6 h. The reaction mixture was divided into three equal parts and was fractionated by gel filtration chromatography using Bio-Gel P-2 Gel (1.6 cm I.D. x 190 cm, Bio-Rad). Fractions containing Fru-Man were recovered and concentrated under reduced pressure. Reducing sugars were decomposed by the incubation with 2 M NaOH at 100 °C for 10 min. The reaction solution was neutralized by adding Amberlite IR120 H⁺ form and decolorized by activated carbon. The resulting samples were again subjected to gel filtration chromatography and purified Fru-Man was obtained. The molecular masses of oligosaccharide products were confirmed by ESI-MS using a Thermo Scientific Exacta spectrometer (Thermo Fisher Scientific, Inc.). ¹³C-NMR (126 MHz) spectra were recorded on a Bruker AVANCE I (Bruker). The relative values of the chemical shifts for 6-kestose and Fru-Man were in agreement with those previously reported (47, 48). 6-Kestose: ¹³C-NMR (126 MHz, D₂O) δ (ppm): 106.6 (C-2', C-2''), 95.0 (C-1), 84.0 (C-5'), 83.1 (C-5'), 79.3 (C-3'), 79.0 (C-3'), 77.5 (C-4'), 77.2 (C-4'), 75.4 (C-3), 75.2 (C-5), 73.9 (C-2), 72.1 (C-4), 65.8 (C-6'), 65.4 (C-6'), 64.0 (C-1'), 63.1 (C-6), 62.6 (C-1'), MS: m/z calculated: 527.16 [M+Na⁺], 503.16 [M – H⁻]; found: 527.16, 503.16. Fru-Man: ¹³C-NMR (126 MHz, D₂O) δ (ppm): 104.8 (C-2'), 94.4 (C-1), 82.2 (C-5'), 76.8 (C-3'), 74.7 (C-4'), 74.1 (C-2), 71.9 (C-3), 71.0 (C-5), 67.3 (C-4), 63.3 (C-6'), 61.8 (C-1'), 61.5 (C-6); MS: m/z calculated: 365.11[M+Na⁺], 341.11 [M – H⁻]; found: 365.11, 341.11.

Data availability

The authors indicate that all of the data is contained within the article.

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Author contributions—M. O., T. T., and A. K. conceived and coordinated the study. M. O., R. S., M. T., A. K., J. S., and W. L. performed the experiments and analyzed data. M. O. and A. K. created the manuscript. M. O. and A. K. provided essential equipment and facilities. M. O. and R. S. contributed equally. All the authors reviewed the results and approved the final version of this article.

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Abbreviations—The abbreviations used are: DP, degree of polymerization; EaLsc, levanansucrase from Erwinia amylovora; FF, β-fructofuranosidase; FFZm, Zymomonas mobilis FF; FOS, fructooligosaccharides; Fru-Man, β-D-fructofuranosyl β-D-mannopyranoside; GH, glycoside hydrolase family; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; INO, inulooligosaccharide; LS, levansucrase; LSO, levansucrase from Bacillus subtilis LS; LS64, Bacillus megaterium LS; LS65, Erwinia amylovora LS; LSGd, Gluconacetobacter diazotrophicus LS; LSZm, Zymomonas mobilis LS; LVO, levanooligosaccharide; TLC, thin-layer chromatography; vFm, vFru-Man [E]₀¹; vFru [fructose [E]₀¹; vGlc [glucose [E]₀¹; v1-kestose [1-kestose [E]₀¹; v6-kestose [6-kestose [E]₀¹; 3D, three-dimensional structure; 1-FF, β-(1→2)-transfructosylation; 6-FF, β-(1→6)-transfructosylation.

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