Crystal Structures of *Aspergillus japonicus* Fructosyltransferase Complex with Donor/Acceptor Substrates Reveal Complete Subsites in the Active Site for Catalysis*[^S^]

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Fructosyltransferases catalyze the transfer of a fructose unit from one sucrose/fructan to another and are engaged in the production of fructooligosaccharide/fructan. The enzymes belong to the glycoside hydrolase family 32 (GH32) with a retaining catalytic mechanism. Here we describe the crystal structures of recombinant fructosyltransferase (AJFT) from *Aspergillus japonicus* CB05 and its mutant D191A complexes with various donor/acceptor substrates, including sucrose, 1-kestose, nystose, and raffinose. This is the first structure of fructosyltransferase of the GH32 with a high transfructosylation activity. The structure of AJFT comprises two domains with an N-terminal catalytic domain containing a five-blade β-propeller fold linked to a C-terminal β-sandwich domain. Structures of various mutant AJFT-substrate complexes reveal complete four substrate-binding subsites (−1 to +3) in the catalytic pocket with shapes and characters distinct from those of clan GH-J enzymes. Residues Asp-60, Asp-191, and Glu-292 that are proposed for substrate recognition, general acid/base catalyst, respectively, govern the binding of the terminal fructosyl at the −1 subsite and the catalytic reaction. Mutants D60A, D191A, and E292A completely lost their activities. Residues Ile-143, Arg-190, Glu-292, Glu-318, and His-332 combine the hydrophobic Phe-118 and Tyr-369 to define the +1 subsite for its preference of fructosyl and glucosyl moieties. Ile-143 and Gln-327 define the +2 subsite for raffinose, whereas Tyr-404 and Glu-405 define the +2 and +3 subsites for inulin-type substrates with higher structural flexibilities. Structural geometries of 1-kestose, nystose and raffinose are different from previous data. All results shed light on the catalytic mechanism and substrate recognition of AJFT and other clan GH-J fructosyltransferases.

Fructans are sugars derived from sucrose consisting of a common glucose moiety and several fructose units. Fructans attract interest because of their physiological characteristics, such as preventing dental caries and colon cancer, selectively stimulating the growth of bifidobacteria and lactobacilli, decreasing total cholesterol and triacylglycerol lipids in blood serum, and promoting the resorption of calcium and magnesium ions (1–5). Fructans are widespread in flowering plants, bacteria, and a few fungi (6). In plants, besides their functions as a reserve of carbohydrates, fructans are suggested to be involved in enhancing tolerance to drought and freezing by stabilizing the cellular membranes (7–9). The fructans in bacteria have been postulated to be involved in symbiosis and phytopathogenesis (10). Fructans of various origins are differentiated according to the degree of polymerization (DP),[^3^] the type of linkage between adjacent fructose units, the presence of branches, and the position of glucose. Fructans in bacteria generally with a DP up to 10[^4^] contain the β-(2→6)-linked levans and β-(2→1)-linked inulin that are converted from sucrose by levansucrases and inulosucrases, respectively. In contrast, fructans in plants exhibit diverse structures, which result from a combination of catalytic actions with various enzymes, including sucrosesucrose:1-fructosyltransferase (1-SST), fructan:fructosyltransferase (1-FFT), frucutan:6G-fructosyltransferase (6G-FFT), and sucrose:6G-fructosyltransferase; GH, glycoside hydrolase; FT, fructosyltransferase; 1-SST, sucrose:1-fructosyltransferase; 1-FFT, fructan:fructosyltransferase; 6G-FFT, fructan:6G-fructosyltransferase; 6-SFT, sucrose:6-fructosyltransferase; FOS, fructooligosaccharide; 1-FEH Ia, fructan 1-exohydrolase Ia; aCINV, *Allium cepa* invertase; PEG, polyethylene glycol; NSRRC, National Synchrotron Radiation Research Center; r.m.s.d., root mean square deviation; WT, wild type.

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[^3^]: The abbreviations used are: DP, degree of polymerization; AJFT, A. japonicus fructosyltransferase; GH, glycoside hydrolase; FT, fructosyltransferase; 1-SST, sucrose:1-fructosyltransferase; 1-FFT, fructan:fructosyltransferase; 6-GFT, fructan:6-G-fructosyltransferase; 6-SFT, sucrose:6-fructosyltransferase; FOS, fructooligosaccharide; 1-FEH Ia, fructan 1-exohydrolase Ia; aCINV, *Allium cepa* invertase; PEG, polyethylene glycol; NSRRC, National Synchrotron Radiation Research Center; r.m.s.d., root mean square deviation; WT, wild type.

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Fructan 6-fructosyltransferase (6-SFT) (6–8). Fructans in fungi are mainly fructooligosaccharides (FOS), composed of 1-kestose (DP 3), nystose (DP 4), 1'-fructofuranosyl nystose (DP 5), which are β-(2→1)-linked linear fructans produced by fructosyltransferase or β-fructofuranosidase (5, 11). Among them FOS with DP 3–6 create most interest because of their significant beneficial effects to human beings. The related enzymes have attracted industrial attention for the mass production of FOS.

Fructosyltransferases (FTs, EC 2.4.1.9) expressed in species of Aspergillus, Penicillium, Aureobasidium, and Kluyveromyces are the most studied fungal FT enzymes (5, 12–15). Fungal FT act on sucrose by cleaving the β-(2→1) linkage, releasing glucose, and then transferring the fructosyl group to an acceptor molecule. The natural acceptor substrates of fungal FT include sucrose, 1-kestose, nystose, and raffinose. Previous authors showed that raffinose could serve as a less preferable donor molecule. The natural acceptor substrates of fungal FT include sucrose, 1-kestose, nystose, and raffinose. Previous authors showed that raffinose could serve as a less preferable donor molecule.

The FT from Aspergillus japonicus possesses both hydrolytic and transfructosylating activities. For sucrose at concentration of >100 mM, FT exhibit almost an exclusive transfructosylation activity (12–14).

Fungal and plant FT, along with fructan-degrading enzymes, such as invertases, β-fructofuranosidases, and fructan exohydrolases, are classified in the glycoside hydrolase (GH) family 32 (GH32) based on the similarities of their amino acid sequences (CAZy, available on-line), whereas bacterial FT, such as levansucrases and inulosucrases, are classified into GH family 68 (16). Enzymes of both families GH32 and GH68 comprise clan GH-J according to their folding similarities. In this clan there are six three-dimensional structures available, including four enzymes from microbial origins and two from plants (17): levansucrase and its mutants from Aspergillus awamori (2QQW, 2QQU, and 2QQV) (24), exoinulinase from Cichorium intybus (1UYP and 1W2T) (21, 22), cell-wall invertase (2AC1) (23) and its mutant complexes with sucrose from Arabidopsis thaliana (2QQW, 2QQQ, and 2QQV) (24), exoinulinase from Aspergillus awamori (1W4Y and 1Y9G) (25), and fructan 1-exohydrolase IIa (1-FeH IIa) from Cichorium intybus and its mutant complexes with various substrates (1ST8, 2ADD, and 2AEZ) (26, 27).

No three-dimensional structure of a typical inulin-type FT has been reported. We present here the first crystal structure of Aspergillus japonicus FT (AjFT), an enzyme involved in the production of FOS that are of great industrial interest, and its mutant D191A in complexes with various donor/acceptor substrates. The residues involved in substrate recognition have been determined, and a structural comparison between AjFT and other clan GH-J enzymes with known structures is discussed. These results suggest the catalytic mechanism and structure-functional relation of GH FTs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chemicals were obtained from Sigma unless specified otherwise.

**Expression and Purification of AjFT and Mutants**—To express fructosyltransferase and its mutants (listed below) from *A. japonicus* CB05 (AjFT), the gene of AjFT (access number GU356596) was cloned, and DNA of a full-length AjFT (residues 1–653) was subcloned into a plasmid pET21b (Novagen) using an EcoRI site to obtain the expression vector pET21b-tft. The correct construct was confirmed by sequencing and then transformed into *Escherichia coli* Tuner(DE3) (Novagen) for expression.

Three AjFT mutants (AjFT-D60A, -D191A, and -E292A) were generated with site-directed mutagenesis (QuikChange kit, Stratagene) and the pET21b-tft vector as a template. The forward primers for the mutants are as follows, in which the mutated bases resulting in changes of amino acids are underlined. D60A: 5’-GGC CAG ATC GGC GCC CCC TGC GCG CAC-3’; D191A: 5’-ACC GCC TTC CGC GCT CGT CTT GTC TTC-3’; and E292A: 5’-GGG TTC AAC TTC GCG GCC ACG GGG AAT GTG-3’. The mutated vectors were transformed into *E. coli* Tuner(DE3), and the mutations were confirmed by sequencing.

The cells containing transient expression vectors were cultured in the Luria-Bertani broth (37 °C) to *A.* _600_ 0.6–0.8, and then induced with isopropyl β-D-thiogalactopyranoside (0.05 mM, 20 °C, 12 h). The induced cells were harvested and disrupted by sonication. The supernatant containing soluble AjFT (or mutants) was passed through the nickel column for purification. AjFT (or mutant) was eluted with imidazole (concentration 100–200 mM). The eluted AjFT (or mutants) was desalted with a desalting column, and lyophilized. Each purified protein appeared as a single major band corresponding to a molecular mass of ~80 kDa on SDS-PAGE (10%).

**Enzyme Activity Assay**—The enzymatic activity of AjFT was assayed according to a method (13) using wild-type AjFT (1 µg) or mutants (10 µg) in a reaction mixture (300 µl) containing sucrose (1 mM) or 1-kestose (200 mM) in a Na2HPO4 buffer (50 mM, pH 5.5). The reaction mixtures with the individual enzyme were incubated at 50 °C for desired intervals; the reactions were terminated on heating at 100 °C for 15 min. The reaction products were analyzed on an NH2 column (4.6 × 250 mm, Nacalai) with the high-performance liquid chromatography (Hitachi) at 40 °C, with acetonitrile (85%) as the mobile phase (flow rate 1 ml/min) (14). The products were detected with an Refraction Index detector and identified with standards, including fructose, glucose, sucrose, 1-kestose (Fru β2→1Fru β2→1αGlc), nystose (Fru β2→1Fru β2→1αGlc), and 1F-rattofuranosyl nystose (Fru β2→1Fru β2→1Fru β2→1αGlc) (purchased from Wako).

**Crystallization of Mutant and Wild Type of AjFT**—Among three mutants, only AjFT-D191A was crystallized with satisfactory quality, whereas AjFT-D60A and -E292A tended to be precipitated in most the crystallization conditions. Lyophilized AjFT-D191A was dissolved in a Tris–HCl buffer (20 mM, pH 8.0) to a concentration 10 mg/ml and screened with crystallization kits. The crystallization was performed with the hanging drop, vapor-diffusion method. The initial condition was obtained from the Crystal Screen I kit (Hampton Research) containing polyethylene glycol (PEG) 4000 (w/v, 8%) in a sodium acetate buffer (100 mM, pH 4.6). This condition was optimized to improve the diffraction resolution on replacing PEG 4000 with PEG 3350 and adding LiCl (150 mM). Equal volumes (1 µl) of the protein solution and crystallization rea-
Preparation of Wild-type and D191A Mutant AjFT Substrate Complex Crystals—Crystals of D191A mutant-substrate complexes were obtained on individually soaking with various substrates (200 mM), including fructose, sucrose, raffinose, 1-kestose, and nystose in the crystallization solution at 23 °C for 10 min. A crystal of wild-type AjFT was also soaked with 1-kestose (200 mM) for 10 min to examine whether there exists the bound fructose in a transition state.

Data Collection—All crystals were cryoprotected with glycerol (20%) and frozen in liquid nitrogen before data collection. X-ray diffraction data were collected at beamline 12B2, equipped with a charge-coupled device detector (Q4R, Area Detector Systems Corporation), of SPring-8 in Japan and beamline 13B1, with a charge-coupled device detector (Q315, Area Detector Systems Corporation), of NSRRC in Taiwan. All data were processed using HKL2000 (28). Multiwavelength anomalous diffraction data for a crystal of the Br-derivative AjFT-D191A were collected with x-rays of wavelengths 0.9194 and 0.9060 Å at the inflection and high remote energy, respectively (Table 1).

Structure Determination and Refinement—The crystal structure of AjFT-D191A was first determined by Br-multiwavelength anomalous diffraction phasing. Initial phases were calculated with SOLVE (29) and subsequently improved with RESOLVE (30). Most structure was built using ARP/WARP (31) and further model building was performed with Coot (32). All refinements were performed with CNS v.1.2 (33). The initial structures of wild-type AjFT and mutant complexes were obtained with molecular replacement using the model of AjFT-D191A and refined with CNS v.1.2. The models of glucose, sucrose, raffinose, and 1-kestose were obtained from the HicUp server (available on-line) and manually fitted into the difference maps with coefficients $|F_o| \text{(substate-bound D191A)} - |F_c| \text{(refined D191A)}$. Among these substrates, the coordinate of nystose was not available in the HicUp server and PDB, and was manually built into the electron densities, initially based on a 1-kestose model.

Model Validation—The correctness of stereochemistry of the models was verified using PROCHECK (34). The root mean square deviation (r.m.s.d.) from ideality in ranges 0.0066–0.0101 Å for bond distances, and 1.4000–1.6257° for angles of all structures calculated with CNS showed a satisfactory stereochemistry. In a Ramachandran plot all main-chain dihedral angles of residues are in the most favored and additionally allowed regions with only glycine exceptions. All crystallographic data and refinement statistics are summarized in Table 1.

RESULTS

Overall Structure of AjFT—The activity assay shows that the recombinant wild-type AjFT (AjFT-WT) retains its fructosyltransferase activity and can produce FOS with DP 3–6 (supplemental Fig. S1). The crystal structures of AjFT-WT and its mutant D191A (AjFT-D191A) were determined at resolution of 2.0 and 1.8 Å, respectively (Table 1). The structures are essentially identical upon superimposition except that γ-C of Asp-119 moves toward Ala-191 by 0.5 Å in AjFT-D191A, and the orientation of the side-chain carboxylate group of Asp-119 also rotates ~60° relative to the AjFT-WT. The r.m.s.d. between the structures of AjFT-WT and AjFT-D191A is 0.42 Å for all atoms. The electron densities of residues before Ser-20 at the N terminus and residues after Arg-653 at the C terminus are invisible, because they were either flexible on the molecular surface or digested by contaminated endogenous proteases during purification. The crystals of space group P2₁2₁2₁ have one AjFT molecule per asymmetric unit. The crystal structure of AjFT comprises 632 residues that fold into two domains, an N-terminal five-blade β-propeller (residues 21–468), and a C-terminal β-sandwich (480–653) domain, which are linked by a 9-residue short α-helix (469–479) (Figs. 1A and 2). Several small helices, of which many have a 3₁₀ configuration, are found interspersed between connected β-strands on the molecular surface.

The five-blade β-propeller domain contains five repeats of radially oriented blades (numbered I through V), enclosing a deep central cavity with the first strand of each blade facing inside. The five-blade β-propeller in AjFT shares a similar fold with the enzymes of GH32 and GH68 but is distantly related to the enzymes of GH43 and GH62 (17). The structure alignment of the five-blade β-propeller domain in AjFT with other GH32 enzymes, invertase from Thermotoga maritima (PDB: 1W2T, residues 1–295) and fructan 1-exohydrolase Ila (1FEH Ila) from Cichorium intybus (1ST8, 1–340), gave r.m.s.d. values of 2.28 and 2.40 Å for 285 and 299 Ca atoms, respectively, using TM align (35) with small sequence identities (22–23%), whereas the superimposed structures of AjFT and GH68 levan-sucrase from Bacillus subtilis (1OY) exhibited a larger r.m.s.d. of 3.69 Å for 298 Ca atoms despite greater sequence identity (35%) (Figs. 1B and 2).

As for the C-terminal β-sandwich domain, the structure consists of two major six-stranded anti-parallel β-sheets (Fig. 1A). The relative orientations of the N-terminal (β-propeller) and C-terminal (β-sandwich) domains are stabilized through multiple hydrogen bonds and hydrophobic interactions. The alignment of C-terminal β-sandwich domain with the program DALI (36) onto other β-sandwich structures revealed top-four structural similarities with β-sandwich in cell-wall invertase from Arabidopsis thaliana (PDB: 2OXB; DALI Z-score: 18.9; r.m.s.d.: 2.8 Å, sequence identities: 22%) (23), fructan 1-exohydrolase Ila from Cichorium intybus (PDB: 2ADD, DALI Z-score: 18.2; r.m.s.d.: 2.7 Å, sequence identities: 20%) (26, 27), exoinulinase from Aspergillus awamori (PDB: 1Y9G; DALI Z-score: 17.4; r.m.s.d.: 3.4 Å; sequence identities: 21%) (25), and invertase from Thermotoga maritima (PDB: 1UYP; DALI Z-score: 17.0; r.m.s.d.: 2.9 Å; sequence identities: 24%) (21) (supplemental Fig. S2).
Table 1

Statistics of diffraction data and structure refinement

| Data collection | A/JFT-D191A:Br-MAD | A/JFT-D191A | A/JFT-WT | A/JFT-WT, glucose | A/JFT-D191A-substrate complex |
|-----------------|---------------------|-------------|-----------|-------------------|-------------------------------|
| Space group     | P2₁,2,2             | P2₁,2,2     | P2₁,2,2   | P2₁,2,2           | P2₁,2,2                       |
| Cell dimensions (Å) |                     |             |           |                   |                               |
| a               | 98.51               | 98.51       | 98.41     | 98.92             | 97.95                         |
| b               | 111.52              | 111.52      | 110.78    | 110.92            | 110.25                        |
| c               | 66.12               | 66.12       | 66.51     | 66.80             | 129.89                        |
| Wavelength (Å)  | 0.9194*             | 0.9060b     | 0.915     | 0.915             | 0.915                         |
| Resolution (Å)  | 20–2.8*             | 20–2.8*     | 20–2.8*   | 20–2.8*           | 20–2.8*                       |
| Rsym(%)         | 4.6 (24.3)*         | 4.7 (25.8)* | 6.9 (33.5) | 8.4 (35.3)        | 7.3 (43.0)                    |
| Completeness    | 4.9 (100)*          | 4.9 (100)*  | 9.9 (99.0)| 9.9 (99.0)        | 98.0 (99.0)                   |
| Redundancy      | 6.0 (6.1)*          | 6.0 (6.1)*  | 6.2 (6.7) | 5.6 (5.7)         | 6.7 (5.5)                     |
| Total observation | 113,112*           | 113,430b    | 446,639   | 261,143           | 674,106                       |
| Unique reflections | 18,852*           | 18,905b     | 67,098    | 46,257            | 101,087                       |
| Refinement      |                     |             |           |                   |                               |
| Resolution      | 20.1–1.8            | 20.8–2.0    | 20.8–2.0  | 20.8–2.0          | 20.8–2.0                      |
| Rwork/Rfree (%) | 22.1/24.4           | 22.8/23.9   | 22.6/25.5 | 22.4/26.4         | 21.5/25.6                     |
| No. of atoms    | Protein             | 4,883       | 4,880     | 9,766             | 4,883                         |
|                 | Ligand/ion          | 24          | 23        | 34                | 23                            |
|                 | Water molecules     | 324         | 305       | 349               | 228                           |
|                 | B-factors (Å²)      | 8.4 (24.6)  | 9.5 (5.2) | 7.3 (43.0)        | 50.2 (4.8)                    |
|                 | Protein             | 18.74       | 18.89     | 24.86             | 23.46                         |
|                 | Ligand/ion          | 24.61       | 24.61     | 36.02             | 36.02                         |
|                 | Water               | 1,481       | 1,481     | 24.23             | 24.23                         |
| r.m.s.d.        | Bond lengths (Å)    | 0.0072      | 0.0070    | 0.0066            | 0.0101                        |
|                 | Bond angles (°)     | 1.4811      | 1.400     | 1.4111            | 1.6257                        |

* Infection.
* High-remote.
* Values in parentheses are for highest resolution shell.

| Values of Rsym are the weighted mean of all measurements of I(h).
| Rwork = Σ|I(h)|-|F(h)|/|I(h)|, where |I(h)| is the observed and calculated structure factor amplitudes of reflection h, respectively.
| Rfree is calculated with 10% of randomly chosen reflections omitted from refinement.

The side chains of these acidic residues are spaced 4.9–5.8 Ǻ from each other. The hydrogen bond network greatly stabilizes the catalytic triad as described in the side chain of Asp-60 forms hydrogen bonds with three water molecules (OD1-water_157, 2.79 Å; OD1-water_294, 2.84 Å; OD2-water_148, 2.71 Å based on A/JFT-WT). Water_294 also forms a hydrogen bond with the amide of Asp-60 (3.35 Å). OE2 of Glu-292 forms a salt bridge (2.95 Å) with NH2 of Arg-190 and hydrogen bonds with water_126 (2.88 Å) and water_190 (2.68 Å), and OE1 forms strong hydrogen bonds with OH of Tyr-369 (2.46 Å) and water_48 (2.77 Å). Water_48 forms hydrogen bonds also with OE2 of Glu-318 (3.38 Å) and NH2 of His-332 (3.17 Å). Asp-191 (OD2) forms hydrogen bonds with NH of Thr-293 (3.03 Å) and a water molecule (water_126, 2.69 Å).

In addition to the three catalytic residues, several polar or charged residues, including Asp-119, His-144, Arg-190, Glu-318, and His-332, hydrophobic residues, including Leu-78, Phe-118, Tyr-369, Ala-370, and Trp-398, and the main-chain carbonyl oxygens of Ile-143 and Tyr-404 surround and form an negatively charged active-site pocket with dimensions width 17 Å and depth 13 Å (Fig. 1, C and D). The amino acids at the loops between the second and third strands of blades I, II, IV, and V, including Gln-57, Asp-80, Gly-81, Leu-141, Pro-142, Ile-143, Glu-327, Val-328, Ser-329, Glu-405, and Gln-406, encompass the entrance of the active-site pocket. Notably, the entrance is partially covered by the negatively charged side chain of Glu-405 (Fig. 1, C and D). A stacking interaction between the imidazole ring of His-332 and the aromatic ring of Tyr-404 was observed with a distance of ~4.0 Å, which is within the range of van der Waals interactions. This π–π interaction not only stabilizes the structural folding of A/JFT but also fixes Tyr-404 to lead the side chain of Glu-405 toward the active-site pocket.

Structures of D191A-Substrate Complexes—To observe the substrate molecules bound in the active site, we selected three inactive mutants, A/JFT-D191A, A/JFT-D60A, and A/JFT-E292A, for crystallization and substrate soaking to prevent rapid substrate processing, but only A/JFT-D191A was crystallized with a quality satisfactory for further soaking experiments. The crystals of A/JFT-D191A complexes with various donor/acceptor substrates, including sucrose, 1-ketose, nystose, and raffinose, were observed, and the corresponding structures were respectively solved (Table 1). All A/JFT-D191A complex crystals contain one A/JFT molecule per asymmetric unit with space group P2₁,2,2. Crystals of A/JFT-WT or A/JFT-D191A complex with fructose were unobtainable despite several soaking or co-crys-
tallization attempts, but soaking AjFT-WT crystals with 1-kestose yielded only a glucose molecule bound in the active site, as described subsequently.

All AjFT-D191A complexes reveal clear electron densities of various substrates binds inside the active-site pocket with the terminal fructose toward the bottom of the active site (Fig. 3, A and B). The carboxylate of Asp-60:OD2 is 3.1–3.6 Å from the fructosyl anomic carbon (C2), and the carboxylate of Glu-292:OE2 is 2.6–3.0 Å from the glycosidic oxygen among these complexes. An apparent feature of these bound substrate molecules is that all the neighboring sugar units do not stack with each other. No significant conformational alterations are observed between the backbone structures of AjFT-D191A with and without the substrates (r.m.s.d. < 0.51 Å).

Substrate (Donor/Acceptor) Binding, Interaction, and Recognition—Superposition of substrates in the active-site pocket from various structures of AjFT complex with sucrose, 1-kestose, nystose, and raffinose are shown in Fig. 3C. All positions and orientations of the terminal fructosyl moieties binding at the −1 subsite (numbers follow the nomenclature of Davies et al. (38)) and the sugar moieties (fructose or glucose) binding at the +1 subsite are nearly identical, likewise the binding residues, which provide the hydrogen bonds (Fig. 4). The positions of sugar moieties at the +2 subsite exhibit deviations among the complexes. The orientation of the sugar moiety at the +3 subsite is directed toward the loop between strands 2 and 3 of blade I in the nystose complex (Fig. 3C). The measurements of the dihedral angles (φ, γ, and ω) (39) for each substrate (from the
terminal fructose at the −1 subsite to the glucose) are presented in Table 2. The linkage conformations of the inulobiose component at the end of 1-kestose differ in \( \text{AjFT} \) and 1-FEH IIa (2AEZ). The conformation of fructooligosaccharide binding in \( \text{AjFT} \) differs also from the crystal structure of 1-kestose and nystose trihydrate (40, 41). Moreover, a close inspection of each

**Structure of A. japonicus Fructosyltransferase Complexes**

![Diagram of A. japonicus Fructosyltransferase Complexes](image)

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monosaccharide structure of all substrates reveals no notable distortion of the sugar rings, suggesting that the bound sugars are in the ground state at the cleavage site (42).

The interactions between the substrates and surrounding residues in the active-site pocket, described in the previous section, are dominated by hydrogen bonds, of which some are mediated with water. The network of hydrogen bonds is depicted in Fig. 4 and summarized in Table 3. Each hydroxyl of sugars present at the −1 and +1 subsites can form at least one direct or water-mediated hydrogen bond with the corresponding binding residues. In contrast, the sugar moieties at the +2 and +3 subsites seem bound less tightly, with only few hydrogen bonds formed between hydroxyls and the residues.

The enzyme-substrate complexes reveal several essential residues for substrate recognition, of which some are conserved in all GH32 members, whereas others are conserved among the fungal FT (Fig. 2). Asp-60, Asp-191, and Glu-292 are responsible for the stabilization of the terminal fructosyl moiety. The side chains of Arg-190, Glu-292, Glu-318, and His-332, the carbonyl oxygen of Ile-143, and the amide nitrogen of Trp-145 are responsible for stabilizing the fructosyl or glucosyl moiety at the +1 subsite (Fig. 4). The carbonyl oxygen of Tyr-404 and the side chain of Glu-405 stabilize the sugar moiety at the +2 subsite and are responsible for the formation of the inulin-type fructooligosaccharides. The galactosyl moiety in raffinose is stabilized by Ile-143 and Gln-327, which form another +2 subsite, resulting in a minor tilt of the glucosyl moiety and hence a longer distance from general acid/base catalyst Glu-292:OE2 to the hydroxyl of the glucosyl moiety (Figs. 3C and 4E).

The active site of A/JFT contains also several conserved hydrophobic residues that provide hydrophobic interactions with substrates, in which Phe-118 and Leu-78 interact with C6 hydroxyl of sugars present at the −1 subsite and are responsible for the stabilization of the fructosyl or glucosyl moiety at the +1 subsite.
The hydrophobic forces, stacking interactions, and hydrogen bonds were proposed to be the dominant interactions in general protein–carbohydrate complexes (43, 44), but no ring-stacking interaction between aromatic residues and substrates is found in any AjFT-D191A complex structure.

Our preliminary diffraction data of AjFT-D191A complex with 1F-fructofuranosyl nystose at a low resolution also confirm these subsites from \(-1\) to \(+3\) in the active site, although the two terminal sugar moieties show partially imperceptible densities as the last glucose moiety protrudes from the binding pocket and is exposed to the molecular surface of AjFT. Thus, we conclude that the four subsites \(\text{H}11002\), \(\text{H}11001\), \(\text{H}11001\), and \(\text{H}11001\) are the complete subsites in the active site of AjFT.

**TABLE 2**
The dihedral angles for each substrate are measured from the terminal fructosyl group to glucose

| Substrate (AjFT) | Nystose (AjFT) | 1-Kestose (AjFT) | 1-Kestose (1-FEH IIa) | 1-Kestose (crystal)* | Nystose (crystal)* |
|------------------|----------------|------------------|-----------------------|---------------------|-------------------|
| Relative moieties | \(-1\) to \(+1\) | \(+1\) to \(+2\) | \(-1\) to \(+1\) | \(-1\) to \(+1\) | \(-1\) to \(+1\) |
| \(\phi\) | 0.21 | -39.60 | 9.39 | -19.56 | -41.18 |
| \(\psi\) | 153.91 | 136.19 | 168.65 | -169.55 | -169.61 |
| \(\omega\) | 11.22 | 47.82 | 15.43 | -61.48 | -64.47 |
| \(\phi\) | -38.27 | -108.31 | -39.61 | -54.65 | -65.90 |
| \(\psi\) | 104.47 | 105.70 | 89.82 | 95.95 | 84.64 |

* The crystal structures of 1-kestose and nystose are referenced (40, 41).

**Structure of A. japonicus Fructosyltransferase Complexes**

FIGURE 4. Interactions between substrates/inhibitors and residues in the active site of various AjFT complexes. A, inulin-type substrates (left) and raffinose (right) used in this study are shown in chemical structures. B, AjFT-D191A complex with sucrose (GF as the donor). C, AjFT-D191A with 1-kestose (GF2 as a donor, GF as an acceptor). Note that the side-chain carboxylate group of Glu-405 is rotated to interact with both sugar moieties at \(\text{H}11001\) and \(\text{H}11001\). D, AjFT-D191A with nystose (GF3 as a donor, GF2 as an acceptor). E, AjFT-D191A complex with raffinose (a suitable donor). F, AjFT-WT complex with glucose (glucose as an inhibitor). Substrate molecules are shown in ball and stick (with the carbon in green and oxygen in red). The bound water molecules are shown in cyan. The surrounding amino acids are presented in stick (with the carbon in magenta, nitrogen in blue, and oxygen in red). Hydrogen bonds between substrate atoms, water molecules, and neighboring polar atoms of residues are shown as dashed lines. Some bound water molecules are conserved in the structures among AjFT complexes. The interacting distances are summarized in Table 3.
Structure of A. japonicus Fructosyltransferase Complexes

Table 3

Atomic interactions of substrates, waters, and amino acids in the active site of A/JFT

P. atom represents the residue and water atoms in proteins. The distances to Asp-119 in wild-type enzymes are estimated and presented in parentheses.

| Substrate | Atom | Nystose (3LEM) | 1-Kestose (3LDR) | Sucrose (3LDK) | Glucose (3LF1) | Raffinose (3LIH) |
|-----------|------|---------------|-----------------|---------------|---------------|-----------------|
| Fructose  | C2   | D60OD2        | D60OD2          | D60OD2        | D60OD2        | D60OD2          |
|           | O1   | D60OD1        | D60OD1          | D60OD1        | D60OD1        | D60OD1          |
|           | Water | 113           | Water           | Water         | Water         | Water           |
|           | O3   | R190NE        | R190NE          | R190NE        | R190NE        | R190NE          |
|           | D191OD2 | 3.07         | D191OD2         | D191OD2       | D191OD2       | D191OD2         |
|           | E292OE2 | 2.65       | E292OE2         | E292OE2       | E292OE2       | E292OE2         |
|           | Water | 9             | Water           | Water         | Water         | Water           |
|           | O4   | D119OD1       | D119OD1         | D119OD1       | D119OD1       | D119OD1         |
|           | C6   | L78CD1        | L78CD1          | L78CD1        | L78CD1        | L78CD1          |
|           | F118CD1 | 3.92       | F118CD1         | F118CE1       | F118CD1       | F118DC1         |
|           | O6   | L78CD1        | L78CD1          | L78CD1        | L78CD1        | L78CD1          |
|           | Water | 61            | Water           | Water         | Water         | Water           |
|           | Water | 117           | Water           | Water         | Water         | Water           |
| Glucose   | O1   | Water_27      | Water_56        | Water_56      | Water_157     | Water_312       |
|           | O3   | E318OE2       | E318OE2         | E318OE2       | E318OE2       | E318OE2         |
|           | Water | 27            | Water           | Water         | Water         | Water           |
|           | O4   | H143O         | H143O           | H143O         | H143O         | H143O           |
|           | R190NH2 | 3.21       | R190NH2         | R190NH2       | R190NH2       | R190NH2         |
|           | Water | 9             | Water           | Water         | Water         | Water           |
|           | Water | 102           | Water           | Water         | Water         | Water           |
|           | O6   | Water_34      | Water_24        | Water_24      | Water_17      | Water_22        |
| Glucose   | O3   | Y404O         | E405OE1         | E405OE1       | E405OE1       | E405OE1         |
|           | Water | 169           | Water           | Water         | Water         | Water           |
|           | Water | 187           | Water           | Water         | Water         | Water           |
| O2       | O2   | E405OE2       | Water_34        | Water_34      | E292OE1       | E292OE1         |
|           | Water | 110           | Water           | Water         | Water_157     | Water_312       |
|           | O3   | E405OE2       | Y404O           | E405OE2       | Water_143     | Water_172       |
|           | Water | 8             | Water           | Water         | Water         | Water           |
|           | O4   | I143O         | I143O           | I143O         | I143O         | I143O           |
|           | R190NH2 | 3.21       | R190NH2         | R190NH2       | R190NH2       | R190NH2         |
|           | Water | 172           | Water           | Water         | Water         | Water           |
|           | O5   | Water_117     | Water           | Water         | Water         | Water           |
|           | O6   | L141CD1       | Water_24        | Water_24      | Water_12      | Water_312       |
| Galactose | O1   | Water_22      | Water           | Water         | Water         | Water           |
|           | O2   | L141CD1       | Water           | Water         | Water         | Water           |
|           | O6   | L141CD1       | Water           | Water         | Water         | Water           |

* The distances to Asp-191 were inferred from superimposed wild-type A/JFT with A/JFT-D191A complex.

see “Experimental Procedures”) to examine whether the fructose exists in a transition state to bind in the active site. The crystal of A/JFT-WT complex was transformed from the original space group P2₁2₁2₁ to P2₂₁2₂₁ containing two molecules per asymmetric unit, which differs from the crystals of A/JFT-WT and all A/JFT-D191A complexes. The result showed, however, that only a glucose molecule was found to bind at the transfructosylation active site. The fructosyl moiety resides under the glucose moiety based on structures of A/JFT-D191A with sucrose and A/JFT-WT with glucose (Figs. 1D, 4B, and 4F). This suggests that the binding of glucose to the A/JFT-WT requires the release of sucrose by either transfructosylation or hydrolysis of 1-ketose and re-binding of sucrose for the final cleavage.
DISCUSSION

Proposed Catalytic Mechanism of AjFT—The general mechanism of glycoside hydrolase with the retaining reaction is a double-displacement mechanism involving two steps: glycosylation involves protonation of the glycosidic oxygen followed by nucleophilic attack on the anomeric carbon of the donor substrate to form fructosyl-enzyme intermediate; deglycosylation transfers the fructosyl group to the acceptor through the general-base-mediated nucleophilic attack and the release of the product and the enzyme. In the latter step, the water or fructan serves as an acceptor for the hydrolysis or transfer, respectively.

During the two-step reaction, a covalent glycosyl-enzyme intermediate is formed and hydrolyzed via oxocarbenium ion-like transition states (46, 47).

According to the present complex structures of AjFT-substrates, only one substrate-binding site was observed, supporting this double-displacement mechanism. The three catalytic residues, Asp-60 (nucleophile), Asp-191 (transition-state stabilizer), and Glu-292 (general acid/base catalyst), are located at appropriate distances and orientations from the substrates for their proposed catalytic roles in the active site. The exact topological orientations of residues responsible for the determination of the +1 ~ +3 subsites produce the acceptor specificity and a greater transfructosylation/hydrolysis ratio of AjFT relative to other GH32 enzymes. The reaction scheme for AjFT is depicted in Fig. 5.

Sucrose/fructooligosaccharide binds to the active site in a ground state, at which its glycosidic oxygen is protonated by Glu-292 and transformed into a transition state. Subsequently, a nucleophilic attack is performed by the carboxyl group of Asp-60, forming a fructosyl-enzyme intermediate, followed by the binding of the acceptor, such as sucrose, 1-kestose, or nystose. Fructose is eventually transferred to the acceptor, thus releasing 1-kestose or nystose or 1\textsuperscript{1}-fructofuranosyl nystose and the substrate-free enzyme.

According to the schematic mechanism, once the fructosyl-enzyme intermediate is formed, sucrose competes with water and other fructooligosaccharides to receive the fructose. This mechanism is consistent with the observation that an increased concentration of sucrose increases the ratio of transfructosylation to hydrolysis (14).

Structural Comparison of the Active-site Pocket of AjFT with Other GH32 and GH68 Enzymes—Regarding the closely related enzymes of GH32 and GH68, five structures of enzyme-substrate complexes have been reported. They share only ~22% primary sequence identity with AjFT but with a somewhat similar three-structural fold (Fig. 2). To compare these enzymes with AjFT, superposition was performed by pair alignment of the catalytic triad of each wild-type enzyme, followed by the superposition of each mutant-substrate complex to its corresponding wild-type enzyme. Inspection of the molecular surfaces of the active-site pocket and the substrate in each complex reveals the differentiation of the shape and size of the pockets (Fig. 6), but the deepest part of the active-site pocket, which is

4 P. Chuankhayan, C.-Y. Hsieh, Y.-C. Huang, Y.-Y. Hsieh, H.-H. Guan, Y.-C. Hsieh, Y.-C. Tien, C.-D. Chen, C.-M. Chiang, and C.-J. Chen, unpublished data.
Inhibitors

A, AjFT with nystose (3LEM, nystose). The 57QIGDPC motif is colored in pink, loops connecting the second and the third β-strands of blades II, IV, and V are colored in orange, green, and cyan, respectively. B, exoinulinase from A. awamori with fructose (119G, fructose); C, invertase from T. maritima with raffinose (1UYP, 1W2T, raffinose); D, cell wall invertase from A. thaliana with sucrose (2AC1, 2QQV, sucrose); E, fructan exohydrolase 1-FEH IIa from C. inbutus with 1-kestose (1ST8, 2AEZ, 1-kestose); F, levansucrase from B. subtilis with raffinose (1OYG, 3BYN, raffinose). The molecular surfaces are shown near the active-site pockets with the oxygen in red, nitrogen in blue, and carbon in gray. The superimposed substrates are shown in ball and stick. Structures were superimposed by pairwise alignments with the catalytic triads of these wild-type enzymes first, and subsequently the mutant complexes were aligned with the corresponding wild-type enzymes for substrate positions.

The sequence alignment and structural analysis of AjFT and other clan GH-J members reveal several distinct areas around the active-site pocket, described as follows. The first major divergence is found in the first β-strand of blade I around the nucleophile residue Asp. The sequence of the WMNDP Ng motif in GH32 invertases and fructan exohydrolases is not conserved in AjFT and other GH32 transferases. In A. thaliana cwINV (AtcwINV), Trp-20 (within this motif) and Trp-47 located at blade I combine with Trp-297 and Tyr-279 at the blade V to form a large hydrophobic patch that connects blades I and V and surrounds the active-site pocket as revealed from the selected enzymes of GH32 hydrolases (Fig. 6, B–E). In AjFT, the sequence is 57QIGDPC with the smaller Gln-57, Ile-58, and sulfoxidyl Cys-62 replacing the corresponding residues of bulky Trp, Met, and amide-containing Asn, respectively. Hence, the first β-strand shifts away from the pocket and the loop connecting the second and third β-strands of blade V moves near the pocket, altering the shape for the active-site pocket in this region (Fig. 6A).

Previous mutagenesis studies at this motif region reveal its effects on the substrate specificity and the type of catalytic reaction. Replacement of Asn-84 (structurally equivalent to Gly59 in AjFT) with Ser, Ala, or Gln turns onion 6G-FFT into 1-SST, indicating that Asn-84 determines the product specificity (48). Mutants W161Y and N166S of onion invertase in this region showed enhanced transfructosylation activities (49). A similar result was observed based on the mutations (W23Y and/or N25S) at this WMNDPGN motif of wheat vacuolar invertase (50). Replacing Trp-23 with a small or hydrophilic residue was...
thought to contribute to the formation of the specific acceptor site for transfructosylation.

The replacements of amino acids at this motif likely alter the environment that affects the hydrophobic interactions and hydrogen bonds between adjacent \( \beta \)-strands at blades I and V and thus produce the tilt of the nucleophilic residue (Asp-60 in \( \text{A} \text{jF} \)) and the shift of the loop (residues 401–416 in \( \text{FT} \)). The position and orientation of galactosyl moiety of raffinose in \( \text{AJFT} \) is different from those in others due to hydrogen bond network between the hydroxyls of galactosyl moiety to neighboring residues. In \( \text{A} \text{jF} \), substrates with fructosyl group at the +1 subsite together with fructose were superimposed. Shown are 1-kestose in \( \text{AJF} \) (3LDR, pink), 1-kestose in \( \text{C. inbutus} \) 1-FEH IIa (2AEZ, blue), and fructose in \( \text{A. awamori} \) exoinulinase (1Y9G, cyan). The position and orientation of 1-kestose in \( \text{AJF} \) are also different from those in 1-FEH IIa.

A negatively charged residue Asp-119 is found near the nucleophile Asp-60 with distance 2.7 Å in \( \text{AJF} \). Although this residue cannot form a hydrogen bond with Asp-60, it might enhance the nucleophilic efficiency. An inspection of other clan GH-J amino acid sequences reveals that the structurally equivalent residue is a serine or threonine (Fig. 2). In \( \text{B. subtilis} \) levan-sucrase, Ser-164 shares a hydrogen bond with nucleophile Asp-86 and maintains the position and orientation of Asp-86. The mutant S164A of \( \text{B. subtilis} \) levan-sucrase exhibits a significant decrease in \( k_{\text{cat}} \) despite the greater enzyme stability and affinity for sucrose (51). A similar result has been shown for the mutant S173A in \( \text{B. megaterium} \) levan-sucrase (52).

The second notable difference is that the two longer loops (residues 119–132 and 320–330), connecting the second and the third \( \beta \)-strands of blades II and IV, respectively, are unique in \( \text{AJF} \). A stretch of lined-up hydrophobic residues forms a part of the boundary and the entrance of the active-site pocket, resulting in a pocket in \( \text{AJF} \) deeper than that of other enzymes (Figs. 1 and 6). The access of water to the general acid/base catalysis (Glu-292 in \( \text{A} \text{jF} \)) to receive the bound fructosyl group is hence much restricted, which might be the reason that \( \text{AJF} \) functions mainly as a transferase, whereas the others exhibit as hydrolases. Within the described loop at blade II, the main chain of Ile-143, together with side chains of Glu-318 and His-332, located, respectively, at the end of the second strand and the start of the third strand of blade IV, form the +1 subsite. The positions and orientations of these residues determine the preference of fructosyl- and glucosyl-groups at the +1 subsite. The main chain of Ile-143 combines also with the side chain of Glu-327 on the other loop (at the blade IV) to form another +2 subsite to stabilize of the galactosyl moiety of raffinose.

The third distinct difference occurs at the loop (residues 401–416) connecting the second and third \( \beta \)-strands of blade V in \( \text{AJF} \). The ring stacking between His-332 and Tyr-404 stabilizes the orientations of His-332, Tyr-404, and even Glu-405 as mentioned previously, exposing the NH\(_2\) of His-332, main-chain oxygen of Tyr-404 and side-chain carboxylate of Glu-405 to the active-site pocket. The exposure of these polar groups in the active site enables \( \text{AJF} \) to form the +2 and +3 subsites for the formation of an inulin-type oligosaccharide.

Structured and Mechanistic Implications—The results from our work might provide structural insights to explain how \( \text{AJFT} \) functions. A similar scheme is applicable to other FTs, such as 1-SST, 1-FFT, 6-SFT, or 6G-FFT. For an FT, not only the specificity of the sugar moiety at the +1 subsite but also the presence of the +2 subsite determines the type of transfructosylation activity. Using sucrose as both donor and acceptor substrates, 1-SST and inulosucrases might have +1 and +2 subsites for 1-kestose formations that are similar to \( \text{AJFT} \). 1-FFT, using fructan as donor/acceptor substrates, exhibits a different preference for sugars at the +1 subsite from \( \text{AJFT} \). For 6-SFT and 6G-FFT that form a 2→6 linkage for product formation, a +1 subsite with a disparate substrate specificity and a +2 subsite by mimicking the binding of the galactosyl moiety of raffinose as in \( \text{AJFT} \) would be expected.

According to various \( \text{AJFT} \)-substrate complexes, the +1 subsite is governed by several residues described in the preceding section, especially residues Glu-318/His-332 that form hydrogen bonds to O3 of the sugar moiety (fructosyl or glucosyl group) and Ile-143 that forms a hydrogen bond to O4 of the sugar moiety. These residues determine the donor/acceptor specificity. The +2 subsite for the formation of an inulin-type product is provided by residues Tyr-404 and Glu-405 located at blade V. Residues Ile-143 and Gln-327, located at the rim of the active-site pocket near the contact between blades II and III, provide another +2 subsite responsible for binding raffinose, which could mimic the formation of a product of levan or neo type.

The mutagenesis in search of the important residues responsible for substrate recognition and the type of catalytic reaction has been studied in plant GH32 members and GH68 microbial levan-sucrases (51–58). The superimposed structures show that Glu-318 in \( \text{AJFT} \) is at a position equivalent to Asp-239 in AtcwINV, which has been shown to be critical for binding and stabilizing sucrose (Fig. 2) (24). Mutation of Asp-239 to Ala
converted AtcwINV into a fructan 1-exohydrolase (54). Moreover, substitutions of Asn-340/Trp-343 of perennial ryegrass 6G-fructosyltransferase at the positions to Asp/Arg formed the enzyme into 1-SST (Fig. 2) (55). The Asp/Arg(Lys) pair located at the loop connecting the second and third strands of blade IV (corresponding to Glu-318/His-332 in AjFT) and the hydrogen bond network created by this D/R pair were thus suggested for recognition of sucrose as a substrate (53–55). The equivalent residue at this position is Phe-233 in fructan 6-exo-hydrolase of the sugar beet Beta vulgaris. The F233D mutant similarly exhibits a β-fructofuranosidase activity against sucrose and levans, indicating the role of this residue in the recognition of donor substrates (56). Arg-360 of acINV, equivalent residue at this position is His-825 in 6-SFT, 463. The Asp/Arg/His pair located at the loop connecting the second and third strands in blade V. This residue position, located at the rim of the active site pocket, is near the structurally related Tyr-404 that forms hydrogen bonds with fructose (or glucose) at the +2 subsite in AjFT.

For 6-SFT or levansucrase, no residue critical for the formation of the β-(2→6) linkage was identified. However, Asn-252, located at blade II of B. megaterium levansucrase, and Arg-370 in AjFT, are related to the synthesis of polymer versus oligosaccharide (48, 49). According to the raffinose binding in AjFT, the polar groups at the rim of the active-site pocket near the contact between blades II and III might be the critical location for seeking particular residues responsible for activity of this kind. AjFT expressed in E. coli is not glycosylated, whereas AjFT expressed in A. japonicus CB05 or P. pastoris produces glycosylation of molecular mass of ~20–30 kDa.4 The glycosylation affecting the substrate specificity and the activity of chicory fructan 1-exohydrolase is reported (59), but the non-glycosylated AjFT retains its transfructosylation activity and specificity. In AjFT, all nine predicted N-glycosylation sites are distant from the active-site pocket. Glycosylation might contribute the stability or polymerization of this enzyme, which requires further investigations. Mutations that alter the transfructosylation-versus-hydrolysis property, the substrate preference, and the product divergence can be designed based on our results and the above discussion. Mutagenesis studies are in progress.

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