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Food Biochemical Study on Fructans and Related Synthesis Enzymes

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Abstract: There are more than 500 kinds of oligosaccharide occurring in nature, and all of these are synthesized by chemical and enzymatic reactions. Various studies have recently identified physiological and physical applications of such oligosaccharides in agricultural chemistry, nutrition and medicine. New technologies for the production of oligosaccharides from natural resources have been developed in the food industry and several oligosaccharides are now produced on a large scale as ingredients in animal feed. We have investigated the isolation of fructo-oligosaccharides, as well as the enzymatic synthesis of oligosaccharides that have functional activities as tertiary functional ingredients in food. We have studied the purification and characterization of several fructosyltransferases from asparagus roots and onion bulbs, including sucrose: sucrose 1-fructosyltransferase (1-SST), fructan: fructan 1-fructosyltransferase (1-FFT), and the novel enzyme fructan: fructan 6^G-fructosyltransferase (6G-FFT). We have also reported that asparagus 1-FFT synthesized new functional oligosaccharides elongated with one or two additional fructose units by fructosyltransfer from 1-kestose to 4^G-β-D-galactosylsucrose, and this compound selectively stimulates the growth of *Bifidobacteria*. For industrial applications, we attempted the isolation and expression of cDNAs encoding 6G-FFT, 1-FFT and 1-SST from asparagus plants. The cDNAs encoding 6G-FFT, 1-FFT and 1-SST were isolated from a cDNA library of asparagus leaves or roots, and the isolated cDNAs were designated *aoft1*, *aoft2* and *aoft3*, respectively. The deduced amino acid sequences of these cDNAs showed high homology with those of plant fructosyltransferases. Expression of these cDNAs was performed using *Pichia pastoris*. The recombinant protein from *Pichia* transformed with *aoft1* produced 1^F,6^G-di-β-D-fructofuranosylsucrose, neokestose and sucrose from 1-kestose, while the transformant with an empty vector produced no saccharides. These results confirmed that 6G-FFT was expressed in *P. pastoris*. Similarly, the recombinant protein from *Pichia* transformed with *aoft2* produced nystose from 1-kestose and the recombinant protein with *aoft3* produced 1-kestose from sucrose. These results confirmed that 1-FFT and 1-SST were expressed in *P. pastoris*, and that the recombinant proteins had enzymatic properties similar to those of 6G-FFT, 1-FFT and 1-SST from asparagus roots. We then examined the conversion of substrate specificity from 6G-FFT to 1-FFT by point mutations in the β-fructosidase motif. The asparagine in this motif in *aoft1* was changed to serine, and the mutant recombinant protein was characterized. We found that this amino acid substitution in wild-type *aoft1* changed the substrate specificity from 6G-FFT to 1-FFT. Finally, we studied the activities of the main enzymes involved in the synthesis and hydrolysis of fructo-oligosaccharides during the post-harvest life of onion bulbs and asparagus spears, and discuss the mechanisms triggering these enzyme activities, as well as the mechanisms by which fructo-oligosaccharides contribute to the quality and perishability of the vegetables.

Key words: fructan, fructo-oligosaccharide, fructosyltransferase, post-harvest, *Pichia pastris*

In our recent studies, we have investigated the enzymatic synthesis of novel non-digestible oligosaccharides that exhibit a range of activities as tertiary functional ingredients in foods. Non-digestible oligosaccharides, such as fructo-oligosaccharides,[1)] inulo-oligosaccharides[2,3)] and fructosylxylosides,[4)] derived from sucrose, inulin and xylose have been found to have several useful functions; for example, they do not elevate blood glucose or insulin concentrations,[5,6)] have no hypocholesterolemic effects in blood,[6)] promote the absorption of calcium[7)] and magnesium,[7,8)] selectively stimulate the growth of *Bifidobacteria*[6)] and have probiotic effects in mice.[9)]

We have been focusing on the isolation of fructo-oligosaccharides[10−12)] and fructo-polysaccharide,[13)] and the enzymatic synthesis of oligosaccharides in asparagus roots[14)] and onion bulbs.[15)] The structures and enzymatic pathway of fructo-oligosaccharides in asparagus roots and onion bulbs[16)] are shown in Fig. 1.

Although this scheme was presented at the first fructan symposium held at Bonn in 1988 and was published in the *Journal of Plant Physiology* in 1989, the scheme now includes new information. In onion bulbs, fructo-oligosaccharides were recently confirmed to be synthesized by a two-enzyme system comprising sucrose: sucrose 1-fructosyltransferase (1-SST) and fructan : fructan 6^G-fructosyltransferase (6G-FFT) together with fructan : fructan 1-fructosyltransferase (1-FFT) in recent works.[17,18)]

Fructo-oligosaccharides are reportedly synthesized by a three-enzyme system in asparagus roots: 1-SST (EC 2.4.1.99),[19)] 1-FFT (EC 2.4.1.100)[20)] and 6G-FFT (EC

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Abbreviations: 1-SST, sucrose : sucrose 1-fructosyltransferase; 1-FFT, fructan : fructan 1-fructosyltransferase; 6G-FFT, fructan : fructan 6^G-fructosyltransferase; 1-KHE, 1-kestose hydrolyzing enzyme.

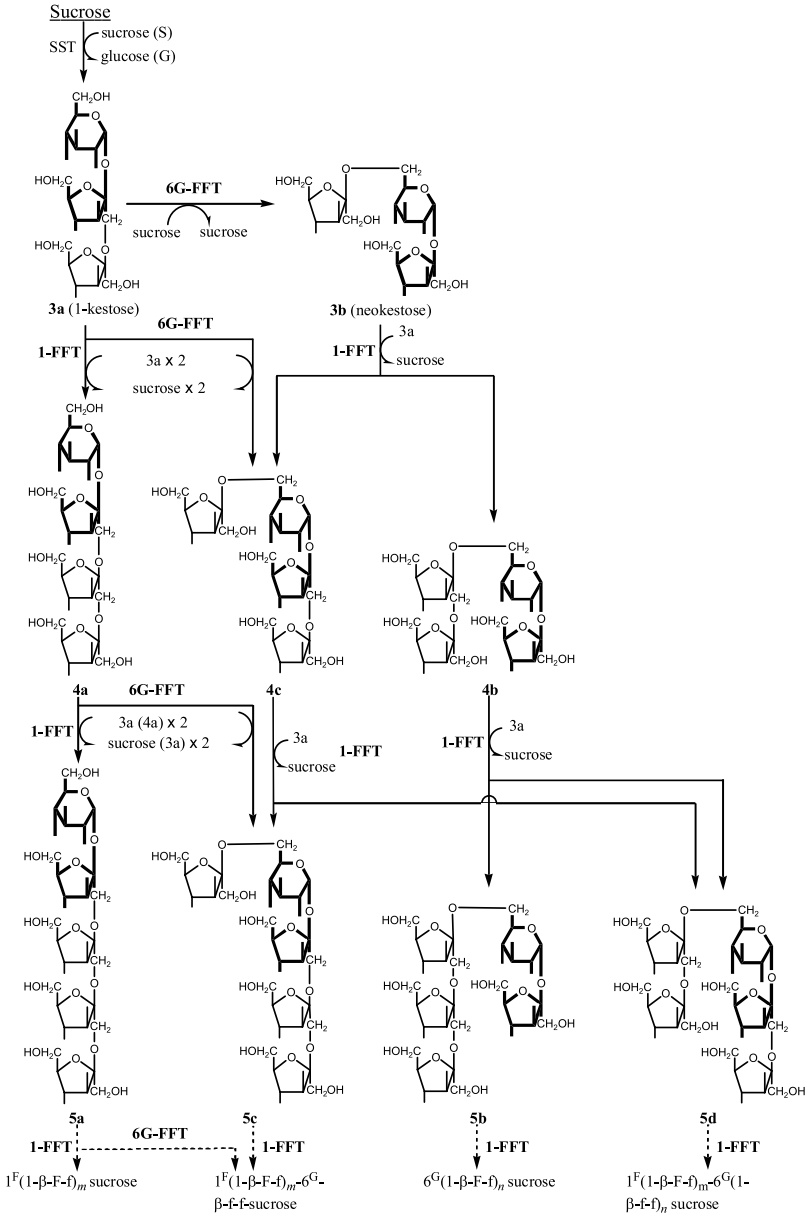

Fig. 1. Pathway of enzymatic synthesis of fructo-oligosaccharides in asparagus and onion plants.[16)]

G, glucose; F, fructose; S, sucrose; Inulin type oligosaccharides, 1^F(1-β-D-fructofuranosyl)$_m$ sucrose [3a(1-kestose, 1-kes): m=1; 4a(nystose, nys): m=2; 5a(fructosylnystose, f-nys): m=3; 6a: m=4; 7a: m=5]; Inulin neotype oligosaccharides, 1^F(1-β-D-fructofuranosyl)$_m$-6G(1-β-D-fructofuranosyl)$_n$ sucrose [3b(neokestose, n-kes): m=0, n=1; 4b: m=0, n=2; 4c: m=1, n=1; 5b: m=0, n=3; 5c: m=2, n=1; 5d: m=1, n=2].

2.4.1.243, entry proposed by Shiomi).[21–23)] We also reported the purification and characterization of 1-SST,[24)] 1-FFT[17)] and 6G-FFT[17)] from the onion. We found that asparagus 1-SST[19)] or 1-FFT[25)] catalyzed fructosyltransfer from 1-kestose to non-reducing terminal fructosyl residues in some oligosaccharides, while 6G-FFT catalyzed fructosyltransfer from inulin type oligosaccharides to the glucose residues of oligosaccharides to produce inulin neotype oligosaccharides.[21,23)]

In this paper, we first report several oligosaccharides synthesized by the actions of 1-SST, 1-FFT and 6G-FFT from asparagus roots and onion bulbs. We then describe the cloning and expression of genes encoding 1-SST, 1-FFT and 6G-FFT from asparagus as an initial study into utilizing the genes for production of oligosaccharide derivatives in the food industry. Finally, we discuss the metabolism of fructo-oligosaccharides in post-harvest onion and asparagus plants.

1. Enzymatic preparation of fructo-oligosaccharides using fructosyltransferases (1-SST, 1-FFT and 6G-FFT)

Fructosyltransferases from microorganisms, such as *Aspergillus niger*,[26)] are currently used to prepare fructo-oligosaccharides in the Japanese food industry. In this case, fructosyltransferases produce fructo-oligosaccharides with a DP higher than 1-kestose; thus, the enzymes are unsuitable for preparation of 1-kestose. In contrast, asparagus 1-SST[19)] and *Eurotium*[27)] or *Scopulariopsis*[1)] fructosyltransferase can catalyze fructosyltransfer with sucrose to produce 1-kestose. We isolated and crystalyzed large amounts of 1-kestose for use as a food additive using the *Eurotium* enzyme. When asparagus 1-SST is applied to the production of fructo-oligosaccharides, only 1-kestose is obtained.

The synthesis of fructosyl-raffinose, -stachyose and -verbascose by 1-SST from asparagus root or by fructo-

syltransferase from *Eurotium* is shown in Fig. 2. Raffinose, stachyose and verbascose are known to be present in soy beans. When extracted, these oligosaccharides are used in Japan as functional saccharide food additives, known as soy oligosaccharides. However, these saccharides have low solubility in water and lower promotion activity for *Bifidobacteria* than fructo-oligosaccharides. In order to improve the function of these saccharides, we attempted to synthesize new oligosaccharide derivatives of raffinose, stachyose or verbascose elongated with one unit of fructose.[28] Three oligosaccharides were synthesized from raffinose, stachyose and verbascose, and sucrose with asparagus 1-SST or *Eurotium* fructosyltransferase in large quantities, and were then isolated to study their physiological effects in rats and microorganisms.

We have also been investigating the purification and properties of asparagus1-FFT. We reported that asparagus 1-FFT[20] preferentially catalyzed fructosyltransfer from 1-kestose to neokestose to produce aspargosin-like saccharides via neokestose derivatives. In the study, we found that 1-FFT also catalyzed fructosyltransfer from 1-kestose to the fructosyl residue of terminal sucrose in other oligosaccharides, thereby producing oligosaccharides elongated with several units of fructose. Therefore, we attempted to synthesize oligosaccharides from 1-kestose and lactosucrose using purified asparagus 1-FFT. Lactosucrose elongated with several units of fructose were produced[25] using asparagus 1-FFT, as shown in Fig. 3. These fructosyllactosucroses improve intestinal conditions, thereby having a beneficial effect on overall health.

It is thought that inulin neotype oligosaccharides are preferentially hydrolyzed to inulin oligosaccharides by β-fructofuranosidase. For example, oligo β-fructofuranosidase from *Bifidobacterium adolescentis* G7 was previously reported to hydrolyze neokestose more rapidly than 1-kestose, and that *Bifidobacteria* utilize neokestose more effectively than 1-kestose.[29] Therefore, inulin neotype oligosaccharides converted from inulin type oligosaccharides are expected to be preferentially utilized by *Bifidobacteria*. We thus attempted to convert inulin type oligosaccharides to inulin neotype oligosaccharides using purified asparagus 6G-FFT as shown in Fig. 4. Anion exchange chromatograms of inulin neotype oligosaccharides produced from inulintype oligosaccharides using asparagus 6G-FFT are shown in Fig. 5.

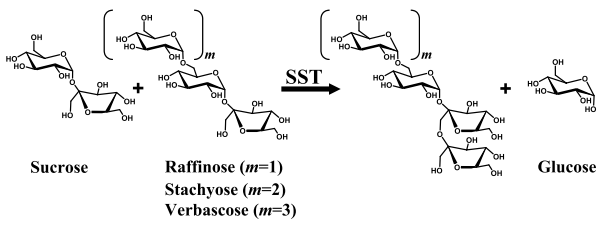

Fig. 2. Synthesis of fructosyl-raffinose , -stachyose and -verbascose with sucrose : sucrose 1-fructosyltransferase (1-SST) from *Eurotium repens* or onion seeds.

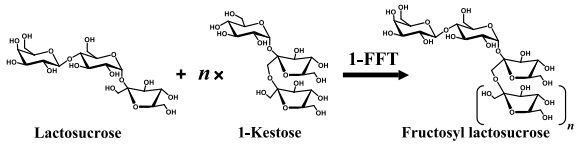

Fig. 3. Synthesis of fructosyl lactosucrose with fructan : fructan 1-fructosyltransferase (1-FFT) from asparagus roots.

The upper part is the chromatogram for a reaction mixture containing inulin type oligosaccharides (from DP 4 to DP 10 or more) used as substrate with 6G-FFT at time 0. The lower part is the chromatogram for reaction products formed from inulin type oligosaccharides with 6G-FFT at 24 h. Inulin neotype oligosaccharides 3b, 4c, 4b, 5b, 5d, 5c, 6 in, 7 in, 8 in and 9 in were observed.

A chromatogram of inulin neotype oligosaccharids produced from 1-kestose using asparagus 6G-FFT is shown in Fig. 6. At 0 h, only the substrate 1-kestose was detected. At 24 h, inulin neotype oligosaccharides 3b, 4c, 4b, 5c, 5d and 5b were detected.

2. Molecular characterization of asparagus fructosyltransferase

1) Cloning and expression of genes encoding fructosyltransferases

The cloning and expression of genes encoding 6G-FFT, 1-FFT and 1-SST were then studied for potential applications in the food industry. cDNAs encoding asparagus

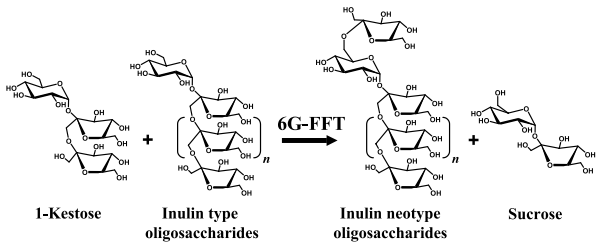

Fig. 4. Synthesis of inulin neotype oligosaccharide with fructan : fructan 6^G-fructosyltransferase (6G-FFT) from asparagus plants.

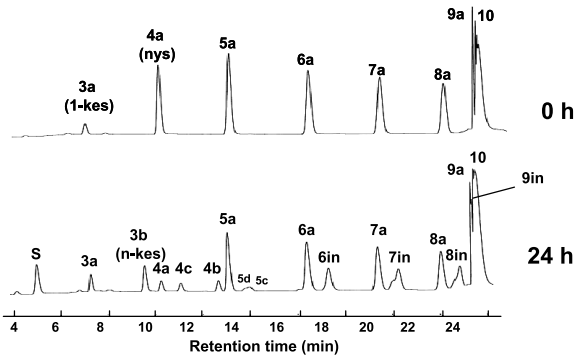

Fig. 5. Anion exchange chromatogram of inulin neotype oligosaccharides produced from inulin type oligosaccharides using fructan : fructan 6^G-fructosyltransferase (6G-FFT) from asparagus leaves.

A mixture of 6G-FFT (25 μL, 0.54 U), McIlvaine buffer (pH 5.5, 25 μL), 5% inulin type oligosaccharides [1^F(1-β-D-fructofuranosyl)$_{1-9}$ sucrose] and toluene (a trace amount) was incubated at 30°C for 96 h. After the reaction was stopped by heating at 100°C for 3.5 min, the reaction mixture was diluted 100 times with distilled water and an aliquot (10 μL) was injected to HPAEC. Inulin type oligosaccharide: 1^F(1-β-D-fructofuranosyl)$_m$ sucrose [3a (1-kestose): m=2; 5a (fructosylnystose): m=3; 6a: m=4; 7a: m=5; 8a: m=6; 9a: m=7, 10: $m \geq 8$]; Inulin neotype oligosaccharides, 1^F(1-β-D-fructofuranosyl)$_m$-6^G (1-β-D-fructofuranosyl)$_n$ sucrose [3b(neokestose: m=0, n=1; 4b: m=0, n=2; 4c:, m=1, n=1; 5c: m=2, n=1; 5d: m=1, n=2; 6in–9in: $m+n$=4–7).

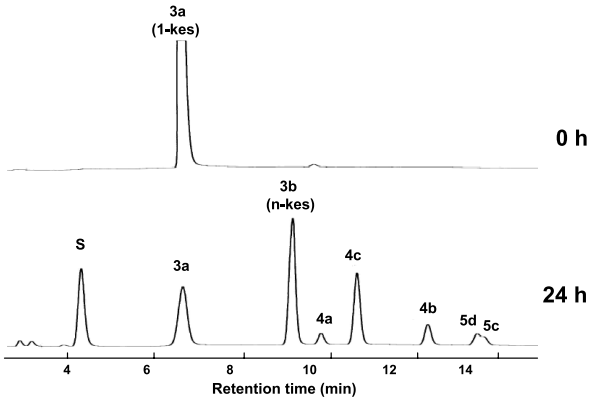

Fig. 6. Anion exchange chromatogram of inulin neotype oligosaccharides produced from 1-kestose using fructan: fructan 6^G-fructosyltransferase (6G-FFT) from asparagus leaves.

fructosyltransferases were obtained by degenerate PCR and cDNA library screening. The obtained cDNAs (*aoft1*, *aoft2* and *aoft3*) resembled other plant fructosyltransferases and invertases.

Isolated *aoft1* consisted of 2201 bp, and contained an open reading frame (ORF) of 1833 bp and a poly(A) sequence at the 3′-end. The ORF encoded a polypeptide of 610 amino acids, as shown in Fig. 7. The molecular mass and *p*I of the deduced polypeptide (designated AoFT1) were calculated to be 68,311 Da and 5.4, respectively. The deduced polypeptide had six potential *N*-glycosylation sites. The primary sequence of *aoft1* showed the highest identity, 68%, with onion 6G-FFT.[23,30] A recombinant protein was obtained by expression of *aoft1* in *P. pastoris* in order to investigate its enzymatic properties.

When reacting recombinant AoFT1 protein with 100 mM 1-kestose for 1 h, the initial products were 4c and sucrose, indicating that the recombinant protein catalyzed fructosyltransfer from 1-kestose to the 6th OH of the glucosyl residue of another 1-kestose, with liberation of sucrose, as shown in Fig. 8. After incubation for 2 h, neokestose was produced. This protein catalyzed fructosyltransfer from 1-kestose to the 6th OH of the glucosyl residue of the liberated sucrose. In addition to oligosaccharides of the inulin neotype resulting from 6G-FFT activity, prolonged incubation of recombinant AoFT1 protein with 1-kestose produced a small amount of nystose, indicating that the protein also has weak 1-FFT activity. The reaction mixture at 24 h also contained 4b, 5c and 5 d. These oligosaccharides were previously reported to be synthesized from 1-kestose by 6G-FFT[16,21,22] and 1-FFT[16,20] activities in asparagus and in onion.[15]

Consequently, the AoFT1 recombinant protein expressed in *P. pastoris* was confirmed to be 6G-FFT.

Isolated *aoft2* consisted of 2190 bp, and contained an ORF of 1875 bp and a poly(A) sequence at the 3′-end. The ORF encoded a polypeptide of 624 amino acids, as shown in Fig. 9. The molecular mass and *p*I of the deduced polypeptide (designated AoFT2) were calculated to

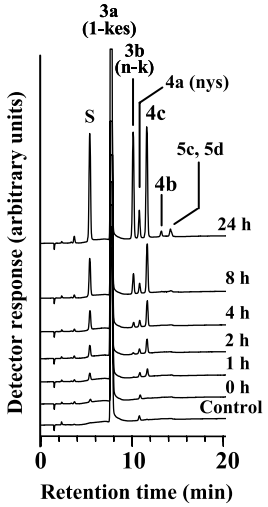

Fig. 8. High performance anion-exchange chromatograms of the reaction products formed by recombinant protein AoFT1.

A reaction mixture containing AoFT1 was incubated with 100 mM 1-kestose. As a control, reaction mixtures containing protein extract obtained by using the empty vector were incubated with each substrate for 24 h. S, sucrose; 1-kes, 1-kestose; n-k, neokestose; nys, nystose; 4c, 1^F, 6^G-di-β-D-fructofuranosylsucrose; 4b, 6^G(1-β-D-fructofuranosyl)$_2$sucrose; 5c, 1^F(1-β-D-fructofuranosyl)$_2$-6^G-β-D-fructofuranosylsucrose; 5d, 1^F-β-D-fructofuranosyl-6^G(1-β-D-fructofuranosyl)$_2$sucrose.

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        10          20          30          40          50          60          70
MATSLQAPIL  GSRPPRRTLR  FLSFALFSAL  VLVVASFSSR  KSESGSGLRS  GSVEPEYAWT  NQMLTWQRAG
        80          90         100         110         120         130         140
FHFRTVKNYM  NDPSGPMYYK  GWYHLFYQHN  PNYAYWGDIS  WGHAVSRDLL  NWFHLPVAVK  PDRWYDIYGV
       150         160         170         180         190         200         210
WTGSITVMPD  DGRVVMLYTG  GTKEKYQIMS  VAMAADPSDP  LLVEWVKYDE  VNPVLRPPPG  IGLTDFRDPN
       220         230         240         250         260         270         280
PIWYNTTDST  WQLVIGSKND  SLQHTGIAMV  YTTKDFINLT  LLPGVLHSVD  HVGMWECVDL  FPVASSGPLI
       290         300         310         320         330         340         350
GRGLDRSMML  ADNVKHVLKA  SMNDEWHDYY  AIGSYDVATH  RWVPDDESVD  VGIGMRIDWG  KFYASRTFYD
       360         370         380         390         400         410         420
PVKERRVMWG  YVGETDSGDA  DVAKGWASFQ  GIPRTVLFDV  KTGTNVLTWP  IEEVESLRMT  RKDFSDIVVN
       430         440         450         460         470         480         490
KGSTVELHVG  DANQLDIEAE  FEMDKDALET  AIEADIGYNC  SSSGGAVSRG  VLGPFGLFVL  ANQDLTELTA
       500         510         520         530         540         550         560
TYFYVSRATD  GSLHTHLCHD  EMRSSKANDI  VKRVVGGTFT  VLDGELLSLR  ILVDHSIVES  FAQGGRTSAT
       570         580         590         600         610
SRVYPTEAIY  ERARVFLFNN  ATGATITAKA  VKVWQMNSTS  NQYYPFTSSN
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Fig. 7. Deduced amino acid sequence of *aoft1*.

AoFT1, deduced amino acid sequence of asparagus fructan: fructan 6^G-fructosyltransferase (accession no. AB084283). Potential *N*-glycosylation sites in the sequence of AoFT1 are marked by underlines. Double underlined sequences indicate the conserved region in the GH 32 family.

be 68,837 Da and 4.97, respectively. The deduced polypeptide had five potential *N*-glycosylation sites. The primary sequence of *aoft2* showed highest identity, 64%, with asparagus 6G-FFT.[23] Recombinant AoFT2 protein was obtained by expression of *aoft2* in *P. pastoris* to investigate its enzymatic properties.

When reacting the recombinant AoFT2 protein with 100 mM 1-kestose for 1 h, the initial products were nystose and sucrose, as shown in Fig. 10. Prolonged incubation of AoFT2 protein with 1-kestose also produced fructosylnystose. Inulin neotype fructo-oligosaccharides such as neokestose 4c were not detected.

These results indicate that AoFT2 has 1-FFT activity, but not 6G-FFT activity. AoFT2 protein did not show any 1-SST or 6-SFT activity in the reaction with 100 mM sucrose as a sole substrate (date is not shown). Consequently, AoFT2 protein expressed in *P. pastoris* was confirmed to be 1-FFT and was secreted into the culture broth.

Isolated *aoft3* consisted of 2107 bp, and contained an ORF of 1887 bp and a poly(A) sequence at the 3′-end. The ORF encoded a polypeptide of 628 amino acids, as

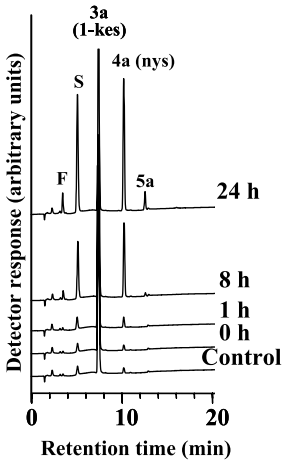

Fig. 10. High performance anion-exchange chromatograms of the reaction products formed by recombinant protein AoFT2.

A reaction mixture containing AoFT2 was incubated with 100 mM 1-kestose. As a control, reaction mixtures containing protein extract obtained by using the empty vector were incubated with each substrate for 24 h. S, 3a, 4a and 5a represent the same saccharides as listed in Figs. 1 and 8.

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        10         20         30         40         50         60         70         80
MGSPDLESHA PLVREAALES RPPRRNLGLL LLGPLVASLL ALVYFLGDKP GSSQGLGSSS GSDEDEFPWT DRMLKWHHTA
        90        100        110        120        130        140        150        160
FHFQPPRNFM SDPSGPIYYR GWYHFFYQHN TNAAYWGHIA WGHAATPDLL NWVHLPVAVY PDHWYDIEGD WTGSVAALPD
       170        180        190        200        210        220        230        240
GRVVMLFTGG VGAVGNELAQ VVNVAWAADP DDPLLTRWVK QEGNPVLVSP PGIGLKDFRD PNPAWYDSSS STWYVLVGSK
       250        260        270        280        290        300        310        320
NDSLSHTGIA LVYTTTDFLS YTLLPGILHS VDIVGMWECT DLYPVSVSGP STHLGLENSV PPGENVKHVL KAGLNDEWHD
       330        340        350        360        370        380        390        400
YYAIGTYDRE GNKWTPDDES LDVGIGLRYD WGKFYASRTF YDPVKRRRVL WGYVGETDTR SVDVQKGWAS VEGLPRTVLF
       410        420        430        440        450        460        470        480
DVKTGSNLLT WPAEEVESLR SSSKNFSNIA IAAGSTVHLD VEDANQLDIE AEFVIKKEEL ELAIQADVNY NCSTSDGASQ
       490        500        510        520        530        540        550        560
RGLLGPFGLL VLANQDLSEQ TATYFYVGRG TDGSLQTHLC QDELRSSKAN QITKRVVGHT VPVLDDETLT LRILVDHSIV
       570        580        590        600        610        620
ESYAQGGRAS TTSRVYPTQA IYEDAKVFLF NNATGATVIA KSVKIWQMSP TSNRSHGYPG SQAL
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Fig. 9. Deduced amino acid sequence of *aoft2*.

AoFT2, the deduced amino acid sequence of asparagus fructan : fructan 1-fructosyltransferase (accession no. AB115554). Potential *N*-glycosylation sites in the sequence of AoFT2 are marked by underlines. Double underlined sequences indicate the conserved region in the GH32 family.

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        10         20         30         40         50         60         70         80
MASPSDLESP PTLSAQLLES RPPRSKLRLV ALTLTAAAFL VALALFLADG SASRFVSGLA RKLRSDPIKE HDYPWTNEML
        90        100        110        120        130        140        150        160
TWQRSGFHFQ PAKNFQSDPN AAMYYKGWYH FFYQYNPTGT AWDYTISWGH AVSRDLIHWL HLPMAMVPDH WYDAKGVWSG
       170        180        190        200        210        220        230        240
YSTLLPDGRV IVLYTGGTPE LVQVQNLAVP ADASDPLLLK WKKSSVNPIL VPPPGIGTSD FRDPFPIWYN ETDSNWHVLI
       250        260        270        280        290        300        310        320
GSKDSNHHGI VLLYKTKDFF NFTLLPSLLH TSTQSVGMFE CVDLYPVATG GPLSNRGLEM SVDLSNGGIK HVLKASMDEE
       330        340        350        360        370        380        390        400
RHDYYAIGTF DLDSFKWTPD DPSIDVGVGL RYDWGKFYAS KTFFDTEKQR RILWGYVGEV DSKDDDKMKG WATLQNIPRT
       410        420        430        440        450        460        470        480
ILLDTKTQSN LIIWPVEEVE DLRTDGNIFN DIKIGAGSSV QLDIGAASQL DIEAEFELDN SALDGAIEAD VTYNCSTSGG
       490        500        510        520        530        540        550        560
AANRGLLGPF GLLVLANQDL TEQTATYFYV SRGTDGDLRT HFCQDELRSS KAGDIVKRVV GSVVPVLHGE TWSLRILVDH
       570        580        590        600        610        620        630
SIIESFAQRG RAVATSRVYP TEAIYNKARL FLFNNATDAK VTAKSVKIWH MNSTHNHPFP GLESLFES
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Fig. 11. Deduced amino acid sequence of *aoft3*.

AoFT3, the deduced amino acid sequence of asparagus sucrose : sucrose 1-fructosyltransferase (accession no. AB115555). Potential *N*-glycosylation sites in the sequence of AoFT3 are marked by underlines. Double underlined sequences indicate the conserved region in the GH32 family.

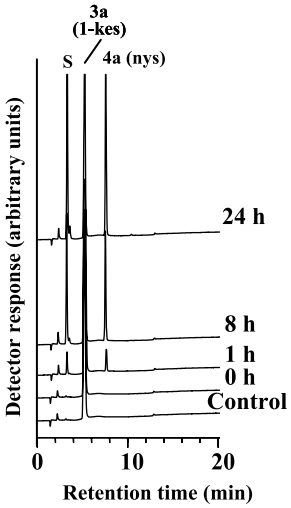

Fig. 12. High performance anion-exchange chromatograms of the reaction products formed by recombinant protein AoFT3.

A reaction mixture containing AoFT3 was incubated with 100 mM sucrose. As a control, reaction mixtures containing protein extract obtained by using the empty vector were incubated with each substrate for 24 h. G, glucose; S and 3a represent the same saccharides as listed in Fig. 8.

shown in Fig. 11. The molecular mass and *p*I of the deduced polypeptide (designated AoFT3) were calculated to be 70,073 Da and 5.4, respectively. The deduced polypeptide had five potential *N*-glycosylation sites.

The primary sequence of *aoft3* showed the highest identity, 71%, with onion 1-SST.[31] Recombinant AoFT3 protein was obtained by expression of *aoft3* in *P. pastoris* to investigate its enzymatic properties.

When reacting AoFT3 protein with 100 mM sucrose for 1 h, the initial products were 1-kestose and glucose, as shown in Fig. 12. Prolonged incubation of AoFT3 with 1-kestose also produced small amounts of nystose. Inulin neotype fructo-oligosacchrides, such as neokestose 4c, were not detected. These results indicate that AoFT3 has 1-SST activity. The AoFT3 protein expressed in *P. pastoris* was confirmed to be 1-SST.

2) *Conversion of 6G-FFT to 1-FFT by point mutation in a β-fructosidase motif.*

Asparagus fructosyltransferases also belong to glycoside hydrolase family 32, which includes invertase and fructosyltransferase from plants, and invertase, inulinase and levanase from bacteria and fungi.[32] Recombinant AoFT1, AoFT2 and AoFT3 proteins contained some of the conserved amino acid sequences observed in various fructosyltransferases and invertases, as shown in Fig. 13.

Two catalytic motifs were confirmed to be NDPN and EC in the primary sequence of yeast invertase by affinity labeling and site-directed mutagenesis.[33,34] The Asp (D) and Glu (E) residues in each motif in yeast invertase were identified as nucleophiles and proton donors, respectively. AoFT1, AoFT2 and AoFT3 possess the putative catalytic residues conserved among other plant enzymes belonging to this family, and the sequences around these residues were also highly conserved.

The asparagine in the β-fructosidase motif in AoFT1 was changed to a serine, similarly to AoFT2. Site-directed mutant of the amino acid residue was performed and the mutant recombinant protein was characterized. When 100

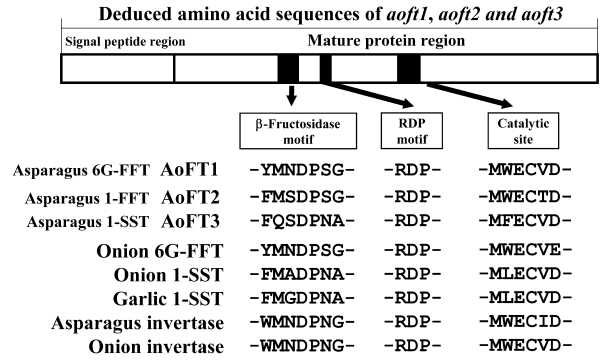

Fig. 13. Comparison of amino acid sequences of highly conserved regions of asparagus fructosyltransferases with those of plant fructosyltransferases and invertases.

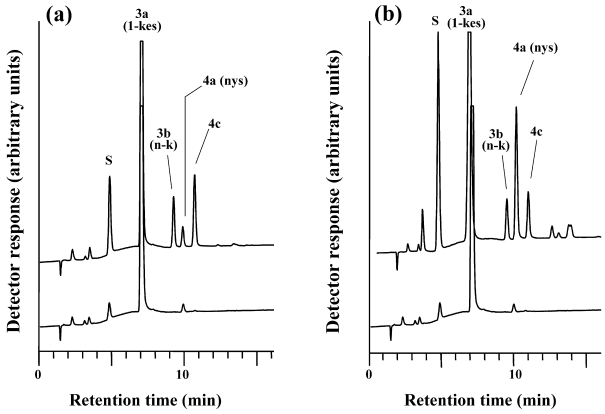

Fig. 14. High performance anion-exchange chromatograms of the reaction products formed from 100 mM 1-kestose by recombinant 6G-FFT wild type (a) or mutant N81S (b). S, 3a, 3b, 4a, 4b and 4c represent the same saccharides as listed in Fig. 8.

mM 1-kestose was given as a sole substrate, AoFT1 mutant protein (N81S) mainly produced nystose, while wild-type AoFT1 produced 4c or neokestose, as shown in Fig. 14. These results suggest that amino acid substitution in the β-fructosidase motif of wild-type AoFT1 altered substrate specificity, from 6G-FFT to 1-FFT, and was similar to those observed in onion bulbs.[35]

3. *Post-harvest metabolism of fructo-oligosaccharides in onion and asparagus plants*

Alongside the studies on fructo-oligodsaccharide accumulation during seed development[36] and bulbing,[37] we also studied the main enzyme activities involved in the synthesis and the hydrolysis of fructo-oligosaccharides during the pre-harvest[38] (growth), and post-harvest[38,39] (storage) life of onion bulbs and asparagus spears,[40] although present knowledge is insufficient to clearly explain the mechanisms triggering enzymes or the mechanisms by which fructo-oligosaccharides contribute to bulb quality and perishability.[39]

Storage of onion bulbs for 6 months causes a decrease[41,42] in the contents of glucose, fructose and sucrose, as shown by Fig. 15. Fructan contents also decrease significantly after this period, and highly polymerized fructans (DP 5 to 12) were strongly hydrolyzed. However, tri and tetra saccharides appear to play a regulatory role between the highly polymerized and disaccharides by regulating fructan hydrolase activity and preventing excessive

accumulation of fructose and sucrose, as reported previously.[41–44] To illustrate the kinetics of fructo-oligosaccharide degradation during bulb storage, the comparative ratio of degrading enzymes to synthesizing enzymes was determined during storage, as shown in Fig. 16. The ratio was low at the beginning of storage, ranging between 0.31 and 0.45, and it peaked at 2.29 during week 12, before decreasing abruptly to values 2-fold higher than those observed during the beginning of the experiment (between 0.75 and 1.26). These results indicate that fructan degradation is controlled by the balance between the different fructo-oligosaccharides of the bulbs.[45,46] Linear regression of fructo-oligosaccharide content of three onion cultivars is shown by Fig. 17. Linear regressions were similar under the three temperature regimens. As shown by the equations for the linear regression curves, the coefficient of regression (R^2) ranged between 0.950 and 0.987, 0.947 and 0.999, and 0.917 and 0.978 for the Yellow Spanish (*YS*), Red Amposta (*RA*) and Tenshin (*Ts*) cultivars, respectively. This indicates that, in the range studied, mobilization of fructo-oligosaccharides during re-growth of onion bulbs occurs independently of temperature.[47]

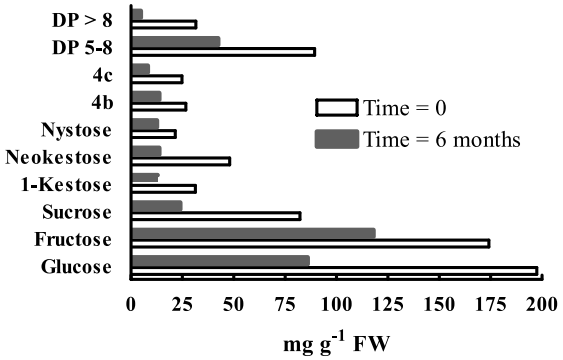

Fig. 15. Effect of storage time on contents of glucose, fructose, sucrose and fructo-oligosaccharides in onion bulbs kept 6 months at 20°C.

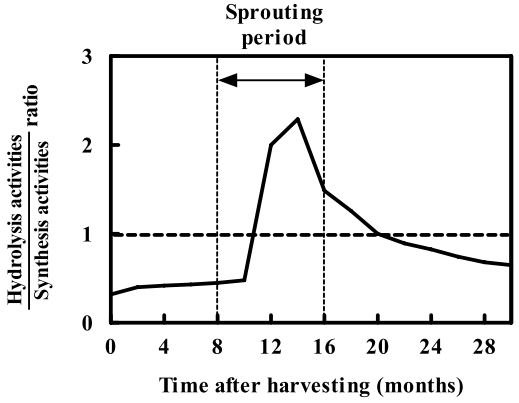

Fig. 16. Profile of the estimated ratio of the enzyme activities of the hydrolysis to those of the synthesis in onion bulb tissues under different temperatures.

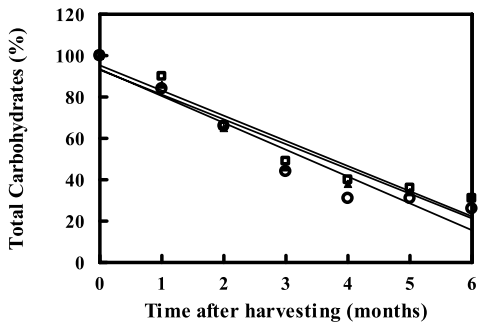

Fig. 17. Linear regression lines and coefficient of determination (r^2) of the total carbohydrates *versus* keeping time after harvesting at different temperatures
□, 10°C; ▲, 15°C; ○, 20°C

Studies on fructo-oligosaccharide degradation in onion bulbs of different cultivars during long-term storage at different temperatures have shown that hydrolysis of fructo-oligosaccharides is more rapid at the beginning of storage, and that hydrolysis increases with temperature. The results also showed that low temperatures did not slow down hydrolysis, even though fructo-oligosaccharide hydrolysis occurs more rapidly at high temperatures. The various data obtained to date suggest that degradation of fructo-oligosaccharides in stored onions may be more strongly influenced by the physiological process of sprouting, and may depend on the demand by growth tissues.

Comparatively extensive literature reported on the metabolism of fructo-oligosaccharides in asparagus plants during growth, while post-harvest life literature reported mainly on saccharides.[40] However, nothing has been reported for the metabolism of fructo-oligosaccharides except the work of Shiomi *et al*.[48] The results showed that only short chain fructo-oligosaccharides (1-kestose and nystose) are present and their contents in the spears is very low compared to those of glucose, fructose and sucrose. They also showed the metabolizing enzymes of these fructo-oligosaccharides play a role in the balance of sugars between the bottom and the top of the spears where high catabolic activities were observed.[48] These results suggest that high fructo-oligosaccharede content in asparagus spears may extend the rapid decline of sugars in the top portion.

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フルクタンと関連糖質およびその合成酵素の食品生化学的研究

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現在，天然と化学合成由来のオリゴ糖は500種以上を数え，わが国ではプレバイオティクス効果やミネラル吸収促進効果等を有する機能性糖質素材として20数種が開発されている．フルクタンの仲間であるフルクトオリゴ糖もその一種であり，1-ケストース，ニストースは結晶品として*Aspergillus*，*Arthrobacter*，*Aureobasidium*，*Penicillium*，*Eurotium*，*Scopulariopsis*などの微生物起源のβ-fructofuranosidaseやfructosyltransferaseを用いて工業的に製造されている．このようなフルクトオリゴ糖やフルクタンは植物にも多く存在し，植物にとって貯蔵炭水化物として重要な役割を果たしている．ユリ科植物であるアスパラガスやたまねぎにはイヌリン型フルクタンとシュクロースおよびイヌリン型フルクタンのグルコース分子の6位炭素の水酸基にフルクトースあるいはフルクトース鎖が結合したイヌリンネオ型フルクタンが存在している．これらの糖の合成経路を精査したところアスパラガスではイヌリン型フルクタンはシュクロースから1-ケストースを生成するsucrose 1-fructosyltransferase (1-SST) や1-ケストースからニストースを生成する1^F-fructosyltransferase (1-FFT) により合成され，イヌリンネオ型のフルクタンはシュクロースおよびイヌリン型フルクタンのグルコース分子の6位炭素の水酸基へ選択的にフルクトースを転移する新規6^G-fructosyltransferase (6G-FFT；新規登録，EC2.4.1.243) により，すなわち3種の酵素により合成されることがわかった．しかし，たまねぎのフルクトオリゴ糖合成は1-SSTと1-FFT活性を有する6G-FFTの2種の酵素が関与しており，アスパラガスの場合と異なっていた．このたまねぎ6G-FFTと微生物由来1-SST類似酵素のハイブリッドシステムによりシュクロースからイヌリンネオ型オリゴ糖への高効率合成に成功した．イヌリンネオ型のフルクタンは，イヌリン型のものと比べてビフィズス菌の利用性が高いことから，このネオシリーズ糖の食品素材としての利用に興味がもたれる．現在までにこの糖を合成する微生物由来の酵素が見出されておらず，アスパラガス由来の6G-FFTの有効利用が期待される．さらに1-SST，1-FFT，6G-FFTのような転移位置の選択性の高い植物酵素も工業的に利用することができれば浸透圧がより低く，消化管にやさしい新規な構造を有するフラクトオリゴ糖を効率よく生産できると考えられる．したがって高等植物起源のfructosyltransferaseの大量生産技術を確立するためにアスパラガス，たまねぎcDNAライブラリーから1-SST，1-FFTおよび6G-FFTをコードするcDNAを単離し，その塩基配列を解析するとともにメタノール資化性酵母による組み換え酵素の発現を行い，さらに部位特異性変換による6G-FFTの機能改変を試みた．得られた組み換え酵素の性質はアスパラガス根の精製1-SST，1-FFT，6G-FFTの性質と各々同様であった．このように植物酵素遺伝子の食品工業への基礎的利用技術が確立された．一方，アスパラガス，たまねぎの貯蔵中におけるフルクトオリゴ糖代謝を調査し休眠打破との関連性も提案した．