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# Purification, Cloning and Functional Characterization of Fructan: Fructan 1-Fructosyltransferase from Edible Burdock (*Arctium lappa* L.)

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**Abstract: A fructan:fructan 1-fructosyltransferase (1-FFT) was purified from edible burdock for the first time, and a cDNA encoding 1-FFT was isolated from the plant. Two 1-FFTs named 1-FFTa and 1-FFTb were purified from extract of edible burdock by ammonium sulfate precipitation, followed by chromatographies on DEAE-Sepharose CL-6B, Toyopearl HW-55S and Sephadex G-100 columns. Inulin-type fructan such as nystose and fructooligosaccharides with higher DP were produced from 1-kestose by purified 1-FFTs. The general properties of both purified enzymes were very similar to each other. The purified 1-FFTa and 1-FFTb showed a single band by native PAGE and two bands, relative molecular masses of about 46,000 and 17,500 for 1-FFTa and 46,000 and 17,000 for 1-FFTb, by SDS-PAGE, respectively. The N-terminal sequences of the 46,000 peptides of both enzymes were the same, and those of the 17,500 and the 17,000 peptides were also identical. Based on the sequences, the 1-FFT cDNA named *alft1* was cloned. The *alft1* encoded a polypeptide of 617 amino acids. The relative molecular mass and p*I* of the mature protein region of deduced polypeptide were calculated to be 60,213 and 4.89, respectively. The characteristics of recombinant protein produced by *Pichia pastoris* closely resembled those of the native 1-FFTs purified from edible burdock. In this study, we demonstrated that *alft1* encoding edible burdock 1-FFT was involved in the elongation of fructosyl chains of fructooligosaccharides in edible burdock.**

**Key words: fructan, fructooligosaccharides, fructosyltransferase, edible burdock**

Fructans (polyfructosylsucrose) are important storage carbohydrates in plants such as Poaceae (*e.g.*, wheat and barley), Asteraceae (*e.g.*, chicory and Jerusalem artichoke) and Liliaceae (*e.g.*, onion and asparagus).[1,2] Inulin-type fructan [$1^F$(1-β-D-fructofuranosyl)$_m$ sucrose], which is a β-2,1 linked fructose-oligomer or -polymer terminated by glucose, is mainly accumulated in Asteraceae plants. The saccharide is synthesized by sucrose : sucrose 1-fructosyltransferase (1-SST, EC 2.4.1.99) and fructan:fructan 1-fructosyltransferase (1-FFT, EC 2.4.1.100). 1-SST synthesizes 1-kestose (1-β-D-fructofuranosylsucrose, 1-kestotriose), an inulin-type trisaccharide, from two molecules of sucrose by fructosyltransfer.[3–6] 1-FFT elongates fructose chains of inulin-type fructans by fructosyltransfer from 1-kestose to another 1-kestose or fructan.[3,7–9]

We have already studied fructooligosaccharides and fructan metabolizing enzymes in asparagus[4,7,10–12] and onion.[13] Although three enzymes, 1-SST, 1-FFT and $6^G$-FFT (fructan:fructan $6^G$-fructosyltransferase, EC. 2.4.1.243), were involved in the synthesis of fructooligosaccharides in asparagus,[11,12] a two-enzyme system, 1-SST and $6^G$-FFT coupled with 1-FFT activity, was revealed in onion bulbs.[13,14]

In our preliminary study, we found 1-FFT activity in the root of edible burdock (*Arctium lappa* L.), an Asteraceae plant which accumulates inulin-type fructan, and is eaten as a root vegetable rich in dietary fiber in Japan. Although fructooligosaccharide is accumulated in edible burdock, information on the fructan metabolism in the edible burdock is very limited. The synthesis of inulin and fructooligosacchrides in edible burdock is not clear. In this study, we have reported the purification, characterization and cloning and functional analysis of a 1-FFT involved in the synthesis of inulin type fructan from edible burdock.

## MATERIALS AND METHODS

***Plant materials.*** Edible burdock roots (*A. lappa* L.) had been freshly harvested on November from an experimental field of Makubetsu Agricultural Co-operative, Hokkaido, Japan and then stored in soil 1 m deep underground to May. After being washed with water the roots of edible burdock were stored at −40 or −80°C until use.

***Substrates and measurement of enzyme activity.*** Saccharides as substrates were prepared as follows. Crystalline 1-kestose [3a: $1^F$-β-D-fructofuranosylsucrose, 1-kestotriose] and nystose [4a: $1^F$(1-β-D-fructofuranosyl)$_2$ sucrose, 1, 1-kestotetraose] were prepared from sucrose using *Scopulariopsis brevicaulis* β-fructofuranosidase.[15] The standards 5a, 6a, 7a and 8a [$1^F$(1-β-D-fructofuranosyl)$_m$ sucrose, $m$= 3, 4, 5, 6] were prepared from Jerusalem artichoke tubers in our laboratory. The reaction mixture, 25 μL of enzyme in 10 mM sodium phosphate buffer (pH 6.5), 50 μL of 200 mM 1-kestose in distilled water, 25 μL

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of 100 mM sodium phosphate buffer (pH 6.5) and a small amount of toluene, was incubated at 30°C. The reaction was stopped by boiling for 3 min. One unit of 1-FFT activity was defined as the amount of enzyme which produced 1 μmol of nystose per min under the above reaction conditions. For analysis of reaction products, high performance anion-exchange chromatography (HPAEC) was done on a DX300 chromatograph (Dionex Corp., Sunnyvale, USA) with a CarboPac PA-1 anion exchange column (Dionex Corp.) and a pulsed amperometric detector (PAD) as described previously.[16)]

In the experiment on optimum pH, Britton-Robinson buffer (pH 3.0–10.0) was used, and the reaction was stopped by addition of 900 μL of 150 mM sodium hydroxide. For pH-stability profiles, the reaction mixture, 10 μL of enzyme and 20 μL of Britton-Robinson buffer (pH 3.0–10.0), was incubated for 24 h at 4°C, and then the reaction mixture was adjusted to pH 6.5 and was incubated with 1-kestose at 30°C for 1 h. The reaction was stopped by boiling for 3 min. In the experiment on temperature-stability profiles, enzyme solutions were pre-incubated with 0.1 M sodium phosphate buffer (pH 6.5) for 15 min at 25, 30, 35, 40, 45, 50, 55 or 60°C, and then the pre-incubated solution was cooled to 0°C. After the pre-incubation, the mixtures were incubated with 1-kestose at 30°C for 1 h. To examine the effects of metal salts and chemicals on the activity, a mixture of enzyme (0.5 U/mL, 15 μL), 200 mM 1-kestose (50 μL) and 100 mM sodium phosphate buffer (pH 6.5, 25 μL) was incubated at 30°C for 1 h in the presence of water, a metal salt or a chemical (1 mM; 0.1 mM *p*-CMB). After the reaction was stopped by heating at 100°C for 3 min, the mixture was subjected to HPAEC. All the experiments were done in duplicate.

***Quantitative determination of proteins.*** Proteins were determined by measuring $A_{280}$ with reference to $E^{1\%}_{1cm}$ (extinction coefficient) = 9.38 in aldolase.[17)]

***Purification of 1-FFT from edible burdock.*** All operations throughout the purification were performed at 0–4 °C. Roots of edible burdock (200 g each, total 2 kg) were chopped, and then homogenized in 10 mM sodium phosphate buffer (pH 6.5, 400 mL each) containing 2 mM dithiothreitol (DTT). The homogenate was filtered through cheesecloth and centrifuged for 30 min at 10,000 × *g*. The supernatant (4940 mL for 2 kg of edible burdock) was treated with solid ammonium sulfate to give 30% saturation, and stored overnight at 2°C. After centrifugation at 10,000 × *g* for 30 min, ammonium sulfate was added to the supernatant up to 80% saturation, and stored at 2°C. After centrifugation at 10,000 × *g* for 30 min, the precipitate was dissolved in 10 mM sodium phosphate buffer (pH 6.5) containing 2 mM DTT, and then the solution was dialyzed for 2 days against the same buffer. This fraction was applied to a column (4 × 35 cm) of DEAE-Sepharose CL-6B equilibrated with 10 mM sodium phosphate buffer (pH 6.5) containing 2 mM DTT. The absorbed proteins were eluted with a linear gradient of 0–1 M sodium chloride in the same buffer. The active fraction was dialyzed overnight against 100 mM sodium phosphate buffer (pH 6.5) containing 2 mM DTT and 30% saturation of ammonium sulfate. The dialyzate was loaded onto a column (1.6 × 30 cm) of Toyopearl HW55S equilibrated with the same buffer. The elution was achieved with a linear gradient from 30 to 0% saturation of ammonium sulfate in the same buffer. Each active fraction was dialyzed overnight against 50 mM sodium phosphate buffer (pH 6.5) containing 100 mM sodium chloride and 2 mM DTT. The dialyzate was concentrated to 0.5 mL by ultrafiltration on VivaSpin concentrator cutting off at 10 kDa (VivaScience, UK). The concentrated solution was filtered on a column (1.6 × 70 cm) of Sephadex G-100 equilibrated with the same buffer.

***Native- and SDS-polyacrylamide gel electrophoresis (PAGE) and analysis of N-terminal amino-acid sequence.*** Native-PAGE was performed with Phast Gel Gradient 10–15 by a Phast system (GE Healthcare, UK). SDS-PAGE for N-terminal amino-acid sequences was conducted according to the method of Laemmli.[18)] Proteins in the gel were stained with Coomassie Brilliant Blue R-250. After SDS-PAGE of the purified enzyme, the protein band was blotted onto a polyvinylidene difluoride (PVDF) membrane. The N-terminal amino-acid sequences were analyzed using a HP G1005A protein sequencing system (Hewlett-Packard Company, USA).

***Molecular cloning of 1-FFT cDNA from edible burdock.*** From 1.0 g of edible burdock root powder, which was ground in liquid nitrogen, total RNA was prepared using an RNeasy Plant Mini Kit (Qiagen K.K., Tokyo, Japan). The first-stranded cDNA was synthesized, using a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Corporation, Carlsbad, USA). The cDNA was used for degenerate PCR as a template. Degenerate PCR was done using a primer set of p52Fw and p21Rv, which were designed according to amino-acid sequences of the purified enzymes (Table 1). The PCR consisted of an initial 2-min denaturation step (94°C) followed by 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min and then a final step at 72°C for 7 min. The PCR was done using Blend *Taq* polymerase (Toyobo Co., Ltd., Osaka, Japan). The PCR product was subcloned in pGEM-T plasmid, selected in *Escherichia coli* DH5α cells (Toyobo Co., Ltd.). The plasmid was purified by a GenElute Plas-

**Table 1.** PCR primers for cDNA cloning and construction of expression vector.

| Primer name | DNA sequence (5′ to 3′) |
|---|---|
| p52Fw | ACNGCNTTYCAYTTYCARCC* |
| p21Rv | CANGCRTARTCRTCRTTNGC* |
| Gene Racer 5′ primer | CGACTGGAGCACGAGGACACTGA |
| 5′ GSP | CACGGCGTGACCCCATGACATATTGCCC |
| Gene Racer 3′ primer | GCTGTCAACGATACGCTACGTAACG |
| 3′ GSP | CCCATCTACTTCAATGGCCGGTGGAGG |
| FFT_Fw | CTGAACTCACTTACCTCATT |
| FFT_Rv | GCAACATTCTTCCTAAACAGATC |
| V78FwPstI | AAG**CTGCAG**TGCAGCCGTCCGCTG** |
| F618RvKpnI | GA**GGTACC**GAAAAAGGATAAGGCTGGATAA G** |

*R=(A, C), N=(A, T, G, C), Y=(C, T). **Bold characters indicate the sequence of restriction sites.

mid Mini-Prep Kit (Sigma Aldrich Inc., USA), and its insert DNA was sequenced. The insert was denoted as DegeFFT.

On the resulting DNA sequence of DegeFFT, gene-specific primers (5′ GSP and 3′ GSP) were designed, and used for 5′ and 3′ RACEs to amplify the 5′- and 3′-flanking regions of DegeFFT. RACE was done using Gene Racer Kit (Invitrogen Corporation). A template cDNA was synthesized from the total RNA prepared from roots of edible burdock described above. The 5′ and 3′ RACEs consisted of an initial 2-min denaturation step (94°C) followed by 35 cycles (40 cycles in the case of 3′ RACE) of 94°C for 15 s, 63°C for 30 s and 68°C for 1.5 min and then a final step at 68°C for 10 min. The 5′ and 3′ RACE were done using KOD plus (Toyobo Co., Ltd.). After adenine residue was attached to RACE products by an A-addition kit (Qiagen K.K.), these products were subcloned into pGEM-T vector, and then its insert DNA sequenced by the same procedures mentioned above.

On the resulting DNA sequence of the 5′ and 3′ RACEs products, gene-specific primers (FFT_Fw and FFT_Rv) were designed, and used for PCR to amplify the DNA consistent with the open reading frame of the target gene. The PCR was done using KOD plus and 1st strand cDNA prepared from the total RNA described above. The PCR consisted of an initial 2-min denaturation step (94°C) followed by 35 cycles of 94°C for 15 s, 50°C for 30 s and 68°C for 1.5 min and then a final step at 68°C for 10 min. The amplified DNA fragments named FFTm were sequenced by the same procedures mentioned above. Full length cDNA was compiled by overlapping the sequences of FFTm and RACE PCR products (Fig. 1). The full length cDNA was named *alft1*. Oligonucleotide sequences used in the cloning procedure are listed in Table 1. The nucleotide sequence of full length cDNA has been submitted to GenBank, EMBL and DDBJ Nucleotide Sequence Databases under accession number AB479464.

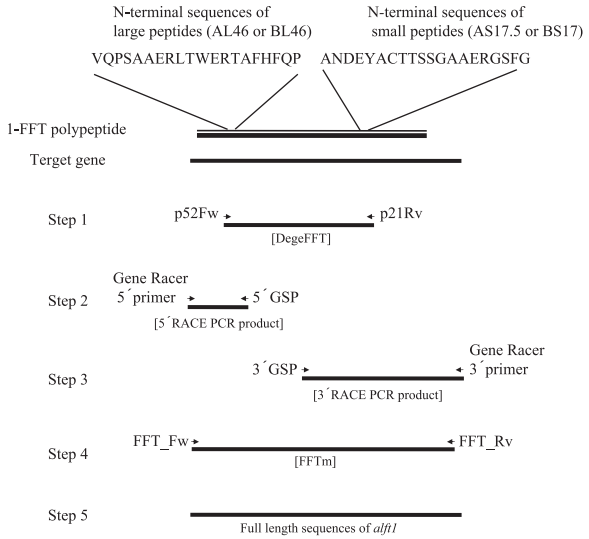


**Fig. 1.** Cloning procedures of cDNA encoding 1-FFT from edible burdock.

About 1000 bp of DNA named DegeFFT was amplified from first strand cDNA of edible burdock by PCR with p52Fw and p21 Rv (Step 1). The outside region of the 5′ end of the DegeFFT was amplified by RACE PCR with Gene Racer 5′ primer and 5′ GSP (Step 2). The outside region of the 3′ end of the DegeFFT was amplified by RACE PCR with Gene Racer 3′ primer and 3′ GSP (Step 3). Amplification of the partial sequence encompassing the mature protein region (named FFTm) was performed by PCR with FFT_Fw and FFT_Rv (Step 4). Full length cDNA was compiled by overlapping the partial sequences of *alft1* and RACE PCR products (Step 5).

***Expression of recombinant proteins in a methylotrophic yeast.*** The isolated cDNA, named *alft1*, was expressed in the methylotrophic yeast *Pichia pastoris* with the secretory expression vector pPICZαB (EasySelect *Pichia* Expression Kit, Invitrogen Corporation). To construct expression plasmids named pPic_alft1, a DNA fragment containing a gene sequence of *alft1* corresponding to the mature protein region was amplified by PCR using V78FwPstI and F618RvKpnI as primers. These primers also had recognition sequences of *Pst*I and *Kpn*I in forward and reverse primers, respectively (Table 1). The PCR conditions were 1 cycle of 94°C for 2 min, 30 cycles of 94°C for 15 s, 50°C for 30 s and 68°C for 1 min, followed by 1 cycle of 68°C for 5 min using KOD plus and FFTm as template DNA. The PCR products were digested with *Pst*I and *Kpn*I followed by ligation into pPICZαB plasmid vector. The resulting plasmids were sequenced to ensure no alternation of sequence in comparison with that of the original *alft1*.

Transformation and cultivation of *P. pastoris* were performed according to the instructions of the manufacturer with minor modification. *P. pastoris* X-33 was transformed with 20 μg of the *Pme*I-linearized vectors by electroporation, and transformants were selected on YPDS (yeast extract pepton dextrose sorbitol)-Zeocin agar plates. A freshly prepared single colony was inoculated in 5 mL of BMGY (buffered glycerol-complex medium, pH 6.0), and cells were grown at 29°C in a shaking incubator at 200 rpm for 24 h. The cells were collected by centrifugation, resuspended as a cell pellet to an $A_{600}$ of 1.0 in 15 mL of induction medium (buffered methanol-complex medium, BMMY, pH 6.0, containing 2% methanol) and incubated at 29°C for 72 h under aerobic conditions, adding 300 μL of methanol to the culture at intervals of 24 h. The culture was centrifuged and the supernatant was obtained.

The supernatant was concentrated to 1.0 mL. After it desalted with 15 mL of 10 mM sodium phosphate buffer (pH 6.5), the desalted solution was concentrated by ultrafiltration on a VivaSpin concentrator cutting off at 10 kDa (VivaScience). The concentrate was filled up to 1.0 mL with the same buffer and was used as an enzyme solution. All the experiments were done in duplicate.

## RESULTS AND DISCUSSION

***Purification of 1-FFT from edible burdock.***

The enzyme was purified from edible burdock by successive chromatographies using DEAE-Sepharose CL-6B, Toyopearl HW55S and Sephadex G-100 columns. The chromatogram of Toyopearl HW55S is shown in Fig. 2. Edible burdock 1-FFT was separated to two fractions by hydrophobic chromatography, using a Toyopearl HW55S column. Each active fraction was separately chromatographed on a column of Sephadex G-100. The active fractions were named 1-FFTa and 1-FFTb and regarded as

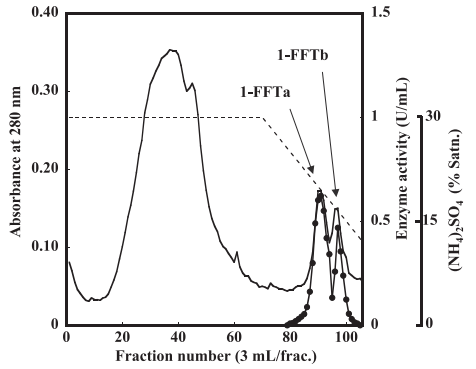


**Fig. 2.** Toyopearl HW55S column chromatography of 1-FFT from edible burdock (*A. lappa* L.).

Closed circles, 1-FFT activity (U/mL); solid line, $A_{280}$; dotted line, $(NH_4)_2SO_4$ (% saturation). The enzyme was applied to a column (ϕ1.6 × 32 cm) of Toyopearl HW-55S equilibrated with 100 mM sodium phosphate buffer (pH 6.5) containing 2 mM DTT and 30% saturation with ammonium sulfate. The elution was done with a liner gradient of 30–0% ammonium sulfate in the same buffer at 20 mL/h.

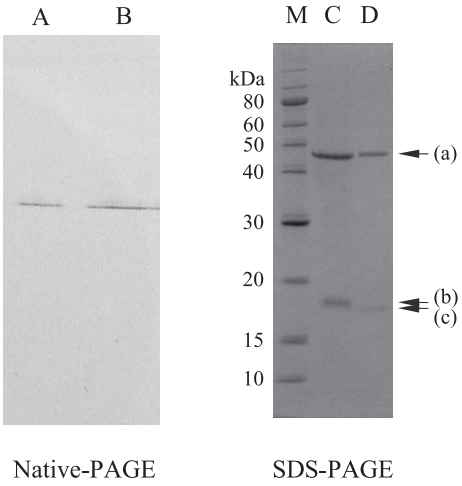


**Fig. 3.** Native- and SDS-PAGE analyses of 1-FFT from edible burdock (*A. lappa* L.).

Native-PAGE: lane A, 1-FFTa; lane B, 1-FFTb. SDS-PAGE: lane M, protein marker (APRO Life Science, Japan); lane C, 1-FFTa; lane D, 1-FFTb. The $M_r$ of the bands, (a), (b) and (c) were estimated to be about 46,000, 17,500 and 17,000. These bands were named AL46 (or BL46), AS17.5 and BS17.

purified enzymes.

Purification procedures of 1-FFTa and 1-FFTb are summarized in Table 2. Specific activities of 1-FFTa and 1-FFTb were 4.57 and 8.19 U/mg of protein. Although each protein showed a single band by native PAGE, two bands were detected by SDS-PAGE (Fig. 3). The bands of large peptides from both enzymes were detected in nearly the same position, and relative molecular masses ($M_r$) of the large peptides were estimated to be about 46,000. On the other hand, the small peptides from both enzymes were detected in slightly differential positions, and the $M_r$ of the small peptides from 1-FFTa and 1-FFTb were estimated to be about 17,500 and 17,000, respectively. The two bands of about 46,000 from 1-FFTa and 1-FFTb were named AL46 and BL46, respectively. In the same manner, the two bands of 17,500 and 17,000 from 1-FFTa and 1-FFTb were named AS17.5 and BS17, respectively. The N-terminal sequences of the AL46 and BL46 peptides were the same (VQPSAAERLTWERTAFHFQP), and those of AS17.5 and BS17 peptides were also the same (ANDEYACTTSSGAAERGSFG).

***General properties of the enzymes.***

The enzymatic properties of 1-FFTa and 1-FFTb were investigated. Optimum pH of both 1-FFTa and 1-FFTb was 6.5 (Table 3). Both of the enzymes were stable from pH 4.5 to 7.5. These enzymes were stable up to 50°C, and were completely inactivated at 60°C.

**Table 3.** Summary of some properties of native and recombinant 1-FFT.

| | Native | | Recombinant |
|---|---|---|---|
| | 1-FFTa | 1-FFTb | |
| pH-activity | 6.5 | 6.5 | 6.8 |
| pH-stability | 4.5–7.5 | 4.5–7.5 | 3.0–8.5 |
| Temperature-stability | ≦50 | ≦50 | ≦45 |

Effects of metal salts and chemicals on the activity of 1-FFTa and 1-FFTb were examined (Table 4). The activities were inhibited by $Zn^{2+}$, $Hg^{2+}$, $CuSO_4$ and $AgNO_3$.

The formation of tetrasaccharides and higher saccharides was determined by HPAEC (Fig. 4). When 1-kestose was incubated with the enzymes, nystose and sucrose were the initial products, and were produced proportionally up to 24 h. Production of 5a was also found with an initial lag phase. Furthermore, 6a, 7a and 8a were gradually produced (Fig. 5).

Enzymatic properties of the two purified enzymes were very similar to each other except that the specific activities of the two purified enzymes differ by a factor of two.

***Molecular cloning of edible burdock 1-FFT cDNA.***

Edible burdock 1-FFT cDNA was obtained by PCR with degenerate primers designed on the basis of amino-

**Table 2.** Summary of purification procedures of edible burdock 1-FFTs.

| | Total protein (mg) | | Total activity (U) | | Specific activity (U/mg) | | Recovery (%) | | Purification (-fold) |
|---|---|---|---|---|---|---|---|---|---|
| | 1-FFTa | 1-FFTb | 1-FFTa | 1-FFTb | 1-FFTa | 1-FFTb | 1-FFTa | 1-FFTb | |
| Crude extract | 81500 | | 362 | | 0.004 | | 100 | | 1 |
| $(NH_4)_2SO_4$ 0.3–0.8 satn. | 15000 | | 110 | | 0.007 | | 30.4 | | 1.8 |
| DEAE-Sepharose CL-6B | 93.5 | | 27.0 | | 0.29 | | 7.5 | | 72.5 |
| Toyopearl HW-55S | 3.33 | 2.30 | 10.45 | 4.73 | 3.15 | 2.06 | 2.9 | 1.3 | |
| Sephadex G-100 | 2.16 | 0.62 | 9.88 | 5.08 | 4.57 | 8.19 | 2.7 | 1.4 | |

**Table 4.** Effect of metal salts and chemicals on the activity of native and recombinant 1-FFT.

| Compound | Concentration (mM) | Relative activity (%) | | |
|---|---|---|---|---|
| | | Native | | Recombinant |
| | | 1-FFTa | 1-FFTb | |
| None | 1 | 100.0 | 100.0 | 100.0 |
| $CaCl_2$ | 1 | 96.8 | 97.2 | 98.7 |
| $ZnCl_2$ | 1 | 56.3 | 51.3 | 36.3 |
| $MgCl_2$ | 1 | 97.1 | 95.1 | 101.7 |
| $CoCl_2$ | 1 | 96.1 | 91.4 | 93.1 |
| $AgNO_3$ | 1 | 42.5 | 42.7 | 4.2 |
| $HgCl_2$ | 1 | 12.2 | 12.5 | 4.2 |
| $FeCl_3$ | 1 | 97.7 | 93.1 | 99.1 |
| $CuSO_4$ | 1 | 18.9 | 7.6 | 6.9 |
| $SnCl_2$ | 1 | 85.1 | 97.5 | 98.3 |
| Sodium deoxycholate | 1 | 93.4 | 104.6 | 91.5 |
| SDS | 1 | 98.2 | 110.7 | 86.5 |
| Triton X-100 | 1 | 96.2 | 106.4 | 96.3 |
| Tween 80 | 1 | 104.7 | 109.4 | 98.9 |
| *p*-CMB | 0.1 | 98.3 | 101.2 | 99.5 |

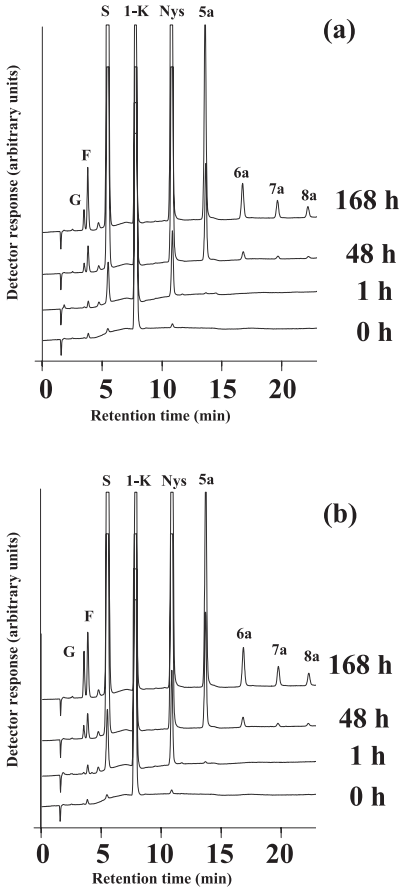


**Fig. 4.** HPAEC analysis of saccharides produced from 1-kestose by 1-FFTa or 1-FFTb purified from edible burdock.

Chromatograms (a) and (b) show saccharides produced from 1-kestose by 1-FFTa and 1-FFTb, respectively. Reaction mixture consisted of enzyme (25 μL), 200 mM 1-kestose (50 μL, 100 mM final concentration), 100 mM sodium phosphate buffer (pH 6.5, 25 μL) and toluene (a trace amount), and was incubated at 30°C. The reaction was stopped by heating in a boiling water bath for 3 min. The reaction mixture was diluted 100 times with distilled water and filtered and an aliquot (25 μL) was applied to HPAEC. G, glucose; F, fructose; S, sucrose; 1-K, 1-kestose; Nys, nystose; 5a, 6a, 7a and 8a, [$1^F$(1-β-D-fructofuranosyl)$_m$ sucrose, $m$= 3, 4, 5 and 6].

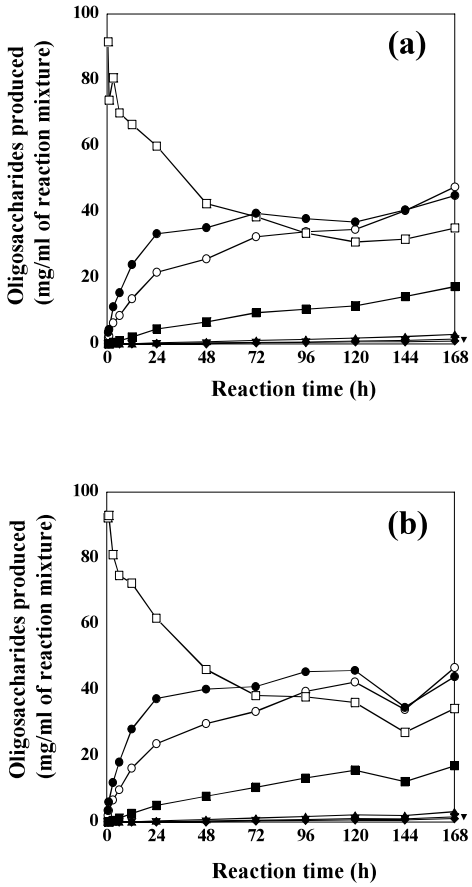


**Fig. 5.** Formation of fructooligosaccharides from 1-kestose by 1-FFTa or 1-FFTb purified from edible burdock.

(a) and (b) indicate formation of saccharides produced from 1-kestose by 1-FFTa and 1-FFTb, respectively. Reaction conditions were described in Fig. 4. Each saccharide is indicated as follows: ○, sucrose; □, 1-kestose; ●, nystose; ■, 5a; ▲, 6a; ▼, 7a; ◆, 8a.

acid sequences of the purified enzyme and the RACE method. The full length cDNA named *alft1* consisted of 2151 bp and contained an open reading frame (ORF) of 1854 bp and a poly (A) sequence at the 3′ end. The ORF encoded a polypeptide of 617-amino acids (Fig. 6). The deduced polypeptide was denoted as AlFT1. The large (AL46 or BL46) and small (AS17.5 or BS17) peptides encoded by *alft1* containing an ORF since the N-terminal sequences of these peptides were identical to AlFT1. Similar results were reported in soluble invertase from carrot (*Daucus carota*)[19] and 1-FFT from chicory (*Chicorium intybus*)[9] and globe thistle (*Echinops ritro*).[20,21] Generally, fructosyltransferases seem to consist of heterodimers containing a large subunit and a small subunit although the reason for the cleavage of purified enzymes is not clear.

The $M_r$ and p$I$ of the mature protein region of AlFT1 were calculated to be 60,213 and 4.89, respectively. The $M_r$ of the mature protein region of AlFT1 was different from the sum of the $M_r$ of AL46 (BL46) and AS17.5 (BS17). The difference in the $M_r$ might be caused by a post-translational modification such as glycosylation. Indeed, the deduced amino-acid sequence contains four potential *N*-glycosylation sites (N-X-S/T). The separation of 1-FFTa and 1-FFTb by hydrophobic chromatography using Toyopearl HW55S column might be affected by glycosylation.

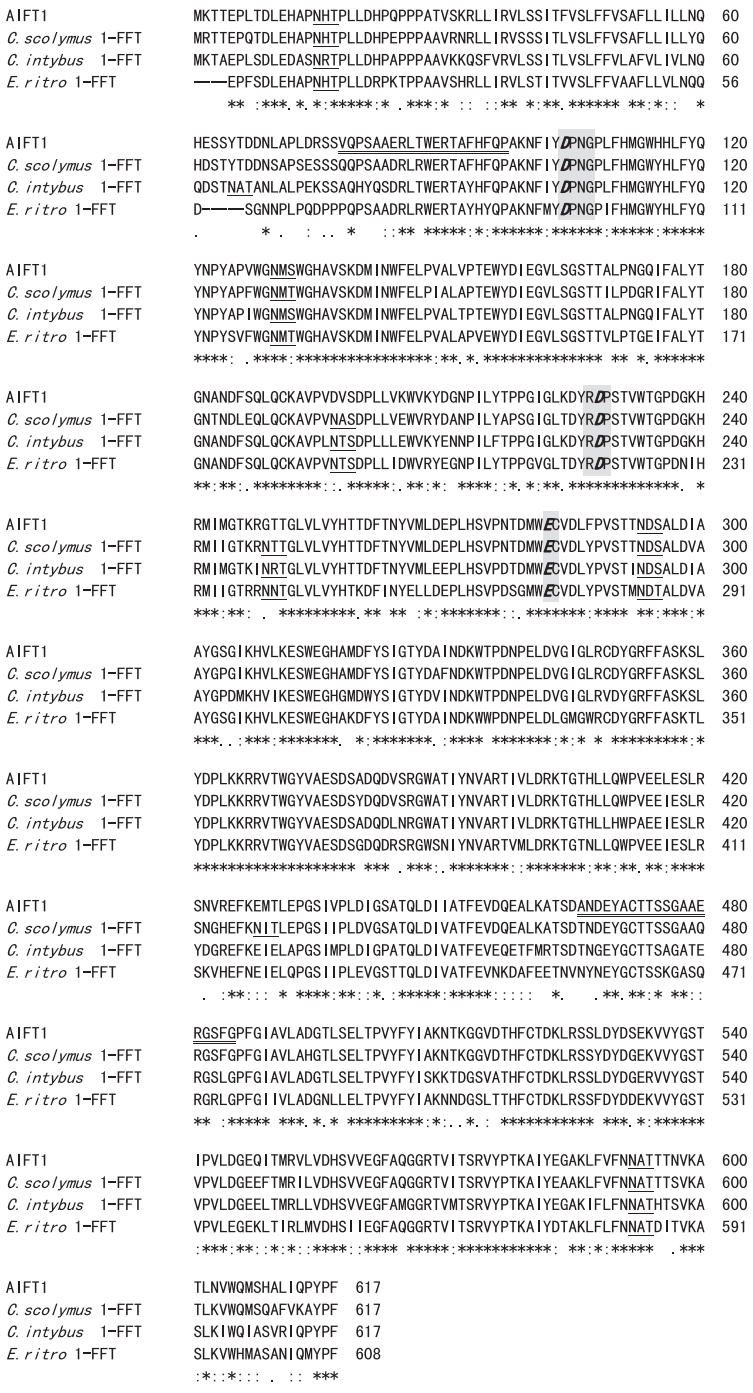


**Fig. 6.** Comparison of deduced amino-acid sequence of *alft1* with those of other Asteraceous 1-FFT.

AlFT1, deduced amino-acid sequence of edible burdock 1-FFT (AB479464); *Cynara scolymus* 1-FFT (AJ000481), *Cichorium intybus* 1-FFT (U84398) and *Echinops ritro* 1-FFT (AJ811624) are aligned. Potential *N*-glycosylation sites in the sequence are underlined. The three carboxylic acids in DPNG, RDP and EC motifs crucial for enzyme activity are bold, italic and shaded in gray. Double underlined sequences indicate the N-terminus of AL46 (or BL46) and AS17.5 (or BS17) peptides. Consensus line: asterisks (*) indicate identical residues; colons (:) indicate conserved substitutions; and periods (.) indicate semi-conserved substitutions.

The N-terminal amino acids of AL46 and BL46 are identical to 78th Val in AlFT1. The first 77 amino acids in AlFT1 probably contain the vacuolar targeting signal because the synthesis of fructan in plants is known to locate in a vacuole,[22,23] and a cleavage site was predicted between the 54th and 55th (or 63th and 64th) amino acids in AlFT1 by signal P server.[24]

In the screening of 1-FFT gene, we could not find other genes encoding a 1-FFT-like protein except for *aleh1*. Therefore, 1-FFTa and 1-FFTb were encoded by *aleh1* although we need the analysis of internal amino-acid sequences of both enzymes or further screening of 1-FFT genes.

AlFT1 belongs to glycoside hydrolase family 32 (GH32), which includes invertases and fructosyltransferases from plant and invertases, inulinases and levanases from bacteria and fungi.[25] AlFT1 contains three conserved amino-acid sequences, such as NDPN (β-fructosidase motif), RDP (RDP motif) and EC (catalytic site), in various fructosyltransferases and invertases (Fig. 6). The Asp (D) and the Glu (E) residues in β-fructosidase and the catalytic site from yeast invertase were identified as a nu-

cleophile and a proton donor, respectively.[26,27] The Asp (D) in RDP motif in levansucrase from *Bacillus subtilis* was identified as a transition state stabilizer.[28] The primary sequence of AlFT1 exhibits high identity with that of asteraceous 1-FFTs such as *Cynara scolymus* (88%), *Cichorium intybus* (82%), *Helianthus tuberosus* (79%) and *Echinops ritro* (76%).

***Heterologous expression of recombinant AlFT1 protein in Pichia pastoris.***

A heterologous expression system using *P. pastoris* is very suitable confirmation of the product translated by encoding the gene of plant fructosyltransferases and invertases.[29] *P. pastoris* does not produce any fructosyltransferases or invertases, and background activities of the enzymes are not detected in culture medium from *P. pastoris*. A recombinant protein was obtained by expression of *alft1* in *P. pastoris*. From the enzyme assay with 1-kestose and nystose as a substrate, 1-FFT activity was detected in the enzyme solution prepared from the culture supernatant of *P. pastoris*. By incubation of recombinant AlFT1 protein with 100 mM 1-kestose for 1, 48 and 168 h, nystose, 5a, 6a, 7a, 8a and other oligosaccharides with higher DP were detected (Fig. 7). Time course of formation of the products from 1-kestose by recombinant 1-FFT were similar to that by native enzymes (data not shown). The optimum pH of recombinant 1-FFT was 6.8. The enzyme was stable in the range of pH 3−8 at 4°C for 24 h. The enzyme was stable up to 45°C for 15 min of incubation. The activity of the recombinant 1-FFT was inhibited by $Zn^{2+}$, $Hg^{2+}$, $CuSO_4$ and $AgNO_3$. The relative residual 1-FFT activity of recombinant enzyme against $AgNO_3$ was 4.2%, whereas those of purified enzymes were about 43% (Table 4). The recombinant and purified 1-FFTs were not affected by the SH reagent. The 1-FFT from other plant such as Jerusalem artichoke and chicory were reported to be not inhibited by other SH reagents, 2-nitrobenzoic acid (DTNB) and iodoacetamide.[8,9] Burdock 1-FFTs seem to be not SH enzyme. As for onion 6G-FFT, detergents such as Tween 80 and Triton X activate the activity.[13] Burdock 1-FFT was not activated by the detergent.

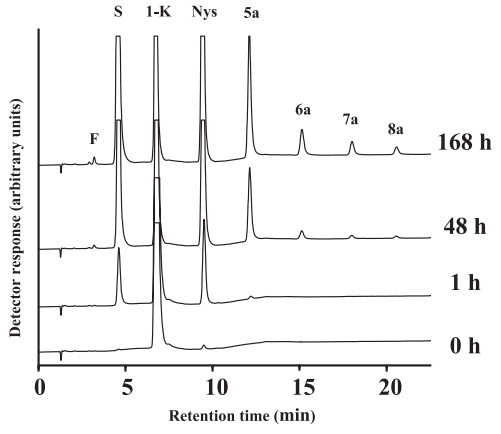


**Fig. 7.** HPAEC analysis of saccharides produced from 1-kestose by recombinant 1-FFT.

Reaction mixture consisted of recombinant enzyme (25 μL), 200 mM 1-kestose (50 μL, 100 mM final concentration), 100 mM sodium phosphate buffer (pH 6.8, 25 μL) and toluene (a trace amount), and was incubated at 30°C. Preparation of HPAEC samples followed the same procedures as in Fig. 4. G, F, S, 1-K, Nys, 5a, 6a, 7a and 8a represent the same saccharides as listed in Fig. 4.

Enzymatic properties of recombinant AlFT1 are summarized with those of native 1-FFTa and 1-FFTb in Table 3. Several plant fructosyltrasferases produced by *P. pastoris* are known to be monomeric proteins, whereas the native enzyme occurs as heterodimers.[21] Furthermore, the difference of *N*-glycosylation between recombinant 1-FFT produced by *P. pastoris* and native 1-FFT was known.[21] From the fact, the differential properties such as enzyme stability and effect of metal salts in native and recombinant 1-FFTs in this study might be affected by difference in sugar chain, monomeric structure or purity of recombinant 1-FFT.

When native and recombinant 1-FFTs were incubated with 100 mM 1-kestose, the formation of nystose, 5a, 6a, 7a and 8a was detected by HPAEC. Moreover, fructans with DP from 9 to 14 were detected by HPAEC (data not shown). Fructooligosaccharides with DP up to 30 were found in edible burdock (our preliminary data). The reason for the difference of maximum DP of fructooligosaccharides between *in vivo* and *in vitro* could be explain the accumulation of relatively high concentration of sucrose in the *in vitro* experiments. Plant 1-FFT was generally known to use sucrose as an acceptor but not to use it as a donor, and acceptor efficiency of sucrose is higher than that of 1-kestose or nystose.[9] Therefore a high concentration of sucrose acts as a competitive inhibitor for fructan elongation.[9,20]

In this study, we demonstrated that *alft1* encoding edible burdock 1-FFT is involved in the elongation of fructosyl chains of fructooligosaccharides in edible burdock.

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## ごぼう (*Arctium lappa* L.) 由来フルクタン: フルクタン 1-フルクトシルトランスフェラーゼの精製，クローニングと機能解析

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ごぼうから初めてフルクタン:フルクタン 1-フルクトシルトランスフェラーゼ (1-FFT) を精製し，それをコードする cDNA をクローニングした．1-FFTa および 1-FFTb と名付けた 2 種の 1-FFT が硫安分画，DEAE-Sepharose CL-6B，Toyopearl HW-55S および Sephadex G-100 の各種クロマトグラフィーを行うことによりごぼう抽出液から精製された．ニストースや高い重合度をもつフルクトオリゴ糖などのイヌリン型フルクタンが 1-ケストースから精製 1-FFT により生成された．両精製酵素の一般性質はよく似ていた．1-FFTa および 1-FFTb 両精製酵素はネイティブ PAGE により単一バンドを示したが，SDS-PAGE では 1-FFTa では分子量が約 46,000 と 17,500 の位置に，1-FFTb では約 46,000 と 17,000 の位置に，それぞれ 2 本のバンドを示した．両酵素の 46,000 のペプチドの N 末端配列は同じ配列であり，両酵素の 17,500 と 17,000 のペプチドの N 末端配列もまた同じ配列であった．これらの配列に基づいて *alft1* と名付けた 1-FFT cDNA をクローニングした．*alft1* は 617 アミノ酸からなるポリペプチドをコードしていた．推定されるアミノ酸配列の成熟タンパク質領域の分子量と等電点は 60,213 および 4.89 と計算された．*Pichia pastoris* で生産された組み換えタンパク質の性質はごぼうから精製した 1-FFT とよく似ていた．本研究において，われわれはごぼう由来 1-FFT とそれをコードする *alft1* がごぼうのフルクトオリゴ糖のフルクトシル鎖の伸長に関与することを示した．