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Cloning and functional characterization of a fructan 1-exohydrolase (1-FEH) in edible burdock (*Arctium lappa* L.)

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Abstract

Background: We have previously reported on the variation of total fructooligosaccharides (FOS), total inulooligosaccharides (IOS) and inulin in the roots of burdock stored at different temperatures. During storage at 0°C, an increase of FOS as a result of the hydrolysis of inulin was observed. Moreover, we suggested that an increase of IOS would likely be due to the synthesis of the IOS by fructosyltransfer from 1-kestose to accumulated fructose and elongated fructose oligomers which can act as acceptors for fructan:fructan 1-fructosyltransferase (1-FFT). However, enzymes such as inulinase or fructan 1-exohydrolase (1-FEH) involved in inulin degradation in burdock roots are still not known. Here, we report the isolation and functional analysis of a gene encoding burdock 1-FEH.

Results: A cDNA, named *aleh1*, was obtained by the RACE method following PCR with degenerate primers designed based on amino-acid sequences of FEHs from other plants. The *aleh1* encoded a polypeptide of 581 amino acids. The relative molecular mass and isoelectric point (*pI*) of the deduced polypeptide were calculated to be 65,666 and 4.86. A recombinant protein of *aleh1* was produced in *Pichia pastoris*, and was purified by ion exchange chromatography with DEAE-Sepharose CL-6B, hydrophobic chromatography with Toyopearl HW55S and gel filtration chromatography with Toyopearl HW55S. Purified recombinant protein showed hydrolyzing activity against β -2, 1 type fructans such as 1-kestose, nystose, fructosylnystose and inulin. On the other hand, sucrose, neokestose, 6-kestose and high DP levan were poor substrates. The purified recombinant protein released fructose from sugars extracted from burdock roots. These results indicated that *aleh1* encoded 1-FEH.

Background

Fructans (polyfructosylsucrose) are important storage carbohydrates in plants such as Poaceae (e.g. wheat and barley), Asteraceae (e.g. burdock, chicory and Jerusalem artichoke), and Liliaceae (e.g. onion and asparagus) [1,2]. Inulin-type fructan [$1^F(1-\beta$ -D-fructofuranosyl) $_m$ sucrose], which is a β -2, 1 linked fructose-oligomer or -polymer terminated by glucose, is mainly accumulated in Asteraceae plants (Figure 1). The inulin-type fructan is synthesized from sucrose 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 chain of inulin-type fructans by fructosyltransfer from 1-kestose to another 1-kestose or fructan [3,7-9]. Levan-type fructan is a β -2, 6 linked fructose-oligomer or -polymer terminated by glucose. Graminan-type fructan is a β -2, 1 and β -2, 6 linked fructose-oligomer or -polymer terminated by glucose. These fructans are mainly found in Poaceae [10-12]. Inulin neoseries -type fructan [$1^F(1-\beta$ -D-fructofuranosyl) $_m$ -6^G(1- β -D-fructofuranosyl) $_n$ sucrose], which has a β -2, 1 linked fructosyl residue(s) on the carbon-6 of terminal glucosyl residue of inulin-type fructan is mainly accumulated in Liliaceae [13-15].

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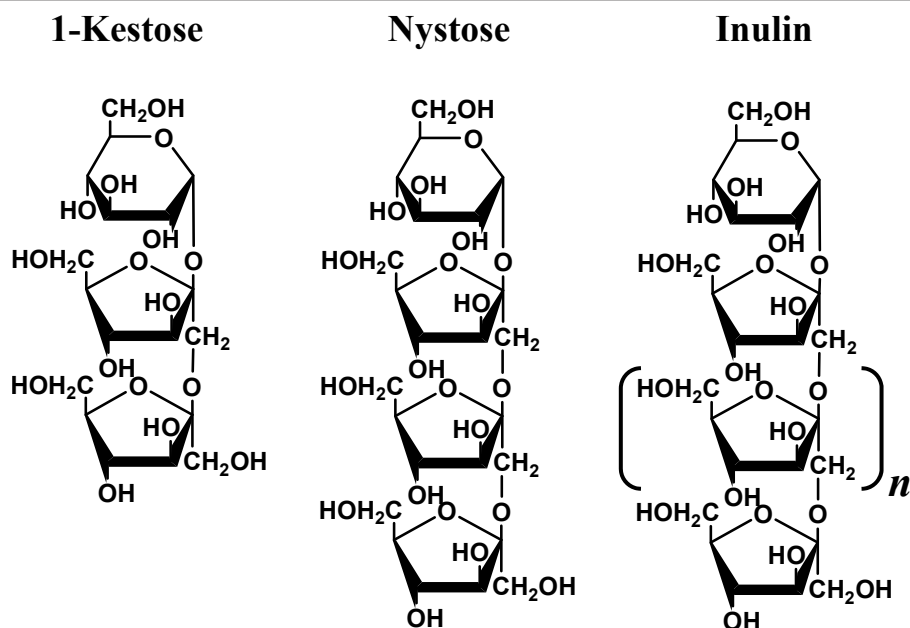


Figure 1 Structures of inulin type fructan; 1-kestose (A), nystose(B) and inulin(C).

Fructan exohydrolases (FEHs) participate in the degradation of plant fructan, and three different types of FEHs such as 1-FEH, 6-FEH and 6&1-FEH have been reported [16-19]. Plant 1-FEH preferentially hydrolyzes the β -2, 1 linkage of terminal fructosyl residue of fructan [16-18]. Plant 6-FEH preferentially hydrolyzes the β -2, 6 linkage of terminal fructosyl residue of fructan [19]. Plant 6&1-FEH can degrade both β -2, 1 and β -2, 6 linkages at the terminal residue [20]. These FEHs are distinguished from invertase (β -fructofuranosidase, EC 3. 2. 1. 26) because the enzymes do not hydrolyze sucrose [21].

Edible burdock (*Arctium lappa* L.) roots are eaten in Japan as it contains much dietary fiber. Edible burdock belongs to the Asteraceae and is known for high content of inulin-type fructan. We have previously reported that an inulin-type fructan accumulated in roots of edible burdock, and we found a cDNA encoding 1-FFT involved in the synthesis of inulin-type fructan from edible burdock [22]. Furthermore, inulooligosaccharides (IOS) as well as inulin-type fructan were detected in burdock roots stored under soil for six month in winter [23], and we assessed the variation of these oligosaccharides and their related metabolizing enzymes stored at different temperatures [24,25]. The IOS and FOS increased progressively during storage at low temperatures [24]. The increase of FOS would likely be due to the hydrolysis of inulin or synthesis by 1-FFT from low DP fructan. One of the reasons for an increase in the IOS was thought to be its biosynthesis by 1-FFT which catalyzes the fructosyltransfer from low DP fructan or inulin to fructose produced by degradation of inulin.

However, the degradation of inulin and fructooligosaccharides in edible burdock was not established. In this study, we demonstrate cloning and functional analysis of a 1-FEH involved in the degradation of an inulin-type fructan of edible burdock.

Results and Discussion

Molecular cloning of edible burdock 1-FEH

Edible burdock 1-FEH cDNA was obtained by the RACE method following PCR using degenerate primers based on the amino-acid sequences of FEHs from other plants (Table 1). A full length cDNA named *aleh1* consisted of 2,063 bp and contained an open reading frame (ORF) of 1746 bp and poly (A) sequence at the 3' end. The ORF

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

Primer name	DNA sequence (5' to 3')
Dp-A	ACIGSITWYCAITTYCARCC*
Dp-C	YTCYTCITYNGGCCAYTG*
Gene Racer 5'primer	CGACTGGAGCACGAGGACACTGA
5Ra-EHa	CACCAGGTCGTATGATACGGCATGCCCCC
Gene Racer 3'primer	GCTGTCAACGATACGCTACTGTAACG
3Ra-EHa	GCGGCAGTGTGATGCACGTGATGAAGGCTG
EHa-FwA	ACTCTCGTACCCAAAG
EHa-Rvl	AGAGCTATCAAACGAAGTCT
EHa-EcoRI.Q34	AG GAATTC AGCGGTTCCAACAACCGTA**
EHa-Xbal	G TTC TAGAGCTGCACCTTTTACGGTCTG**

*N = (A, T, G, C), R = (A, C), S = (C, G), Y = (C, T), W = (A, T), I = inosine.

**Bold characters indicate the sequence of restriction sites.



Figure 2 Comparison of deduced amino-acid sequence of *aleh1* with those of other Asteraceae 1-FEH. AIEH1, deduced amino-acid sequence of *aleh1* (AB611034); Chi_1-FEH2, *Cichorium intybus* 1-FEH IIa (AJ295033); Vh_1-FEH, *Vernonia herbacea* (AM231149) and Chi_1-FEH1, *Cichorium intybus* 1-FEH I (AJ242538) are aligned. Potential N-glycosylation sites in the sequence are underlined. DPNG, RDP and EC motifs are shaded in gray. The three carboxylic acids that are thought to be crucial for enzyme activity are indicated by black inverted triangle. Consensus line: asterisks (*) indicated identical residues; colons (:) indicated conserved substitutions; and periods (.) indicated semi-conserved substitutions. Putative N-terminal amino acid of AIEH1 is indicated with an arrow.

encoded a polypeptide of 581-amino acids (Figure 2). The deduced polypeptide was denoted as AIEH1. The primary sequence of AIEH1 exhibits high identity with that of chicory 1-FEH IIa/IIb (82%) and *Vernonia herbacea* 1-FEH (80%), but only 53% identity with chicory

1-FEH I. Phylogenetic tree analysis based on the deduced amino acid sequences shows that the AIEH1 is present in group of cell wall invertases as well as other FEHs (data not shown) [16-20]. The molecular mass and *pI* of AIEH1 was calculated to be 65,666 and 4.86,

respectively. The amino acid sequence contains three putative *N*-glycosylation sites (N-X-S/T). The AIEH1 contains three regions of amino acid sequences such as NDPN (β -fructosidase motif), RDP (RDP motif) and EC (catalytic site). These regions are conserved in the glycoside hydrolase family 32 (GH32) [26], and the role of the conserved domains of GH32 was identified by Reddy and Maley and by Meng and Fütterer [27-29].

Heterologous expression and purification of recombinant protein

A heterologous expression system using *Pichia pastoris* is very suitable for producing the translation product from the genes of plant fructosyltransferases and invertases [30]. *P. pastoris* does not produce any fructosyltransferases and invertases, background activities of these enzymes were not detected in the culture medium of *P. pastoris*. A recombinant protein was obtained by expression of *aleh1* in *P. pastoris*. From the enzyme assay with 1-kestose as a substrate, hydrolytic activity was detected in the culture medium of *P. pastoris*. The enzyme was purified from the culture medium by ion exchange chromatography with DEAE-Sepharose CL-6B, and hydrophobic and gel filtration chromatographies using Toyopearl HW55S.

The active fraction separated by gel filtration chromatography showed a single band of protein, and the molecular mass was estimated to be about 80,000 by SDS-PAGE. On the other hand, the molecular mass of the purified enzyme deglycosylated by peptide: *N*-glycosidase F (PNGase F) was estimated to be about 65,000 in agreement with the calculated mass of the recombinant protein (Figure 3). Purification procedures used to obtain the recombinant protein are summarized in Table 2. Specific 1-FEH activity of the recombinant protein was 73.4 U/mg of protein.

General properties of the enzyme

Optimum pH of the recombinant AIEH1 was 5.8. The enzyme was stable in the range of pH 5.5-9.0 at 4°C for 24 h (residual activity more than 80%). The enzyme was stable up to 40°C (residual activity more than 90%), and inactivated at 50°C (Table 3) in pre-incubation for 15 min.

To confirm the cleavage type of the recombinant enzyme, the enzyme was incubated with 100 mM sucrose, 1-kestose, nystose and 5% inulin at 30°C for 0, 1, 2, 4, 8 and 24 h. Reaction products were analyzed by high performance anion-exchange chromatography (HPAEC). Sucrose was hardly hydrolyzed. In the reaction mixture of 1-kestose and the enzyme, fructose and sucrose were produced (Figure 4A). When the enzyme was incubated with nystose, fructose and 1-kestose were produced (Figure 4B) and sucrose was also produced by prolonged incubation. A small amount of transferase activity was detected by prolonged incubation of 1-kestose and

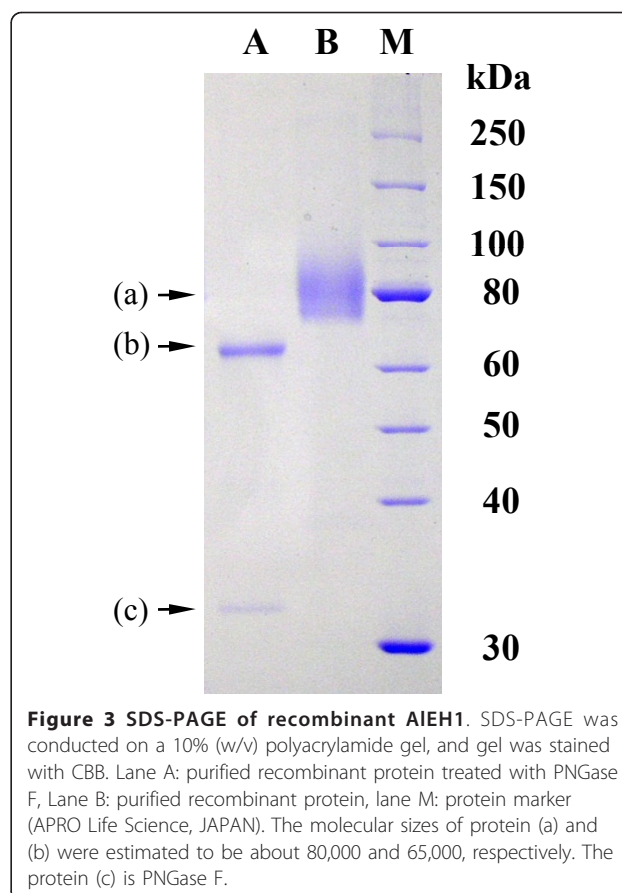


Figure 3 SDS-PAGE of recombinant AIEH1. SDS-PAGE was conducted on a 10% (w/v) polyacrylamide gel, and gel was stained with CBB. Lane A: purified recombinant protein treated with PNGase F, Lane B: purified recombinant protein, lane M: protein marker (APRO Life Science, JAPAN). The molecular sizes of protein (a) and (b) were estimated to be about 80,000 and 65,000, respectively. The protein (c) is PNGase F.

nystose, from which nystose and fructosyl nystose were produced, respectively. In reaction mixtures with inulin, fructose was produced (Figure 4C). IOS was not produced. These results showed that the enzyme is capable of degrading each substrates via an exo-type of cleavage, releasing terminal fructosyl residues as well as 1-FEHs from other plants [16-18].

To examine the substrate specificity of the recombinant enzyme, the enzyme was incubated with several saccharides (Table 4). Sucrose, neokestose, 6-kestose and high DP levan were hardly hydrolyzed. The recombinant 1-FEH showed activity against β -2, 1 type fructans such as 1-kestose, nystose, fructosyl nystose and inulin. From these results, the recombinant enzyme was 1-FEH and was not β -fructofuranosidase or 6-FEH. Therefore, *aleh1* encoded a 1-FEH.

The recombinant 1-FEH hydrolyzed inulotriose 2.5 times slower than 1-kestose although they have β -2, 1 fructofuranosyl linkages.

When the recombinant 1-FEH was incubated for 0, 1 and 24 h with a sugar extract from burdock roots that had been stored in soil for six months (Figure 5), the peak areas of high DP fructan were decreased whereas those of fructose and sucrose were increased. In the reaction

Table 2 Summary of purification procedures of recombinant AIEH1.

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (-fold)
Supernatant	1,027	1,813	1.765	100	1
DEAE-Sepharose CL-6B	55.3	1,003	18.1	55.4	10.3
Toyopearl HW-55S	17.3	469.5	27.1	25.9	15.4
Toyopearl HW-55S	5.9*	433.2	73.4	23.9	41.6

*In final purification step, proteins were measured as described by lowry et al.

mixture of sugar extracts of stored burdock and the recombinant 1-FEH, a decrease in the peak area of inulobiose was observed to be slower than those of other IOS. The reason for slight decrease of the inulobiose might be due to the low activity against inulobiose as shown in Table 4.

Recently, we have reported the variation of total FOS, total IOS and inulin in burdock roots stored at different temperature [24]. During storage in 0°C, increase in FOS derived from the hydrolysis of inulin was observed. Moreover, we suggested that the increase of IOS would likely be due to its synthesis by fructosyltransfer from 1-kestose to accumulated fructose and elongated fructose oligomers which can act as acceptors for 1-FFT. The recombinant protein of *aleh1* showed 1-FEH activity, and produced fructose from sugars of burdock root, fructooligosaccharides and inulin. Therefore, *aleh1* may be encoding the enzyme involved in hydrolysis of inulin and oligosaccharides, and also in provision of fructose as an acceptor for biosynthesis of IOS by 1-FFT in roots of stored burdock. Further study on the expression of *aleh1* in burdock roots during storage at different temperatures is needed for elucidation of the regulation of inulin degradation in burdock roots.

Conclusion

In this study, we described cloning *aleh1* encoding edible burdock fructan 1-exohydrolase and characterization of the recombinant protein of *aleh1* expressed in *Pichia pastoris*. The purified recombinant protein of *aleh1* showed 1-FEH activity which released fructose from 1-kestose, nystose, fructosyl nystose, inulin and sugars extracted from burdock root, but did not show endo-inulinase activity which produced IOS from inulin. Therefore, *aleh1* may be encoding the enzyme not only involved in hydrolysis of inulin but also in provision of fructose as an acceptor for biosynthesis of IOS by 1-FFT in roots of stored burdock.

Table 3 Effects of pH and temperature on 1-FEH activity from recombinant AIEH1

Optimal pH	5.8
pH-stability	5.5~9.0
Temperature-stability (°C)	≤40

Experimental

Plant materials

Edible burdock roots (*Arctium lappa* L.) were freshly harvested in November from an experimental field of the Makubetsu Agricultural Co-operative, Hokkaido, Japan and then stored in soil 1 m deep underground until May. After washing with water the roots were stored at -80°C until use.

Substrates

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 [31]. The standards fructosyl nystose, 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. Sugar extract of burdock roots were prepared from the roots stored for six months [23]. Inulobiose and inulotriose were prepared from stored burdock roots [23]. Sucrose and inulin were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Neokestose was prepared from asparagus roots as described previously [32].

Measurement of enzyme activity

A reaction mixture consisted of 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 of 100 mM sodium phosphate buffer (pH 5.8) and a small amount of toluene was incubated at 30°C. The reaction was stopped by boiling for 3 min. One unit of 1-FEH activity was defined as the amount of enzyme which produced 1 μmol of fructose per min under the above reaction conditions. For analysis of reaction products, HPAEC was done on a DX500 chromatograph (Dionex, USA) with a CarboPac PA-1 anion exchange column (Dionex, USA) and a pulsed amperometric detector (PAD) as described previously [32]. The gradient was established by mixing eluent A (150 mM NaOH) with eluent B (500 mM acetate-Na in 150 mM NaOH) in two ways. System I: 0-1 min, 25 mM; 1-2 min, 25-50 mM; 2-20 min, 50-200 mM, 20-22 min, 500 mM; 22-30, 25 mM. System II: 0-1 min, 25 mM; 1-2 min, 25-50 mM; 2-14 min, 50-500 mM, 14-22 min, 500 mM; 22-30, 25 mM. The

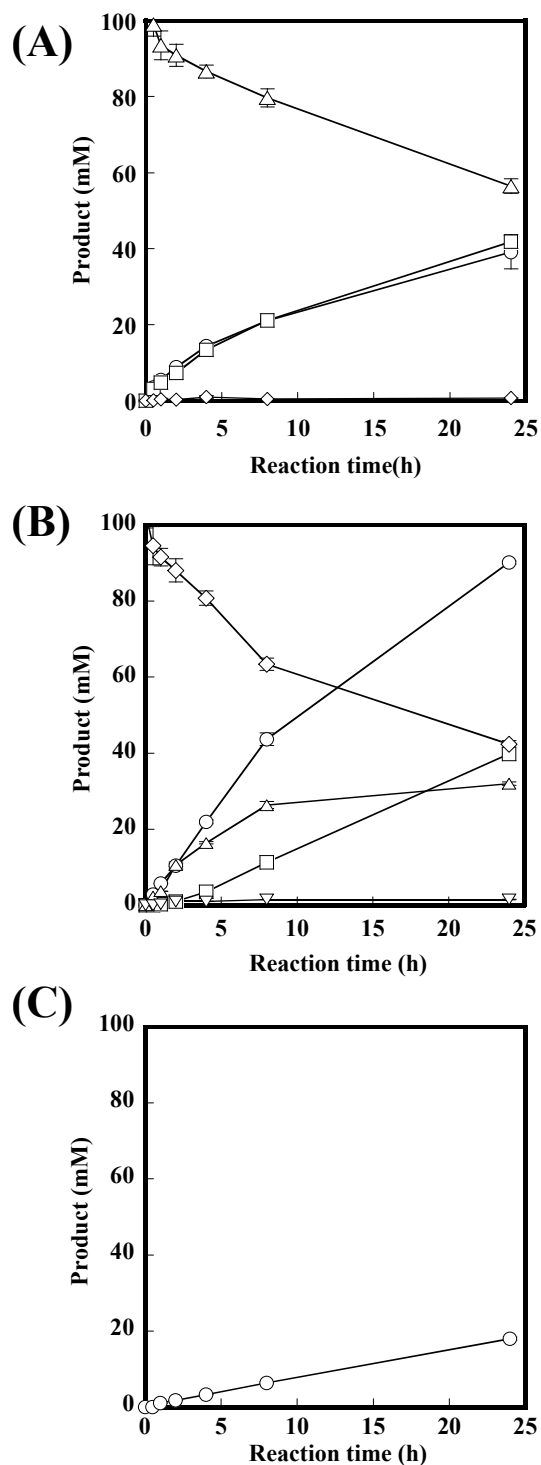


Figure 4 Time courses of saccharides formed from 100 mM 1-kestose (A), nystose (B) or 5% inulin (C) by purified recombinant AIEH1. Symbols of circle, square and triangle indicate fructose, sucrose and 1-kestose, respectively. Symbols of diamond and inverted triangle indicate nystose and fructosylnystose, respectively. Data are means of 3 replications. Standard deviation indicated with bars.

Table 4 Substrate specificity of recombinant AIEH1.

Substrate	DP	Relative activity
Sucrose	2	0.2
Inulobiose	2	12
Inulotriose	3	41
1-kestose	3	100
6-kestose	3	2
Neokestose	3	7
Nystose	4	66
Fructosylnystose	5	97
Inulin		42
Levan		0

Relative activity is expressed as the percentage of the activity with 1-kestose. Final concentration of each substrate in reaction mixture is 10 mM except for levan.

Concentration of the levan in reaction mixture is 0.5%.

established gradient of mixing eluent A with eluent B was: 0-1 min, 95% A-5% B; 1-2 min, 80% A-20% B; 2-20 min, 60% A-40% B; 20-22 min, 100% B, 22-30 min, 95% A-5% B. The flow rate through the column was 1.0 mL/min. The applied PAD potentials for E1 (400 ms), E2 (200 ms) and E3 (400 ms) were 0.05, 0.75 and -0.15 V, respectively, and the output range was 1 μ C).

In the experiment for estimation of optimum pH, McIlvaine buffers with a pH range 3.0-8.0 were used. The reaction was stopped by addition of 900 μ l of 150 mM sodium hydroxide. For pH-stability profiles, the mixture of 25 μ l of Britton Robinson buffer with a pH range 3.0-9.0 and 25 μ l of purified enzyme solution was kept at 4°C for 24 h, and then the mixture was adjusted to pH 5.8, and incubated with 100 mM 1-kestose at 30°C for 30 min. The reaction was stopped in a boiling water bath for 3 min. For temperature stability profiles, enzyme solutions were pre-incubated with 0.1 M sodium phosphate buffer (pH 5.8) for 15 min at 4, 30, 40, 50 or 60°C, and then the pre-incubated solution was cooled to 0°C, and then the mixtures were incubated with 1-kestose at 30°C for 30 min. All the experiments were done in duplicate.

Quantitative determination of proteins

Proteins were determined by measuring absorbance at 280 nm with reference to $E^{1\%}$ (extinction coefficient) = 9.38 in aldolase [33]. The concentration of purified protein was measured by the method described by Lowry *et al* [34].

SDS-polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was conducted according to the method of Laemmli [35]. Proteins in the gel were stained with Coomassie Brilliant Blue R-250.

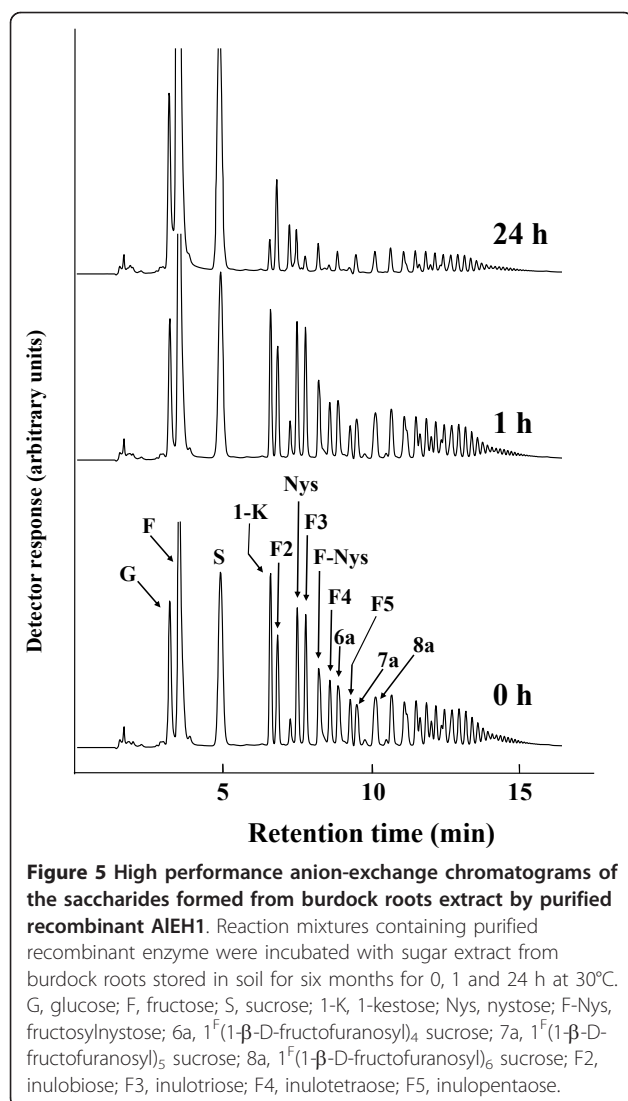


Figure 5 High performance anion-exchange chromatograms of the saccharides formed from burdock roots extract by purified recombinant AIEH1. Reaction mixtures containing purified recombinant enzyme were incubated with sugar extract from burdock roots stored in soil for six months for 0, 1 and 24 h at 30°C. G, glucose; F, fructose; S, sucrose; 1-K, 1-kestose; Nys, nystose; F-Nys, fructosyl-nystose; 6a, 1^F(1-β-D-fructofuranosyl)₄ sucrose; 7a, 1^F(1-β-D-fructofuranosyl)₅ sucrose; 8a, 1^F(1-β-D-fructofuranosyl)₆ sucrose; F2, inulobiose; F3, inulotriose; F4, inulotetraose; F5, inulopentaose.

To estimate molecular mass of polypeptide of recombinant protein, deglycosylated recombinant protein was prepared by PNGase F (NEB) as described in the protocol.

Molecular cloning of 1-FEH from edible burdock

From 1.0 g of edible burdock roots powder, which were ground in liquid nitrogen, total RNA was prepared using RNeasy Plant Mini Kit (Qiagen, USA). The first strand cDNA was synthesized, using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, USA). The cDNA was used for degenerate PCR and RACE as template. Degenerate PCR was done using a primer set of Dp-A and Dp-C, which were designed according to amino-acid sequences of FEHs from other plants. The PCR consisted of an initial 2 min denaturation step (94°C) followed by 35 cycles of 94°C for 30 s (denaturation), 50°C for 30 s (annealing) and 72°C for

1 min (extension) and then a final extension at 72°C for 7 min. The PCR was done using Blend *Taq* polymerase (Toyobo, Japan). The PCR product was subcloned in pGEM-T plasmid, selected in *Escherichia coli* DH5α cells (Toyobo, Japan). The plasmid was purified by GenElute Plasmid Mini-Prep Kit (Sigma Aldrich, USA), and its insert DNA was sequenced. The insert was denoted as DegeEHa.

On resulting DNA sequence of DegeEHa, gene-specific primers (5Ra-EHa and 3Ra-EHa) were designed, and used for 5' and 3' RACE to amplify the 5'- and 3'-flanking regions of DegeEHa. RACE was done using the Gene Racer Kit (Invitrogen, USA). The 5' and 3' RACE consisted of an initial 2 min denaturation step (94°C) followed by 35 cycles of 94°C for 15 s, 63°C for 30 s and 68°C for 70 s and then a final step at 68°C for 10 min. The 5' and 3' RACE were done using KOD plus (Toyobo, Japan). After an adenine residue was attached to the RACE products by A-addition kit (Qiagen, USA), these products were subcloned into pGEM-T vector, and then its insert DNA was sequenced.

Gene-specific primers (EHa-FwA and EHa-RvI) were designed from the DNA sequences of the 5' and 3' RACE products, and subjected to PCR to amplify the DNA corresponding to the open reading frame of target gene. 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 PsEHa were sequenced. Full length cDNA was compiled by overlapping the sequences of PsEHa and RACE PCR products. The full length cDNA was named *aleh1*. Oligonucleotide sequences used for the cloning procedures are listed in Table 1. The nucleotide sequence of full length cDNA has been submitted to GenBank, EMBL and DDBJ (AB611034).

Expression of recombinant proteins in a methylotrophic Yeast

The isolated cDNA, named *aleh1* was expressed in the methylotrophic yeast *Pichia pastoris* with the secretory expression vector pPICZαB (EasySelect *Pichia* Expression Kit, Invitrogen, USA). To construct expression plasmids named pPic_aleh1, a DNA fragment containing a gene sequence of *aleh1* corresponding to the mature protein was amplified by PCR using EHa-EcoRI.Q34 and EHa-XbaI as primers. These primers also had recognition sequences for *EcoRI* and *XbaI* incorporated in forward and reverse primers, respectively (Table 1). The PCR condition was 1 cycle of 94°C for 2 min, 30 cycles of 94°C for 30 s, 52°C for 30 s and 68°C for 1.5 min, followed by 1 cycle of 68°C for 7 min using KOD plus and PsEHa as template DNA. The PCR products were digested with *EcoRI* and *XbaI* followed by ligation into a

pPICZαB plasmid vector. The resulting plasmid pPic_aleh1 carries a Zeocin resistance gene and contains a gene sequence of *aleh1* corresponding to the mature protein in frame behind the α-factor signal sequence. The pPic_aleh1 was sequenced to ensure no alteration of sequence in comparison with that of original *aleh1*.

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 *SacI*-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. The resuspended cell pellet was adjusted to an A_{600} of 1.0 with 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.

Purification of recombinant protein

All operations throughout the purification were performed at 0–4°C. The recombinant protein was purified from the supernatant by DEAE Sepharose CL-6B anion exchange chromatography, Toyopearl HW55S hydrophobic chromatography and Toyopearl HW55S gel filtration chromatography. The supernatant (45 ml) was dialyzed for 1 day against the 10 mM sodium phosphate buffer (pH 6.5). The dialyzate was applied to a column (1.8 × 17 cm) of DEAE-Sepharose CL-6B equilibrated with 10 mM sodium phosphate buffer (pH 6.5). The adsorbed proteins were eluted with a linear gradient of 0–0.5 M sodium chloride in the same buffer. The active fraction was dialyzed overnight against 45% saturated ammonium sulfate in 10 mM sodium phosphate buffer (pH 6.5). The dialyzate was loaded on to a column (1.8 × 17 cm) of Toyopearl HW55S equilibrated with the same buffer. The elution was achieved with a linear gradient from 45% to 0% saturation of ammonium sulfate in the same buffer. Each active fraction was dialyzed overnight against 50 mM sodium phosphate buffer containing 100 mM sodium chloride (pH 6.5). The dialyzate was concentrated to 1.0 ml by ultrafiltration on VivaSpin concentrator cutting off at 30 kDa (Vivascience, U.K.). The concentrated solution was filtered on a column (2.0 × 65 cm) of Toyopearl HW55S equilibrated with the same buffer.

Abbreviations

FOS: fructooligosaccharides; IOS: inulooligosaccharides; 1-FEH: fructan 1-exohydrolase; 1-FFT: fructan:fructan 1-fructosyltransferase; 1-SST: sucrose: sucrose 1-fructosyltransferase; DP: Degree of polymerization; HPAEC: high

performance anion-exchange chromatography; RACE: rapid amplification of cDNA ends.

Acknowledgements

This work was partially supported by a Grant-in-Aid to Cooperative Research from Rakuno Gakuen University. Rakuno Gakuen University Dairy Science Institute, 2009-3.

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Authors' contributions

KU and YI collected data of gene sequences and recombinant protein, and contributed to drafting the manuscript. SO, MY and NS generated study design and drafted manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 9 February 2011 Accepted: 5 April 2011

Published: 5 April 2011

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doi:10.1186/1752-153X-5-16

Cite this article as: Ueno *et al.*: Cloning and functional characterization of a fructan 1-exohydrolase (1-FEH) in edible burdock (*Arctium lappa* L.). *Chemistry Central Journal* 2011 **5**:16.

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