Characterization of recombinant β-fructofuranosidase from Bifidobacterium adolescentis G1

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Abstract

Background: We have previously reported on purification and characterization of β-fructofuranosidase (β-FFase) from Bifidobacterium adolescentis G1. This enzyme showed high activity of hydrolysis on fructo-oligosaccharides with a low degree of polymerization. Recently, genome sequences of B. longum NCC2705 and B. adolescentis ATCC 15703 were determined, and cscA gene in the both genome sequences encoding β-FFase was predicted. Here, cloning of cscA gene encoding putative β-FFase from B. adolescentis G1, its expression in E. coli and properties of the recombinant protein are described.

Results: Using the information of cscA gene from Bifidobacterium adolescentis ATCC 15703, cscA gene from B. adolescentis G1 was cloned and sequenced. The N-terminal amino acid sequence of purified β-FFase from B. adolescentis G1 was identical to the deduced amino acid sequences of cscA gene from B. adolescentis G1. To confirm the translated product of the cscA gene, the recombinant protein was expressed in Escherichia coli. Molecular mass of the purified recombinant enzyme was estimated to be about 66,000 by SDS-PAGE and 60,300 by MALDI TOF-MS. The optimum pH of the enzyme was 5.7 and the enzyme was stable at pH 5.0-8.6. The thermostability of the enzyme was up to 50°C. The $K_m$ (mM), $V_{max}$ (μmol/mg of protein/min), $k_0$ (sec⁻¹) and $k_0/K_m$ (mM⁻¹ sec⁻¹) for 1-kestose, neokestose, nystose, fructosylnystose, sucrose and inulin were 1.7, 107, 107.5, 63.2, and 1.7, 142, 142.7, 83.9, and 3.9, 152, 152.8, 39.2, and 2.2, 75, 75.4, 34.3, and 38, 79, 79.4, 2.1, and 25.9, 77, 77.4, 3.0, respectively. The hydrolytic activity was strongly inhibited by AgNO₃, SDS, and HgCl₂.

Conclusion: The recombinant enzyme had similar specificity to the native enzyme, high affinity for 1-kestose, and low affinity for sucrose and inulin, although properties of the recombinant enzyme showed slight difference from those of the native one previously described.

Background

Bifidobacteria are saccharolytic anaerobes generally present in human intestine. Growth of bifidobacteria is selectively promoted by prebiotics [1]. Fructo-oligosaccharides, such as 1-kestose, nystose and fructosylnystose, consist of β-2,1-linked fructose to sucrose, and they are naturally contained in artichoke tubers [2], chicory roots [3] and burdock roots [4,5]. These saccharides have been produced and commercially manufactured from sucrose with bacterial fructosyltransferase [6] and β-fructofuranosidases (β-FFases) [7-9], and have been on the market as prebiotics. Fructo-oligosaccharides are not hydrolyzed by digestive enzymes of mammalian origin, so they are able to reach large intestine, and to be selectively degraded by the resident microbes, such as bifidobacteria.

We have already reported that B. adolescentis G1 were isolated from feces of human adults, and produce the unique β-FFase which has high affinity toward 1-kestose, nystose and fructosylnystose [10-12]. Recently, genome sequences of B. longum NCC2705 (accession no.
AE014295) and *B. adolescentis* ATCC 15703 (AP009256) were determined, and cscA gene in the both genome sequences encoding β-FFase was predicted [13]. The recombinant β-FFase from *B. infantis* [14], *B. lactis* [15] and *B. longum* [16] have been studied using mixtures of fructo-oligosaccharides (Actilight, Raftilose and Raftiline) as a substrate, although detailed substrate specificity of the enzyme to sole fructo-oligosaccharide remains unclear. In our previous study purification and the sub- 
of the enzyme to sole fructo-oligosaccharide was demonstrated [10,11]. However, we have not revealed the 
strate specificity of β-FFase from *B. adolescentis* yet. This 
study is aimed at cloning of cscA gene from *B. adolescentis* G1 and characterizing the recombinant protein of cscA gene expressed in *Escherichia coli*.

**Results and discussion**

*A* deduced amino acid sequence of cscA gene from *B. adolescentis G1*

The open reading frame (ORF) of cscA gene from *B. adoles- 
centis* G1 consisted of 1,557 nucleotides which encoded a 518 amino acid polypeptide. The molecular mass and pI of the deduced polypeptide were predicted to be 58,119 and 4.89, respectively. N-terminal sequence of purified enzyme was found in the deduced polypep-
tide. The deduced polypeptide of cscA gene from *B. adoles- 
centis* G1 shows high identity that from bifidobacteria (*B. adolescentis* ATCC15703 [accession no. AP009256, 
protein ID BAF39931]: 98%, *B. longum* NCC2705 [acces-
sion no. AE014295, protein ID AAN23970]: 85%, *B. breve* UCC2003 [accession no. AY549965, protein ID 
AAT28190]: 84%, *B. animalis* subsp. *lactis* DSM10140 [accession no. AY509036, protein ID AA87041]: 72%) (Fig. 1). Deduced polypeptides of cscA gene from *B. adoles- 
centis* G1 belong to GH family 32, and confirmed exis-
tence of NDPNG motif and EC motif involved in 
catalysis. Two acidic residues have been identified as 
being related in the catalytic mechanism of yeast 
invertase: Asp-23 as a nucleophile and Glu-204 as an 
acid/base catalyst [17,18]. Asp within NDPNG motif and 
Glu within EC motif were significant part of β-FFase from 
*B. adolescentis* G1 (position of NDPNG: Asn-53-Gly-57, 
EC: Glu-235-Cys-236). Additionally, Asp-309 within RDP 
motif was investigated on *Acetobacter diazotrophicus* 
levansucrase, the motif was found at a conserved posi-
tion, not only in levansases but also in invertase [19]. The 
Asp (D) in RDP motif in levansucrase from *Bacillus sub- 
tilis* was identified as a transition state stabilizer [20]. The 
existence of the motif was likely to keep a common 
functional role for β-FFase. It seems that the motif was 
also important portion for β-FFase from *B. adolescentis* 
G1 (position of RDP: Arg-180-Pro-182). The motifs are in 
highly conserved position in β-fructofuranosidase of 
GH32 [21].

**Heterologous expression and purification of recombinant protein**

To investigate translated product of cscA gene from *B. adolescentis* G1 in detail, recombinant protein was pro-
duced by *E. coli* Rosetta2 (DE3) with pET-G1cscA. The recombinant protein was confirmed in cell extract by 
SDS-PAGE and enzyme assay. The recombinant protein 
was purified from cell extract (total protein 163 mg, total 
enzyme activity 1,480 U, specific activity 9.1 U/mg) (Fig. 
2(a)) by TALON CellThru Resin, DEAE Sepharose CL-
6B, Toyopearl HW-55S. Purification procedures of recombinant protein were summarized in Table 1. Spec-
cific activity of the final purified enzyme was 86.0 U/mg, 
SDS-PAGE analysis of the purified enzyme showed a 
single band with an apparent molecular mass of about 
66,000 (Fig. 2(b)). MALDI-TOF mass spectrum of puri-
fied recombinant protein shows a mass-peak at near 
60,300, in agreement with the calculated mass of recombi-
nant protein including extra sequences from pET32b 
(+ vector (data not shown). The reason of difference 
between calculated mass and actual measurement mass 
by SDS-PAGE is not clear. However, Shirai et al reported 
that acidic proteins (those for which isoelectric points are 
less than 6.0) exhibited mobility smaller than calculated, 
this may be due to negative charge repulsion with SDS 
[22]. In case of our study, the smaller mobility of recombi-
nant protein (Fig. 2) than the mobility from calculated 
mass might be thought by the same reason.

**Effects of pH and temperature**

The effects of pH on hydrolytic activities toward 1-kes-
tose were examined. The optimum pH of recombinant 
protein was observed to be 5.7 (Fig. 3). The characteriza-
tion is similar to other β-FFase from bifidobacteria [14] 
that enzyme reaction proceeds strongly at acidulant con-
dition. The recombinant protein was stable in the pH 
range from 5.0 to 8.6 (residual activity more than 90%) 
(Fig. 4). Although this extend of pH-stability is slightly 
different from the native enzyme [10], it is not poles 

Effect of various metal salts and chemical reagents

The hydrodase activity of the enzyme was not inhibited by 
MgCl2, FeCl3, CoCl2, MnCl2, SnCl2 and CaCl2, deoxy-
cholic acid sodium salt, TritonX-100, Tween80 and ZnCl2, 
had enhanced the enzyme activity. The enzyme activity 
was strongly inhibited by AgNO3, SDS, HgCl2, and was 
partially inhibited by CuSO4. The result was summarized 
in Table 2. They formed mercaptides with free SH 
groups, so inhibited the hydrolysis of 1-kestose, as 
described previously [10].
Figure 1 Deduced amino acid sequence of cssA from *Bifidobacterium*. B. adoG1_CssA, deduced amino acid sequence of cssA gene from *B. adolescentis* G1; B. ado_CssA, *B. adolescentis* ATCC 15703 (AT12aB); B.lon_CssA, *B. longum* NCC2705 (Q8C57); B bre_CssA, *B. breve* UC3 2003 (115293E); Blac_CssA, *B. lactis* (656ac7) are aligned. The three carboxylic acids in NDPNG, RDP and EC motifs are crucial for enzyme activity are shaded in gray. Underlined sequences indicate the N-terminus of purified β-FFast from *B. adolescentis* G1. Consensus line: asterisks (*) indicated identical residues, colons (;) indicated conserved substitutions; and periods (.) indicated semi-conserved substitutions.
Substrate specificity and kinetic parameters

To examine the substrate specificity of the recombinant β-FFase, the enzyme was incubated in 10 mM fructooligosaccharides such as 1-kestose, neokestose, nystose or fructosylnystose at 37°C for 0, 10 min, 1 and 4 h. Reaction products were analyzed by HPAEC (Fig. 6). This enzyme catalyzed hydrolytic reaction on the fructooligosaccharides. In the reaction of 1-kestose and neokestose, fructose and sucrose were produced (Fig. 6(a) and 6(b)). These results indicate that the enzyme hydrolyzed not only terminal β-2,1 fructoside bond of 1-kestose, but also β-2,6 fructoside bond (fru-2,6-glc) of neokestose. When the enzyme was incubated with nystose, fructose and 1-kestose were produced (Fig. 6(c)), with fructosylnystose, fructose and nystose were produced (Fig. 6(d)), with sucrose, glucose and fructose were produced (Fig. 6(e)), with inulin, fructose was produced (Fig. 6(f)). By the prolonged incubation of nystose or fructosylnystose, sucrose and 1-kestose were also produced. This result showed that the enzyme is capable of degrading each substrate via an exo-type of cleavage, releasing terminal fructosyl residues as well as other β-FFase reported from bifidobacteria [14,15]. To estimate the rate parameters of hydrolysis of several substrates, Michaelis constants ($K_m$) and maximum velocities ($V_{\text{max}}$) for each substrate were measured.

Table 1: Summary of purification procedure recombinant β-FFase.

| Procedure               | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Purification (-fold) | Recovery (%) |
|-------------------------|--------------------|------------------------|------------------------------|----------------------|--------------|
| Crude extract           | 163                | 1,480                  | 9.08                         | 1.00                 | 100          |
| Metal affinity resins   | 30.0               | 600                    | 20.0                         | 2.20                 | 40.5         |
| DEAE-Sepharose CL-6B    | 11.3               | 900                    | 79.7                         | 8.78                 | 60.8         |
| Toyopearl HW-55S        | 3.65               | 314                    | 86.0                         | 9.47                 | 21.2         |
$k_m$, $V_{max}$, $k_0/k_m$ are listed in Table 3. These parameters indicated that the recombinant enzyme had high affinity and high rate for hydrolyzing FOS, especially for 1-kestose, neokestose and nystose. The hydrolyzing activity against sucrose and inulin was lower than formers, so the enzyme was thought mainly as relating FOS degradation.

The recombinant protein indicated a higher relative efficiency for fructo-oligosaccharides such as 1-kestose, neokestose and nystose than sucrose and inulin. The result is in similar to the kinetic parameters from native enzyme [11]. Janer et al reported that recombinant β-FFase from B. lactis (72% identity with amino acid sequence from B. adolescentis G1 β-FFase) showed high affinity to low molecular fructans (Raftilose, DP < 5) compared to low or high polymerized inulin (Raftiline LS or HP) or sucrose [15]. These observations were very similar to our result. Some properties (pH-optima, pH-stability, temperature-stability, effects of various metal salts or chemical reagents) are different between the recombinant and native enzymes, but the characterizations are similar each other. We investigated substrate specificity for neokestose in this study, although it was not used in the previous research. Neokestose ($\beta$-D-fructofuranosyl (2→6) -α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) and its related fructo-oligosaccharides are contained in onion bulbs and asparagus roots. Neokestose and the related saccharides were reported to be synthesized by fructan:fructan 6G-fructosyltransferase from the vegetables [23,24]. Recombinant β-FFase from B. adolescentis G1 preferentially hydrolyzed neokestose rather than 1-kestose. The enzyme hydrolyzes not only terminal $\beta$-2,1 fructoside bond of 1-kestose or nystose, but also $\beta$-2,6 fructoside bond (fru-2-6-glc) of neokestose. The result indicated that neokestose had potentiality to

**Table 2: Effects of various metal salts or chemical reagents on recombinant β-FFase.**

| Reagent                          | Relative activity (%) |
|----------------------------------|-----------------------|
| None                             | 100                   |
| AgNO$_3$                         | 0                     |
| CuSO$_4$                         | 51.8                  |
| MgCl$_2$                         | 99.1                  |
| FeCl$_3$                         | 110.6                 |
| CoCl$_2$                         | 105.1                 |
| MnCl$_2$                         | 100.2                 |
| SnCl$_2$                         | 99.2                  |
| ZnCl$_2$                         | 121.1                 |
| CaCl$_2$                         | 102.1                 |
| HgCl$_2$                         | 0                     |
| SDS                              | 0                     |
| Deoxycholic acid sodium salt     | 136.9                 |
| TritonX-100                      | 137.8                 |
| Tween 80                         | 137.7                 |

A reaction mixture containing 25 μl of enzyme solution, 25 μl of 0.2 M sodium phosphate buffer (pH 5.7), 10 μl of each chemical (10 mM), 15 μl of Milli-Q water and 25 μl of 40 mM 1-kestose was incubated at 37°C for 10 min.
Figure 6 High performance anion-exchange chromatograms of the reaction products formed from fructo-oligosaccharide by recombinant β-FFase. Recombinant β-FFase was incubated with 10 mM 1-kestose (a), neokestose (b), nystose (c), fructosylnystose (d), sucrose (e) or inulin (f) for up to 4 h at 37°C. The reaction mixtures were analyzed by high performance anion-exchange chromatography (HPAEC). Glc, Glucose; Fru, Fructose; Suc, Sucrose; 1-K, 1-kestose; N-k, Neokestose; Nys, Nystose; F-nys, Fructosylnystose; Inu, Inulin.
be a good efficient prebiotics, which can promote the human health due to growth of bifidobacteria in the gut. Our result exhibited that the recombinant β-FFase from \textit{B. adolescentis} G1 had unique properties to hydrolyze preferably low DP fructo-oligosaccharides as well as native one. We suppose that research about β-FFase efficiently to hydrolyze fructo-oligosaccharides is to be of much help for developing more effective prebiotics, probiotics and synbiotics.

**Conclusion**

In this article, cloning the \textit{cscA} gene from \textit{Bifidobacterium} \textit{adolescentis} G1 and characterization of the recombinant protein of \textit{cscA} gene expressed in \textit{Escherichia coli} were described. Molecular mass of the purified recombinant enzyme was estimated to be about 66,000 by SDS-PAGE and 60,300 by MALDI TOF-MS. The optimum pH of the enzyme was 5.7 and the enzyme was stable at pH 5.0-8.6. The thermostability of the enzyme was up to 50°C. The $K_m$ (mM), $V_{max}$ (μmol/mg of protein/min), $k_0$ (sec$^{-1}$) and $k_0/K_m$ (mM$^{-1}$ sec$^{-1}$) for 1-kestose, neokestose, nystose, fructosylnystose, sucrose and inulin were 1.7, 107, 107.5, 63.2, 1.7, 142, 142.7, 83.9, 3.9, 152, 152.8, 34.3, 2.2, 75, 75.4, 34.3, and 2.2, 75, 75.4, 34.3, respectively. The hydrolytic activity was strongly inhibited by AgNO$_3$, SDS, HgCl$_2$. The recombinant enzyme had similar specificity to the native enzyme, high affinity for 1-ke stose, and low affinity for sucrose and inulin, although properties of the recombinant enzyme showed slight difference from those of the native one previously described.

**Experimental**

**Bacterial strains, plasmids and culture condition**

\textit{B. adolescentis} G1 was cultured in GAM broth (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan), and then, it was used for the extraction of genomic DNA. \textit{Escherichia coli} DH5α was used as host cell with the plasmid pGEM-T vector (Promega, Madison, WI, USA) for cloning and sequencing. \textit{Escherichia coli} Rosetta2 (DE3) (Novagen, Madison, WI, USA) was used as host cell with the plasmid pET-32b (+) vector (Novagen) for protein expression. \textit{E. coli} strains were grown in Luria Bertani (LB) medium supplemented with 100 μg/ml carbenicillin.

**Analysis of N-terminal amino acid Sequences**

N-terminal sequences of β-FFase purified from \textit{Bifidobacterium} \textit{adolescentis} G1 was determined by ABI 477A protein sequencer/120A PTH analyzer system.

**Gene cloning**

After grown \textit{B. adolescentis} G1 in GAM broth, the cells were collected by centrifugation (1,700 × g, 10 min, 4°C) and the supernatant was removed, the precipitate was resuspended by adding 20 mM Tris-HCl buffer (pH 8.0), and centrifugated (1,700 × g, 10 min, 4°C). Genomic DNA was extracted from the precipitate by DNeasy Tissue Kit (Qiagen, Courtaboueuf, France).

Based on \textit{cscA} gene encoding β-FFase from \textit{Bifidobacterium} \textit{adolescentis} ATCC 15703 [accession no. AP009256, protein ID BAF39931], a 1,659 bp of DNA including \textit{cscA} gene from \textit{B. adolescentis} G1 was amplified by PCR using \textit{B. adolescentis} G1 genomic DNA as a template and using G1FFase1-for (5'-CCCAACAATTCATAACCCAG-3') and G1FFase2-rev (5'-TTCCCATATACCCCTTGCTA-3') as primers. PCR condition was: initial step of denaturation by 94°C for 2 min, followed by 30 cycles of 94°C for 15 sec, 57°C for 30 sec and 68°C for 90 sec, and then a final step at 68°C for 10 min using of KOD-plus- (Toyobo, Osaka, Japan). After adenine was attached to the PCR products by A addition kit (Qiagen), these products were ligated into pGEM-T vector using T4 DNA ligase (Promega) and \textit{E. coli} DH5α was transformed by the resulting vector. The transformants were grown and harvested, and the plasmids were isolated by Sigma GenElute Plasmid Mini-Prep Kit (Sigma-Aldrich, St. Louis, MO, USA), and its insert DNA was sequenced. The plasmid was named pGEM-G1cscA.

**Expression of a recombinant protein in \textit{E. coli}**

For construction of expression vector, \textit{cscA} gene was amplified by PCR using pGEM-G1cscA as a template. Primers used were fphaseNtEcoRI-for (5’-TCCGAATTC-3’) and fphaseNtEcoRI-rev (5’-GGTCGACATATCCACCACATTGC-3’) as primers. PCR condition was: initial step of denaturation by 94°C for 2 min, followed by 30 cycles of 94°C for 15 sec, 57°C for 30 sec and 68°C for 90 sec, and then a final step at 68°C for 10 min using of KOD-plus- (Toyobo, Osaka, Japan). After adenine was attached to the PCR products by A addition kit (Qiagen), these products were ligated into pGEM-T vector using T4 DNA ligase (Promega) and \textit{E. coli} DH5α was transformed by the resulting vector. The transformants were grown and harvested, and the plasmids were isolated by Sigma GenElute Plasmid Mini-Prep Kit (Sigma-Aldrich, St. Louis, MO, USA), and its insert DNA was sequenced. The plasmid was named pGEM-G1cscA.

**Table 3: Rate parameters of recombinant β-FFase.**

| Substrate     | $K_m$ (mM) | $V_{max}$ (μmol/mg of protein/min) | $k_0$ (sec$^{-1}$) | $K_m/k_0$ (mM$^{-1}$ · sec$^{-1}$) |
|---------------|-----------|-----------------------------------|-------------------|-----------------------------------|
| 1-Kestose    | 1.7       | 107                               | 107.5             | 63.2                              |
| Neokestose   | 1.7       | 142                               | 142.7             | 83.9                              |
| Nystose      | 3.9       | 152                               | 152.8             | 39.2                              |
| Fructosylnystose | 2.2   | 75                                | 75.4              | 34.3                              |
| Sucrose      | 38        | 79                                | 79.4              | 2.1                               |
| Inulin       | 25.9      | 77                                | 77.4              | 3.0                               |

*a), mM; b), μmol/mg of protein/min; c), sec$^{-1}$; d), mM$^{-1}$ · sec$^{-1}$
GATGACTGGGCTTTACTCGGGA-3') and ffasεCtXhol-
rev (5'-TTGCTCGAGTTCCGATCCGATCGACT-
TCAT-3'). These primers had recognition sequence of
EcoRI and Xhol, respectively. PCR condition was: initial
step of denaturation by 94°C for 2 min, followed by 30
cycles of 94°C for 15 sec, 57°C for 30 sec and 68°C for 60
sec, and then a final step at 68°C for 7 min using of KOD-
plus-. The PCR product was digested with EcoRI and
Xhol, followed by ligation into pET-32b (+) vector cleaved
with the same restriction enzyme using Quick T4 DNA
ligase (New England Biolabs, Inc., Ipswich, MA). Finally,
E. coli Rosetta2 (DE3) was transformed by the ligated pET
vector. Resulting plasmid was named pET-G1cscA. The
nucleotide sequence of the plasmid was analyzed by ABI
3730 × 1 sequencer (Applied Biosystems, Foster City,. CA,
USA), and confirmed it had no error. The transformants
were selected on LB agar plates containing 100 μg/ml carbenicillin.
All operations were done at 4°C. The cells were sus-
dpended in 8 ml wash buffer (50 mM NaH2PO4, 0.3 M
NaCl, pH 7.5) containing protease inhibitor cocktail
EDTA-free (Roche Molecular Biochemicals, Mannheim
Germany), and the suspension was disrupted by ultrason-
cation. Supernatant after centrifugation (12,000 ×
g, 5 min, 4°C), and stored at -80°C until the
preparation of the crude enzyme.

**Purification of recombinant protein**

All operations were done at 4°C. The cells were sus-
pended in 8 ml wash buffer (50 mM NaH2PO4, 0.3 M
NaCl, pH 7.5) containing protease inhibitor cocktail
EDTA-free (Roche Molecular Biochemicals, Mannheim
Germany), and the suspension was disrupted by ultrason-
cation. Supernatant after centrifugation (12,000 ×
g, 5 min, 4°C), and stored at -80°C until the
preparation of the crude enzyme.

**Enzyme assays**

For the measurement of β-FFase activity, 50 μl of 20 mM
1-ketose in distilled water was mixed with 25 μl of 0.2 M
sodium phosphate buffer (pH 5.7) and 25 μl of purified
enzyme solution and incubated at 37°C for 10 min. The
reaction was stopped by boiling for 5 min. One unit of β-
FFase activity was defined as the amount of enzyme
which produced 1 μmol of fructose per min under the
above reaction conditions. For quantification of fructose,
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 ampero-
metric detector (PAD) as described previously [26].

For the determination of optimum pH, McIlvaine bu-
ferr with pH range 3.0-8.5 were used. The reaction was
stopped by adding 900 μl of 150 mM NaOH.

To investigate the pH stability of enzyme, the mixture
of 25 μl of Britton- Robinson buffer with pH range 3.0-
10.0 and 25 μl of purified enzyme solution containing
0.1% BSA was kept at 4°C for 20 h, then the mixture was
adjusted to pH 5.7, and incubated with 10 mM 1-ketose
at 37°C for 10 min. The reaction was stopped by heating
the samples at 100°C for 5 min.

For temperature stability profiles, 25 μl of 0.2 M sodium
phosphate buffer (pH 5.7) and 25 μl of purified enzyme
solution containing 0.1% BSA were mixed, and they were
incubated at 4, 30, 40, 45, 50, 55, 60 and 65°C for 15 min,
respectively, and then, each solution was cooled to 0°C.
The mixtures were incubated with 50 μl of 20 mM 1-kes-
ties (talline 1-kestose [1F-β-D-fructofuranosylsucrose, 1-kestotetraose] were prepared from sucrose using Scopulariopsis brevicaulis β-Fase [27]. Fructosylnystose [1F(1-β-D-fructofuranosyl)₃ sucrose, 1, 1, 1-kestopen-taose] was prepared from Jerusalem artichoke tubers in our laboratory. Sucrose and inulin were purchased from Wako (Wako Pure Chemicals Industries, Osaka, Japan). Neokestose was prepared from asparagus roots as described previously [28]. The structures of the substrates were shown (Fig. 7).

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
TO and KU collected data of gene sequences and recombinant protein, and contributed to drafting the manuscript. KM and MK collected data of native enzyme from bifidobacteria. NS and SO generated study design and drafted manuscript. All authors read and approved the final manuscript.

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References
1. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr 1995, 125:1401-1412.
2. Bacon JSD, Edelman J. The carbohydrates of the Jerusalem artichoke and other compositae. Biochem J 1951, 48:114-126.
3. Ende V, Van den, Mintiens A, Speleers H, Onrusta AA, Van Laere A. The metabolism of fructans in roots of Cichorium intybus during growth, storage and freezing. New Phyto 1996, 132:555-563.
4. Ishiguro Y, Ueno K, Abe M, Onodera S, Fukushima E, Benkeblia N, Shiomi N. Isolation and structural determination of reducing fructooligosaccharides newly produced in stored edible burdock. J Appl Glycosci 2009, 56:139-146.
5. Abe M, Ueno K, Ishiguro Y, Omori T, Onodera S, Shiomi N. Purification, cloning and functional characterization of fructan 1-fructosyltransferase from edible burdock (Arctium lappa L.). J Appl Glycosci 2009, 56:239-246.
6. van Balken JAM, van Dooren T, Tweel WJJ van den, Kamphuis J, Meijer EM. Production of 1-kestose with intact mycelium of Aspergillus phoenicis containing sucrose-1F-fructosyltransferase. Appl Microbiol Biotechnol 1991, 35:16-221.
7. Takeda H, Sato K, Kinoshita S, Sasaki H. Production of 1-kestose by Scopulariopsis brevicaulis. J Ferment Bioeng 1994, 77:386-389.
8. Hirayama M, Sumi N, Hidaka H. Purification and properties of a fructooligosaccharides-producing β-fructofuranosidase from Aspergillus niger ATCC 20611. Agric Biol Chem 1989, 53:667-673.
9. Hidaka H, Adachi T, Tokunaga T, Nakaizumi Y, Kono T. The road of fructooligosaccharide research and business development. In Recent Advances in Fructooligosaccharide Research Edited by: Shiomi N, Benkeblia N, Onodera S. Kerala: Research Signpost Publisher, 2007:375-395.
10. Muramatsu K, Onodera S, Kikuchi M, Shiomi N. Purification and some properties of a β-fructofuranosidase from Bifidobacterium adolescentis G1. Biosci Biotechnol Biochem 1994, 58:1642-1645.
11. Muramatsu K, Onodera S, Kikuchi M, Shiomi N. Substrate specificity and subsite affinities of β-fructofuranosidase from Bifidobacterium adolescentis G1. Biosci Biotechnol Biochem 1994, 58:1642-1645.
12. Muramatsu K, Onodera S, Kikuchi M, Shiomi N. The production of β-fructofuranosidase from Bifidobacterium spp. Biosci Biotechnol Biochem 1992, 56:1451-1454.

Figure 7 Chemical structure of saccharides. The structures of the substrates used in the study are shown.

tose at 37°C for 10 min. The reaction was stopped by heating the samples at 100°C for 5 min.

The effects of metal salts and chemical reagents on the enzyme activity were investigated. The reaction mixture, 25 μl of 40 mM 1-kestose, 25 μl of 0.2 M sodium phosphate buffer (pH 5.7), 10 μl of each metal salt or chemical reagents, 15 μl of distilled water and 25 μl purified enzyme was incubated at 37°C for 10 min. The reaction was stopped by boiling for 5 min.

To measure rate parameters of hydrolysis against different substrates, a reaction mixture containing 50 μl of each substrate at various concentrations, 25 μl of 0.2 M sodium phosphate buffer (pH 5.7) and 25 μl of enzyme solution was incubated at 37°C for 10 min. Molecular activities (kₐ) were calculated by using maximum velocities (Vₘₐₓ) and relative molecular mass of the enzyme.

Substrates
Saccharides as substrates were prepared as follows. Crystalline 1-kestose [1F,β-D-fructofuranosylsucrose, 1-kesto-
13. Schell MA, Karmisantzou M, Snel B, Vilanova D, Berger B, Pessi G, Zvahlen MC, Desiere F, Boek P, Delley M, Pidmore RD, Argioni F: The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci USA 2002, 99:14422-14427.

14. Warhol M, Perrin S, Grill JP, Schneider F: Characterization of a purified β-fructofuranosidase from Bifidobacterium infantis ATCC 15697. Lett Appl Microbiol 2002, 35:462-467.

15. Janer C, Rohr LM, Pelaez C, Lalo M, Cleuix V, Requena T, Meile L: Hydrolysis of oligofructoses by the recombinant β-fructofuranosidase from Bifidobacterium lactis. Syst Appl Microbiol 2004, 27:279-285.

16. Kullin B, Abratt VR, Reid SJ: A functional analysis of the Bifidobacterium longum cscA and cscP genes in sucrose utilization. Appl Microbiol Biotechnol 2006, 72:975-81.

17. Reddy VA, Maley F: Identification of an active-site residue in yeast invertase by affinity labeling and site-directed mutagenesis. J Biol Chem 1990, 265:10817-10820.

18. Reddy A, Maley F: Studies on identifying the catalytic role of Glu-204 in the active site of yeast invertase. J Biol Chem 1996, 271:13953-13958.

19. Batista FR, Hernández I, Fernández JR, Arrieta J, Menéndez C, Gómez R, Tambara Y, Pons T: Substitution of Asp-309 by Asn in the Arg-Asp-Pro (RDP) motif of Acetobacter diazotrophicus levansucrase affects sucrose hydrolysis, but not enzyme specificity. Biochem J 1999, 377:503-506.

20. Meng G, Futterer K: Structural framework of fructosyl transfer in Bacillus subtilis levansucrase. Nat Struct Biol 2003, 10:935-941.

21. Carbohydrate-Active enzymes Database [http://www.cazy.org/]

22. Shirai A, Matsuyama A, Yashiroda Y, Hashimoto A, Kawamura Y, Arai R, Komatsu Y, Horinouchi S, Yoshida M: Global analysis of gel mobility of proteins and its use in target identification. J Biol Chem 2008, 283:10745-10752.

23. Ueno K, Onodera S, Kawakami A, Yoshida M, Shiomi N: Molecular characterization and expression of a cDNA encoding fructan:fructan 6G-fructosyltransferase from asparagus (Asparagus officinalis). New phytologist 2005, 165:813-824.

24. Fujishima M, Sakai H, Ueno K, Takahashi N, Onodera S, Benkeblia N, Shiomi N: Purification and characterization of a fructosyltransferase from onion bulbs and its key role in the synthesis of fructo-oligosaccharides in vivo. New phytologist 2005, 165:513-524.

25. Shiomi N, Onodera S, Chatterton NJ, Harrison PA: Separation of fructooligosaccharide isomers by anion-exchange chromatography. Agric Biol Chem 1991, 55:1427-1428.

26. Takeda H, Sato K, Kinoshita S, Sasaki H: Production of 1-kestose by Scopulariopsis brevicaulis. J Ferment Bioeng 1994, 77:386-389.

27. Shiomi N, Yamada J, Iizawa M: Isolation and identification of fructooligosaccharides in roots of asparagus (Asparagus officinalis L). Agric Biol Chem 1976, 40:567-575.

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