Molecular Cloning and Characterization of the Fructooligosaccharide-Producing β-Fructofuranosidase Gene from *Aspergillus niger* ATCC 20611

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The *fopA* gene encoding a fructooligosaccharide-producing β-fructofuranosidase was isolated from *Aspergillus niger* ATCC 20611. The primary structure deduced from the nucleotide sequence showed considerable similarity to those of two other β-fructofuranosidases from *A. niger*, but the *fopA* gene product had several amino acid insertions and an extra C-terminal polypeptide consisting of 38 amino acids that could not be found in the two others. We could successfully express the *fopA* gene in *S. cerevisiae* and the *fopA* gene product obtained from the culture supernatant of the *S. cerevisiae* transformant had similar characteristics to the β-fructofuranosidase purified from *A. niger* ATCC 20611. However, we could not detect any β-fructofuranosidase activity in either the culture supernatant or cell lysate when the C-terminal truncated *fopA* gene product by 38 amino acids was used to transform *S. cerevisiae*. In western analysis of those samples, there was no protein product that is cross-reacted with anti-β-fructofuranosidase antibody. These results suggested that the C-terminal region of the *fopA* gene product consisting of 38 amino acids was essential for the enzyme production.

Key words: β-fructofuranosidase; fructooligosaccharide; *Aspergillus niger*

β-fructofuranosidases (invertase, EC 3.2.1.26) are widely distributed in the bacterial, fungal, and plant world, and catalyze mainly the following reaction;
Sucrose + Acceptor → Glucose + Fructosyl-Acceptor.
In this reaction, a preference for water as an acceptor gives a hydrolyzed product, but other acceptors, such as sugar and alcohol, give fructosyl transferred products. Therefore, a β-fructofuranosidase intrinsically has both hydrolysis (Uh) and fructosyl transfer (Ut) activity, but the ratio (Ut/Uh) of each enzyme seems to be very different.

A number of β-fructofuranosidase coding genes have been isolated from bacteria, fungi, and plants, and therefore their amino acid sequences have been predicted. On β-fructofuranosidase related enzymes, such as inulinase and levanase, several amino acid sequences have also been deduced from the nucleotide sequences of their genes. A comparison of their amino acid sequences indicated six well-conserved regions that might be important for the enzyme activity.[1] However, the relationship between the strength of fructosyl transfer activity of β-fructofuranosidase and its primary structure remains unknown.

Aspergillus niger ATCC 20611 produces a unique β-fructofuranosidase, which has not only a very high ratio (Ut/Uh) compared to those of other strains belonging to *Aspergillus*, but also a high regiospecificity for fructosyl transfer to the 1-OH group of terminal fructofuranosides when sucrose is used as substrate.[2] Owing to this, the crude enzyme preparation derived from *A. niger* ATCC 20611 has been used for commercial production of fructooligosaccharide (Meioligo®) from sucrose, which is a mixture of oligosaccharides with inulin-type structure and has been shown to have beneficial health effects as a functional food component.[3] Information concerning the structure of this enzyme must be useful to understand how β-fructofuranosidases show fructosyl transfer activity. In this paper, we describe the molecular cloning and characterization of the fructooligosaccaride-producing β-fructofuranosidase gene derived from *A. niger* ATCC 20611.

Materials and Methods

Strains, media, and culture conditions. *A. niger* ATCC 20611 was cultivated for 20 h at 28°C in YPD medium containing 1% yeast extract, 2% polypeptone, and 2% glucose to prepare the chromosomal DNA. *Saccharomyces cerevisiae* MS-161 (*MAT*a *trp1 ura3* Suc⁻) is a stock strain of our laboratories and is not able to assimilate sucrose as a carbon source. This yeast strain was used as host cell for the

expression of the *fopA* gene, which encodes the fructooligosaccharide-producing β-fructofuranosidase of *A. niger* ATCC 20611. Yeast cells were grown in YPD medium. Yeast transformants were selected on SD-U medium (0.67% yeast nitrogen base w/o amino acids, 2% glucose, 0.005% uracil) containing 2% purified agar. For the expression of the *fopA* gene, transformants were cultivated in SD-U medium overnight and a part of the culture was inoculated to a final concentration of 1% into SD-CU medium (0.67% yeast nitrogen base w/o amino acids, 2% glucose, 2% casamino acids, 0.005% uracil). *Escherichia coli* JA221 (*recA1 leuB6 trpE5 hsdR⁻ hsdM⁻ lacI thr thi*) was used for plasmid construction and propagation. *E. coli* XL1-Blue MRA (*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA96 gyrA1 relA1 lac*) was used as a host for phage λDASHII. *E. coli* JM109 (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)* F'[*traD36 proAB lacI�q lacZΔM15*]) was used as a host to prepare single-stranded DNA for sequencing. *E. coli* cells were cultivated in LB medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride, with 50 μg/ml ampicillin added when required).

Plasmids. Plasmids pUC118 and pUC119 were purchased from Takara Shuzo Co., Ltd. (Japan) and used for sequencing. Plasmid pY2831, a yeast expression vector, was constructed from the pYPR2831, which had been reported by Horiuchi *et al.*[4] as described below; the pYPR2831 was digested with *Eco*RI and *Sal*I, treated with Klenow fragment, and recirculized with the *Bam*HI linker (5'-CGGAT-CCG-3').

Analysis of N-terminal and internal peptide sequences of the β-fructofuranosidase. The β-fructofuranosidase of *A. niger* ATCC 20611 was purified as described previously.[5] The purified enzyme was desalted by dialysis and underwent automated Edman degradation with a protein sequencer PSQ-1 (Shimadzu Corporation, Japan). To analyze internal amino acid sequences, the purified enzyme was digested with endoproteinase Lys-C (Promega Corporation, USA). The digests were put through reverse-phase HPLC on a TSKgel ODS 120T column (Tosoh Corporation, Japan) and each of eluted peptides was analyzed with a protein sequencer PSQ-1.

Amplification of a specific fragment of the fopA gene. Two degenerate oligonucleotide (5'-ATC-GCSGAYCAYCCSTTYGC-3' and 5'-TCRTTRTC-SACSACRTTYTC-3'; where S = C or G; Y = C or T; R = A or G) were designed and synthesized. PCR was done with a DNA Thermal Cycler PJ2000 (Perkin Elmer) by using these oligonucleotides and chromosomal DNA from *A. niger* ATCC 20611 under the

following conditions: melting temperature, 94°C for 1 min; annealing temperature, 54°C for 2 min; extension, 72°C for 3 min; and 25 cycles. The resulting DNA fragment was fractionated on and recovered from an agarose gel, and used as a probe for screening of the genomic DNA library described below.

Construction and screening of a genomic DNA library. The chromosomal DNA of *A. niger* ATCC 20611, isolated by the method of Horiuchi *et al.*,[6] was digested with *Eco*RI and fractionated on a 1% agarose gel. Fragments of 10 to 20 kbp were recovered from the gel and ligated to λDASHII phage DNA that had been digested with *Eco*RI and *Hin*dIII. The ligated DNA was packaged *in vitro*, and the phage particles were transfected to *E. coli* XLI-Blue MRA. The genomic DNA library was screened by plaque hybridization by using the PCR fragment as a probe. Plaque hybridization was done with ECL direct nucleic acid labelling and detection systems (Amersham K. K., Japan) according to the manufacturer's instructions.

DNA sequencing. DNA sequencing was done by the dideoxy chain termination method of Sanger *et al.*[7] with an ALFred DNA sequencer (Pharmacia Biotech, Japan).

Deglycosylation of the fopA gene product. Deglycosylation of the *fopA* gene products from *A. niger* and *S. cerevisiae* was done with a Glycopeptidase F De-N-glycosylation Set (Takara Shuzo Co., Ltd., Japan) according to the manufacturer's instruction.

Enzyme assay. The reaction mixture, which contained 500 μl of 20% sucrose, 40 μl of 0.5 M sodium acetate (pH 5.5), and 460 μl of enzyme solution, was incubated for 1 h at 40°C, and the reaction was stopped by heating in boiling water. Fructose and kestose in the reaction mixture were measured by HPLC as described previously.[2] The fructosyl transfer activity and hydrolysis activity were measured from the amounts of kestose and fructose, respectively. For total β-fructofuranosidase activity, the amount of glucose was measured with a Glucose CII-Test Wako (Wako Pure Chemical Industries, LTD., Japan) according to the manufacturer's instructions. One unit of each of these enzymatic activities was defined as the amount of enzyme required to produce 1 μmol of the corresponding saccharide per min under these conditions.

Western analysis. Protein samples to be assayed were separated by SDS-polyacrylamide gel electrophoresis by the method of Laemmli[8] and transferred to Hybond-ECL (Amersham K. K., Japan) by using standard procedures.[9] Detection of the *fopA*

gene product was done with an ECL Western blotting analysis system (Amersham K. K., Japan) according to the manufacturer's instructions. Polyclonal antibody was raised in a rabbit against the purified β-fructofuranosidase.

Preparation of cell lysate. Transformant cells of *S. cerevisiae* were collected by centrifugation, washed two times with the lysis buffer (50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol), and homogenized in the lysis buffer by ultrasonication. After centrifugation, supernatants were used as cell lysates.

Protein measurement. Protein concentrations were measured with a Bio-Rad Protein Assay (Bio-Rad Laboratories, Japan) using bovine gamma globulin as a standard.

DNA manipulations. Lambda phage DNA was isolated by the method of Davis *et al.*[10] Plasmid DNA was isolated from *E. coli* by the alkaline lysis method.[9] *E. coli* was transformed using standard procedures.[9] *S. cerevisiae* MS-161 was transformed by the lithium acetate method of Ito *et al.*[11] Recombinant DNA manipulations were done by standard methods.[9]

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the DDBJ, EMBL, and GenBank databases under the accession number AB046383.

Results

Purification and amino acid sequence analyses of β-fructofuranosidase

Fructooligosaccharide-producing β-fructofuranosidase was purified from *A. niger* ATCC 20611 as described previously.[5] Peptides were prepared by endoproteinase Lys-C digestion of a purified sample. Amino acid sequence analyses of the N-terminal part of the protein and several internal peptides were done and the results are shown in Table 1. A total of 85 amino acids were sequenced.

Cloning and sequence analysis of β-fructofuranosidase gene

We named the gene encoding the fructooligosaccharide-producing β-fructofuranosidase for *fopA* (*f*ructooligosaccharide-*p*roducing β-fructofuranosidase). In order to isolate the *fopA* gene, two degenerate oligonucleotides were designed based on the amino acid sequences of peptides 1 and 3, and used for a PCR experiment. A specifically amplified DNA was cloned into pUC119, and sequencing of this fragment showed it was 788 bp long. The amino acid sequence

Table 1. Amino Acid Sequences of N-terminus and Internal Peptides of the β-Fructofuranosidase from *A. niger* ATCC 20611

Source	Amino Acid Sequence
Purified enzyme	SYHLDT
Peptide 1	LDQGPV<u>IADHPFA</u>VDVTAFR
Peptide 2	VEFSPSMAGFLDWGFSAYAA
Peptide 3	VQTV<u>ENVVDNEL</u>VREEGVSW
Peptide 4	AALLAXGSVTAEEDRTLQTA

Underlined amino acids indicate the sequences used for the design of oligonucleotides. An X indicates an undetermined amino acid.

deduced from the DNA sequence of the PCR fragment included the amino acid sequence of peptide 2 as well as those of peptides 1 and 3, which were used for oligonucleotide design. From these results, we concluded that this PCR fragment encoded a part of the *fopA* gene, and decided to use it as a probe.

Southern blot analysis of the chromosomal DNA digested with several restriction enzymes indicated that there was a single hybridizable band the molecular size of which was about 15 kbp in the *Eco*RI digested sample. A subgenomic DNA library containing *Eco*RI fragments of 10 to 20 kbp was constructed in the λDASHII vector as described in Materials and Methods, and screened by plaque hybridization. All positive clones obtained had the same 15-kbp *Eco*RI fragment judging from restriction enzyme analyses. The region that hybridized with the probe was sequenced. The nucleotide and deduced amino acid sequences of the *fopA* gene are shown in Fig. 1. There is an open reading frame of 1962 bp that encodes 654 amino acids including an N-terminal signal sequence of 19 amino acid residues. The amino acid sequences of the N-terminus and all the internal peptides from the purified β-fructofuranosidase can be identified in the deduced amino acid sequence. Cleavage of this putative signal peptide from precursor results in yielding a mature protein the molecular mass of which is 69061 Da. The difference between the deduced molecular mass of the *fopA* gene product and that of the purified β-fructofuranosidase, which was estimated to be 100 kDa on SDS-PAGE, seems to be due to post-translational modification, *e.g.*, N-glycosylation. Twelve potential N-glycosylation sites are present in the amino acid sequence deduced from the *fopA* gene. To analyze the effects of N-glycosylation on the molecular mass of the β-fructofuranosidase, the purified β-fructofuranosidase was deglycosylated with N-glycosidase F[12] and put through western analysis. As shown in Fig. 3 (lane 2), the molecular mass of the deglycosylated β-fructofuranosidase was 70 kDa, which is in good agreement with the calculated molecular mass.

The 5'-noncoding region of the *fopA* gene contains a typical TATA box that could be involved in transcriptional initiation, and it is present 66 bp up-

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                                                            gagctc  -271
cgtcggcgaaaccccaggcaaaacctcggcgatatcctccgcgcccttcacccgcaaccctggcagtgcgggatttcgccgaaatcgcg  -181
gagaatccatggagattcatcgtatgacgatgcacaagggtctgctctgagcaggacatcacggaagtagcagcagcagcagcagcagca   -91
gcgcaagcaagcgcgcaagcgacatatataaagcgccccatctccccggtggcaagactctcctcgaagccatccaatccaggagcatcaca   -1

ATGAAGCTCACCACTACCACCCTGGCGCTCGCCACCGGCGCAGCAGCAGCAGAAGCCTCATACCACCTGGACACCACGGCCCCGCCGCCG    90
 M  K  L  T  T  T  T  L  A  L  A  T  G  A  A  A  A  E  A  S  Y  H  L  D  T  T  A  P  P  P     30

ACCAACCTCAGCACCCTCCCCAACAACACCCTCTTCCACGTGTGGCGGCCGCGCGCGCACATCCTGCCCGCCGAGGGCCAGATCGGCGAC   180
 T  N* L  S  T  L  P  N* N  T  L  F  H  V  W  R  P  R  A  H  I  L  P  A  E  G  Q  I  G  D     60

CCCTGCGCGCACTACACCGACCCATCCACCGGCCTCTTCCACGTGGGGTTCCTGCACGACGGGGACGGCATCGCGGGCGCCACCACGGCC   270
 P  C  A  H  Y  T  D  P  S  T  G  L  F  H  V  G  F  L  H  D  G  D  G  I  A  G  A  T  T  A     90

AACCTGGCCACCTACACCGATACCTCCGATAACGGGAGCTTCCTGATCCAGCCGGGCGGGAAGAACGACCCCGTCGCCGTGTTCGACGGC   360
 N  L  A  T  Y  T  D  T  S  D  N* G  S  F  L  I  Q  P  G  G  K  N  D  P  V  A  V  F  D  G    120

GCCGTCATCCCCGTCGGCGTCAACAACACCCCCACCTTACTCTACACCTCCGTCTCCTTCCTGCCCATCCACTGGTCCATCCCCTACACC   450
 A  V  I  P  V  G  V  N* N  T  P  T  L  L  Y  T  S  V  S  F  L  P  I  H  W  S  I  P  Y  T    150

CGCGGCAGCGAGACGCAGTCGTTGGCCGTCGCGCGCGACGGCGGCCGCCGCTTCGACAAGCTCGACCAGGGCCCCGTCATCGCCGACCAC   540
 R  G  S  E  T  Q  S  L  A  V  A  R  D  G  G  R  R  F  D  K  L  D  Q  G  P  V  I  A  D  H    180

CCCTTCGCCGTCGACGTCACCGCCTTCCGCGATCCGTTTGTCTTCCGCAGTGCCAAGTTGGATGTGCTGCTGTCGTTGGATGAGGAGGTG   630
 P  F  A  V  D  V  T  A  F  R  D  P  F  V  F  R  S  A  K  L  D  V  L  L  S  L  D  E  E  V    210

GCGCGGAATGAGACGGCCGTGCAGCAGGCCGTCGATGGCTGGACCGAGAAGAACGCCCCCTGGTATGTCGCGGTCTCTGGCGGGGTGCAC   720
 A  R  N* E  T  A  V  Q  Q  A  V  D  G  W  T  E  K  N  A  P  W  Y  V  A  V  S  G  G  V  H    240

GGCGTCGGGCCCGCGCAGTTCCTCTACCGCCAGAACGGCGGGAACGCTTCCGAGTTCCAGTACTGGGAGTACCTCGGGGAGTGGTGGCAG   810
 G  V  G  P  A  Q  F  L  Y  R  Q  N  G  G  N* A  S  E  F  Q  Y  W  E  Y  L  G  E  W  W  Q    270

GAGGCGACCAACTCCAGCTGGGGCGACGAGGGCACCTGGGCCGGGCGCTGGGGGTTCAACTTCGAGACGGGGAATGTGCTCTTCCTCACC   900
 E  A  T  N* S  S  W  G  D  E  G  T  W  A  G  R  W  G  F  N  F  E  T  G  N  V  L  F  L  T    300

GAGGAGGGCCATGACCCCCAGACGGGCGAGGTGTTCGTCACCCTCGGCACGGAGGGGTCTGGCCTGCCAATCGTGCCGCAGGTCTCCAGT   990
 E  E  G  H  D  P  Q  T  G  E  V  F  V  T  L  G  T  E  G  S  G  L  P  I  V  P  Q  V  S  S    330

ATCCACGATATGCTGTGGGCGGCGGGTGAGGTCGGGGTGGGCAGTGAGCAGGAGGGTGCCAAGGTCGAGTTCTCCCCCTCCATGGCCGGG  1080
 I  H  D  M  L  W  A  A  G  E  V  G  V  G  S  E  Q  E  G  A  K  V  E  F  S  P  S  M  A  G    360

TTTCTGGACTGGGGGTTCAGCGCCTACGCTGCGGCGGGCAAGGTGCTGCCGGCCAGCTCGGCGGTGTCGAAGACCAGCGGCGTGGAGGTG  1170
 F  L  D  W  G  F  S  A  Y  A  A  A  G  K  V  L  P  A  S  S  A  V  S  K  T  S  G  V  E  V    390

GATCGGTATGTCTCGTTCGTCTGGTTGACGGGCGACCAGTACGAGCAGGCGGACGGGTTCCCCACGGCCCAGCAGGGGTGGACGGGGTCG  1260
 D  R  Y  V  S  F  V  W  L  T  G  D  Q  Y  E  Q  A  D  G  F  P  T  A  Q  Q  G  W  T  G  S    420

CTGCTGCTGCCGCGCGAGCTGAAGGTGCAGACGGTGGAGAACGTCGTCGACAACGAGCTGGTGCGCGAGGAGGGCGTGTCGTGGGTGGTG  1350
 L  L  L  P  R  E  L  K  V  Q  T  V  E  N  V  V  D  N  E  L  V  R  E  E  G  V  S  W  V  V    450

GGGGAGTCGGACAACCAGACGGCCCAGGCTGCGCACGCTGGGGATCACGATCGCCCGGGAGACCAAGGCGGCCCTGCTGGCCAACGGCTCG  1440
 G  E  S  D  N* Q  T  A  R  L  R  T  L  G  I  T  I  A  R  E  T  K  A  A  L  L  A  N* G  S    480

GTGACCGCGGAGGAGGACCGCACGCTGCAGACGGCGGCCGTCGTGCCGTTCGCGCAATCGCCGAGCTCCAAGTTCTTCGTGCTGACGGCC  1530
 V  T  A  E  E  D  R  T  L  Q  T  A  A  V  V  P  F  A  Q  S  P  S  S  K  F  F  V  L  T  A    510

CAGCTGGAGTTCCCCGCGAGCGCGCGCTCGTCCCCGCTCCAGTCCGGGTTCGAAATCCTGGCGTCGGAGCTGGAGCGCACGGCCATCTAC  1620
 Q  L  E  F  P  A  S  A  R  S  S  P  L  Q  S  G  F  E  I  L  A  S  E  L  E  R  T  A  I  Y    540

TACCAGTTCAGCAACGAGTCGCTGGTCGTCGACCGCAGCCAGACTAGTGCGGCGGCGCCCACGAACCCCGGGCTGGATAGCTTTACTGAG  1710
 Y  Q  F  S  N* E  S  L  V  V  D  R  S  Q  T  S  A  A  A  P  T  N  P  G  L  D  S  F  T  E    570

TCCGGCAAGTTGCGGTTGTTCGACGTGATCGAGAACGGCCAGGAGCAGGTCGAGACGTTGGATCTCACTGTCGTCGTGGATAACGCGGTT  1800
 S  G  K  L  R  L  F  D  V  I  E  N  G  Q  E  Q  V  E  T  L  D  L  T  V  V  V  D  N  A  V    600

GTCGAGGTGTATGCCAACGGGCGCTTTGCGTTGAGCACCTGGGCGAGATCGTGGTACGACAACTCCACCCAGATCCGCTTCTTCCACAAC  1890
 V  E  V  Y  A  N  G  R  F  A  L  S  T  W  A  R  S  W  Y  D  N* S  T  Q  I  R  F  F  H  N    630

GGCGAGGGCGAGGTGCAGTTCAGGAATGTCTCCGTGTCGGAGGGGCTCTATAACGCCTGGCCGGAGAGAAATtgaccgggattagggcat  1980
 G  E  G  E  V  Q  F  R  N* V  S  V  S  E  G  L  Y  N  A  W  P  E  R  N                      654

gcagtgtatgattgcctagcatagcacgattgtaacacaaatcaattacaattccattggtaactcctcgctatgcggtacatgcttagt  2070
atgctttgcttgaaagacaagaccagaaacaggatgagcgattttagtagaagctgctcgctcgtggccgtcgttcaatcccctgcgttg  2160
cgggtgggcgctcctgtgtcccgatggtcaacggggcgggcaggagctgtgaagacatcggcccggg                          2227
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Fig. 1. Nucleotide Sequence of the *fopA* Gene and the Deduced Amino Acid Sequence of the Gene Product.
    Amino acid sequences identified by Edman degradation are underlined. Putative CreA binding sites are double underlined. Asparagine residues marked with asterisks indicate potential N-glycosylation sites.

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FopA   MKLTTTTLALATGAAAAEA-----SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHV
Suc1   ***Q*-ASV*LGS****SPSMQTRASVVIDYNVA*P*******GS**ET******V**PN*******L***********
Sir-1  ***Q*-ASV*LGS****SPSMQTRASVVIDYNVA*P*******GS**ET**S***V**PN*RSVI*ACITPI*PRAS***

FopA   GFLHDGDGIAGATTANLATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSFLPIHWSIPYTRGSET
Suc1   ******S**SS***DD****K*LNQ-*NQV*V***I*********S***S*I*GL*********Y**************
Sir-1  ******S**SS***DD*P**Q*LNQ-*NQV*V***I*********S***N*I*GL*********Y**************

FopA   QSLAVARDGGRRFDKLDQGPVIADHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAV
Suc1   *****SS***SN*T********PGP***YN*******Y**QNPT**S**H------------------S**NT**TVI
Sir-1  *****SS***SN*T********PGP***YN*******Y**QNPT*ES**H-----------------S**NA**TVI

FopA   SGGVHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEVFVTL
Suc1   ***L**K**********YD---PD****F**Q**H*P***T**N-*******A********FS*D*Y*YN*-H*QI*S*I
Sir-1  ***L*EK**********YD---*D*******Q**H*P***T**N-*******A********FS*D*Y*YN*-H*QI*T*I

FopA   GTEGSGLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSKTSGVEVDRYVS
Suc1   *****DQ*V***LT*******VS*N*SR-----NGS*S*T*N**********S*********ST*LP*TK**AP-**FI*
Sir-1  *****D**V***LT*******VS*T*SR-----NGS*S*T*N**********S*********ST*LP*TK**AP-**FI*

FopA   FVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSW--VVGESDNQTARLRTLGITIARETKA
Suc1   Y***S**LF***E****N**N***T*******R*LYIP*****A*A**S*A**QV*SSD*SAG*VE*Q****S*******
Sir-1  Y***S**LF***E****N**N***T*******R*LYIP*****A*A**S*A**QV*SSDGSAG*VE*Q****S*******

FopA   ALLANGSVTAEEDRTLQTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEILASELERTAIYYQFSNESLVVDRS
Suc1   ***SGT*F*-*S****NSSG****KR***E*****S***S******G*G*K***Q**S****S*TV********II****
Sir-1  ***SGT*F*-*SG***NSSG****KR***E*****S***S******G*G*K***Q**S**H*S*TV********II****

FopA   QTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEVYANGRFALSTWARSWYDNSTQIRFFHNGEG
Suc1   N*****R*TD*I**SA*A*********LNG*EQAI******L****S*L*I***********V*----------------
Sir-1  N*****R*TD*I**SA*A*********LNG*EQAI******L****S*LG***********V*----------------

FopA   EVQFRNVSVSEGLYNAWPERN
Suc1   --------------------
Sir-1  --------------------
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Fig. 2. Alignment of the β-Fructofuranosidase Amino Acid Sequences Deduced from the *fopA*, *suc1*, and *sir-1* Genes. Asterisks indicate amino acids identical to those of the *fopA* gene product.

stream of the ATG codon. In addition, there are two consensus binding target sequences for CreA,[13] which plays a major role in carbon catabolite repression, located at 49 and 259 bp upstream of the ATG codon. The existence of CreA binding sites in the 5'-noncoding region of the *fopA* gene is consistent with the expression of the β-fructofuranosidase being repressed by glucose (data not shown).

The deduced amino acid sequence of the *fopA* gene product had high degrees of similarity to the primary structures deduced from the *suc1*[14] and *sir-1*[15] gene (62% and 59% identity, respectively), both of which were isolated from *A. niger* and encoded β-fructofuranosidases (Fig. 2), and also had a significant level of similarity to the primary structure of the sucrose:sucrose 1-fructosyltransferase from *A. foetidus* (22% identity).[16] Among the three β-fructofuranosidases from *A. niger*, sequence similarity

was observed throughout the amino acid sequences, but in the *fopA* gene product there were several amino acid insertions and an extra C-terminal polypeptide consisting of 38 amino acids which could not be found in two other β-fructofuranosidases.

Expression of the fopA gene in S. cerevisiae

To ascertain whether the presumption of the open reading frame of the *fopA* gene is correct, we tried to express it in *S. cerevisiae*. The plasmid pYSUC for yeast transformation was constructed as follows. The coding region of the *fopA* gene was amplified by PCR method using the following primers, 5'-GC-GGATCCAATGAAGCTCACCACTACC-3' and 5'-GCGGATCCCGGTCAATTTCTCTCC-3' as N- and C-terminal side primer, respectively. The amplified DNA fragment was digested with *Bam*HI and inserted into the *Bam*HI site of yeast expression vector
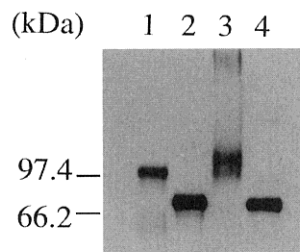
pY2831, which has the promoter and terminator of the glyceraldehyde 3-phosphate dehydrogenase gene of *S. cerevisiae*, yielding the plasmid pYSUC.

S. cerevisiae MS-161, which is not able to assimilate sucrose as a carbon source, was transformed with each plasmid, pY2831 and pYSUC. Transformants harboring plasmid pY2831 did not show any enzymatic activity of *β*-fructofuranosidase in the culture supernatant, but transformants harboring plasmid pYSUC showed significant *β*-fructofuranosidase activity, the fructosyl transfer and hydrolysis activity of which was 10.5 and 0.47 units/ml, respectively. The Ut/Uh ratio of the *fopA* gene product produced by *S. cerevisiae* was 22.3 and higher than that (=14.2) of crude enzyme prepared from *A. niger* ATCC 20611.[2] This may be due to the yeast host strain used for transformation experiment having no endogenous hydrolysis activity for sucrose. The carbohydrate composition of fructooligosaccharides produced by the culture supernatant of the yeast transformant was almost the same as that produced by the *β*-fructofuranosidase prepared from *A. niger* ATCC 20611 (data not shown).

In western analysis of the culture supernatant of the transformant harboring plasmid pYSUC (Fig. 3), a smear signal was detected at more than 100 kDa, indicating that the *fopA* gene product produced by *S. cerevisiae* was heterogeneous in size, mostly larger than the native enzyme produced by *A. niger* (lane 1 and 3). This was due to the high N-glycosylation occurring in *S. cerevisiae*, because N-glycosidase F treatment of the *fopA* gene product produced by *S. cerevisiae* increased its mobility and it migrated as one distinct band, and its size corresponded to that of the deglycosylated native enzyme (lane 2 and 4).

Analysis of C-terminal region of the fopA gene product

As described above, amino acid sequence alignment indicated the *fopA* gene product contained an additional C-terminal region consisting of 38 amino acids, which was not present in the amino acid sequences deduced from the *suc1* and *sir-1* genes encoding *β*-fructofuranosidases of *A. niger*. To analyze the function of the C-terminal region of the *fopA* gene product, we decided to express the 3′-truncated mutant gene in *S. cerevisiae* and constructed the expression plasmid pYSUC-DEL as follows. Using the same N-terminal side primer for plasmid pYSUC construction and a new C-terminal side primer, 5′-GCGGATCCTCATCTCGCCCAGGTGCTCA-3′, the PCR reaction was done to introduce a new termination codon, TGA, just below the 597th amino acid, arginine, for the mature enzyme. The specific amplified 1.87-kb DNA fragment was digested with *Bam*HI and inserted into the *Bam*HI site of the expression vector pY2831, yielding pYSUC-DEL. Hence, yeast transformants harboring plasmid

Fig. 3. Western Blot Analysis of the *β*-Fructofuranosidase Purified from *A. niger* ATCC 20611 and the Culture Supernatant from the *S. cerevisiae* Transformant Expressing the *fopA* Gene.

Lane 1, the purified *β*-fructofuranosidase; lane 2, the purified *β*-fructofuranosidase after treatment with N-glycosidase F; lane 3, the culture supernatant from the *S. cerevisiae* transformant; lane 4, the culture supernatant from the *S. cerevisiae* transformant after treatment with N-glycosidase F. The position and size of the molecular mass markers used are shown on the left.

Table 2. *β*-Fructofuranosidase Activities in the Culture Supernatants and Cell Lysates of *S. cerevisiae* Transformants

Plasmid	*β*-Fructofuranosidase activity	
	Supernatant (U/ml)	Cell lysate (U/mg·protein)
pYSUC	12.4	50.3
pYSUC-DEL	$<2.8 \times 10^{-3}$	$<1.5 \times 10^{-2}$

One unit of *β*-fructofuranosidase was defined as the amount of enzyme required to produce 1 *μ*mol of glucose per min under the conditions described in Materials and Methods.

pYSUC-DEL should express the C-terminal by 38 amino acids truncated *fopA* gene product, the C-terminus of which was equivalent to those of two other *β*-fructofuranosidases of *A. niger*.

S. cerevisiae MS-161 was transformed with pYSUC-DEL and the transformant obtained was cultivated in the same way as the transformant harboring pYSUC. The culture supernatants and cell lysates were prepared from each transformant, and the measurement of total *β*-fructofuranosidase activity and western analysis were done. The results were shown in Table 2 and Fig. 4. In the case of the transformant harboring pYSUC, enzymatic activities and protein products that cross-reacted with anti-*β*-fructofuranosidase antibody were detected in both of the supernatant and cell lysate. On the other hand, in the case of the transformant harboring pYSUC-DEL, no enzymatic activity and protein product cross-reacting with the antibody was detected in both of the supernatant and cell lysate. These results suggest that the C-terminal region of the *fopA* gene product consisting 38 amino acids was essential for the production of it.

Discussion

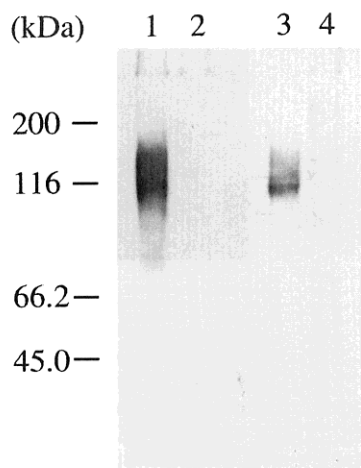In this report, we describe the isolation of the gene (*fopA*) encoding the fructooligosaccharide-produc-

Fig. 4. Western Blot Analysis of the Culture Supernatants and Cell Lysates from the *S. cerevisiae* Transformants Harboring pYSUC and pYSUC-DEL.

Samples were concentrated by ultrafiltration and then put through SDS-PAGE. Lane 1, the culture supernatant from the *S. cerevisiae* transformant harboring pYSUC (200 μl); lane 2, the culture supernatant from the *S. cerevisiae* transformant harboring pYSUC-DEL (200 μl); lane 3, the cell lysate from the *S. cerevisiae* transformant harboring pYSUC (20 μg); lane 4, the cell lysate from the *S. cerevisiae* transformant harboring pYSUC-DEL (20 μg). The position and size of the molecular mass markers used are shown on the left.

ing β-fructofuranosidase from *A. niger* ATCC 20611. Nucleotide sequence analysis indicated that the *fopA* gene had an open reading frame composed of 1962 bp and not interrupted by introns. All of the five amino acid sequences obtained with the sequence analyses of the N-terminus and four internal peptides from the purified β-fructofuranosidase could be identified in the amino acid sequence deduced from the open reading frame. *S. cerevisiae* transformants harboring the plasmid pYSUC, constructed for the expression of the open reading frame, produced a β-fructofuranosidase that had the same catalytic properties as the enzyme derived from *A. niger* ATCC 20611. Moreover, western analysis of the β-fructofuranosidase produced by *S. cerevisiae* transformants and native enzyme showed that the two proteins were different in molecular mass but the same after enzymatic deglycosylation of them, indicating that two proteins had the same polypeptide chain length. Taken together, these data show that this open reading frame certainly encodes the amino acid sequence of the fructooligosaccharide-producing β-fructofuranosidase from *A. niger* ATCC 20611.

Two genes, *suc1* and *sir-1*, have been isolated as β-fructofuranosidase encoding genes from other strains of *A. niger*. The catalytic properties of the two β-fructofuranosidases, especially fructosyl transfer activity/hydrolysis activity (Ut/Uh), remain unknown. Recently we have obtained data that show that *A. niger* NRRL 4337 has the same gene as the *suc1* gene.

The β-fructofuranosidase prepared from *A. niger* NRRL 4337 showed a much lower ratio (=Ut/Uh) than that of *A. niger* ATCC 20611 and could not be used for fructooligosaccharide production.[2] Therefore, the fructosyl transfer activity of the *suc1* gene product might be lower than that of the *fopA* gene product.

Amino acid sequence alignment indicated that the *fopA* gene product contained an extra C-terminal polypeptide consisting of 38 amino acids, which could not be found in the *suc1* and *sir-1* gene products. At first we thought that this extra C-terminal polypeptide plays an important role in fructosyl transfer activity, and then we tried to express and characterize the C-terminal truncated *fopA* gene product by 38 amino acids in *S. cerevisiae*. However we could not express it. From this result, it was suggested that the extra C-terminal polypeptide was not important for the fructosyl transfer activity but essential for the enzyme production itself. There is 62% sequence similarity between the *fopA* and *suc1* gene products, and they are regarded as β-fructofuranosidases belonging to the same family. It is very strange that the polypeptide region essential for the production of the *fopA* gene product does not exist in the *suc1* gene product. So, we looked over the nucleotide sequence of the *suc1* gene in detail and found a candidate for an intron at 1 bp upstream of the deduced termination codon. It is composed of 55 bp and consistent with the consensus sequences of filamentous fungal introns.[17] If our assumption for the existence of intron in the *suc1* gene is correct, the coding region of the *suc1* gene is longer than that reported before[14] by 39 amino acids, the sequence of which has 68% sequence similarity to that of the extra C-terminal polypeptide of the *fopA* gene product. Therefore, it is reasonable that the intron is really present in the *suc1* gene. On the *sir-1* gene, it remains unknown whether an intron is present at the same position as the *suc1* gene or not because the nucleotide sequence of the 3'-noncoding region has not been shown. cDNA analyses of the *suc1* and *sir-1* genes will clarify this ambiguity.

One of our interests is why the *fopA* gene product has strong fructosyl transfer activity. Somiari *et al.* describe how 16 amino acids between positions 61 and 77 of the *sir-1* gene product are different from that reported for the *suc1* gene product, and speculate that the hydrophobicity of this region relates to the transfructosylating properties.[15] This is not applicable to the *fopA* gene product because the amino acid sequence in this region of the *fopA* gene product is identical to that of the *suc1* gene product. The *fopA* gene and its expression system in this study will provide a clue as to the mechanism by which the *fopA* gene product shows strong fructosyl transfer activity.

Acknowledgment

References

1) Gunasekaran, P., Karunakaran, T., Cami, B., Mukundan, A. G., Preziosi, L., and Baratti, J., Cloning and sequencing of the *sacA* gene: Characterization of a sucrase from *Zymomonas mobilis*. *J. Bacteriol.*, **172**, 6727–6735 (1990).

2) Hidaka, H., Hirayama, M., and Sumi, N., A fructooligosaccharide-producing enzyme from *Aspergillus niger* ATCC 20611. *Agric. Biol. Chem.*, **52**, 1181–1187 (1988).

3) Hidaka, H., Hirayama, M., Tokunaga, T., and Eida, T., The effects of undigestible fructooligosaccharides on intestinal microflora and various physiological functions on human health. *Adv. Exp. Med. Biol.*, **270**, 105–117 (1990).

4) Horiuchi, H., Ashikari, T., Amachi, T., Yoshizumi, H., Takagi, M., and Yano, K., High-level secretion of a *Rhizopus niveus* aspartic proteinase in *Saccharomyces cerevisiae*. *Agric. Biol. Chem.*, **54**, 1771–1779 (1990).

5) Hirayama, M., Sumi, N., and Hidaka, H., Purification and properties of a fructooligosaccharide-producing β-fructofuranosidase from *Aspergillus niger* ATCC 20611. *Agric. Biol. Chem.*, **53**, 667–673 (1989).

6) Horiuchi, H., Yanai, K., Okazaki, T., Takagi, M., and Yano, K., Isolation and sequencing of a genomic clone encoding aspartic proteinase of *Rhizopus niveus*. *J. Bacteriol.*, **170**, 272–278 (1988).

7) Sanger, F., Nicklen, S., and Coulson, A. R., DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467 (1977).

8) Laemmli, U. K., Cleavage of structual proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685 (1970).

9) Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., Current protocols in molecular biology., Greene Publishing Associates and Wiley-Interscience, New York (1987).

10) Davis, L. G., Dibner, M. D., and Battey, J. F., Methods in molecular biology., Elsevier, New York, pp. 158–160 (1986).

11) Ito, H., Fukuda, Y., Murata, K., and Kimura, A., Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.*, **153**, 163–168 (1983).

12) Tarentino, A. L., Gomez, C. M., and Plummer, T. H., Deglycosylation of asparagine-linked glycans by peptide:N-glycosidase F. *Biochemistry*, **24**, 4665–4671 (1985).

13) Cubero, B. and Scazzocchio, C., Two different, adjacent and divergent zinc finger binding sites are necessary for CREA-mediated carbon catabolite repression in the proline gene cluster of *Aspergillus nidulans*. *EMBO J.*, **13**, 407–415 (1994).

14) Boddy, L. M., Bergès, T., Barreau, C., Vainstein, M. H., Dobson, M. J., Ballance, D. J., and Peberdy, J. F., Purification and characterisation of an *Aspergillus niger* invertase and its DNA sequence. *Curr. Genet.*, **24**, 60–66 (1993).

15) Somiari, R. I., Brzeski, H., Tate, R., Bieleck, S., and Polak, J., Cloning and sequencing of an *Aspergillus niger* gene coding for β-fructofuranosidase. *Biotechnol. Lett.*, **19**, 1243–1247 (1997).

16) Rehm, J., Willmitzer, L., and Heyer, A. G., Production of 1-kestose in transgenic yeast expressing a fructosyltransferase from *Aspergillus foetidus*. *J. Bacteriol.*, **180**, 1305–1310 (1998).

17) Ballance, D. J., Sequences important for gene expression in filamentous fungi. *Yeast*, **2**, 229–236 (1986).