Gene Cloning and Functional Characterization by Heterologous Expression of the Fructosyltransferase of *Aspergillus sydowi* IAM 2544

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We have purified a fructosyltransferase from conidia of the inulin-producing fungus *Aspergillus sydowi* IAM 2544 and obtained peptide sequences from proteolytic fragments of the protein. With degenerated primers, we amplified a PCR fragment that was used to screen a cDNA library. The fructosyltransferase gene from *Aspergillus sydowi* (EMBL accession no. AJ289046) is expressed in conidia, while no expression could be detected in mycelia by Northern blot analysis of mycelial RNA. The gene encodes a protein with a calculated molecular mass of 75 kDa that is different from all fructosyltransferases in the databases. The only homology that could be detected was to the invertase of *Aspergillus niger* (EMBL accession no. L06844). The gene was functionally expressed in *Escherichia coli*, yeast, and potato plants. With protein extracts from transgenic bacteria and yeast, fructooligosaccharides could be produced in vitro. In transgenic potato plants, inulin molecules of up to 40 hexose units were synthesized in vivo. While in vitro experiments with protein extracts from conidia of *Aspergillus sydowi* yielded the same pattern of oligosaccharides as extracts from transformed bacteria and yeast, in vivo inulin synthesis with fungal conidia leads to the production of a high-molecular-weight polymer.

Fructans are a structurally diverse group of polysaccharides consisting mainly of fructose units which are linked by either β2→1 (inulin type) or β2→6 (levan type) glycosidic bonds (46). In most cases the molecules contain a terminal glucose, as polymerization starts from sucrose, but structural types with an intermittent glucose molecule can also be observed (8). Interest in fructans and fructooligosaccharides has increased constantly since the discovery of beneficial effects in human nutrition. They are regarded as "functional food," which positively influences the composition of the gut microflora (reviewed in reference 34), and there is indication for improved mineral absorption, blood lipid composition, and prevention of colon cancer (44). Besides, fructans are interesting resources for nonfood applications, e.g., the production of biodegradable surfactants (10). For technical applications, fructans with a high molecular mass and a low degree of branching are desirable.

Fructans occur in various bacterial, fungal, and plant species, where they serve different functions. In plants, fructans are synthesized as short-term or long-term storage carbohydrates and are usually of low M_r (>35,000) (30). In bacteria, fructans are produced as part of the exopolysaccharide, have a very high molecular mass, and are in almost all cases of the levan type. They are synthesized by secreted enzymes that use sucrose as substrate for fructosyltransfer to a growing chain (5). Levans impose a high viscosity on aqueous solutions, and their presumed function is to protect cells from desiccation, allow adherence to surfaces (23), or, in the case of plant pathogenic bacteria, to delay recognition of the pathogen by the host defense system (17, 21).

Little is known about the physiological significance of fructans in fungi, although several fungal strains have been identified that synthesize either low- or high-molecular mass fructans. A fructosyltransferase producing the trisaccharide 1-kestose has been cloned from *Aspergillus foetidus* (31), and fructooligosaccharide production has been reported for *Aspergillus niger* (19) and *Fusarium oxysporum* (29). Synthesis of high-molecular-mass inulin was demonstrated for *Penicillium chrysogenum* (28) and *Aspergillus sydowi* IAM 2544 (14, 22).

The fructosyltransferase of *A. sydowi* is particularly interesting because synthesis of different products has been observed under differing experimental conditions. Suspensions of fungal conidia synthesize an inulin of an M_r of about 30×10^6 (14, 22, 48), whereas lyophilized, rehydrated mycelia produce a mixture of oligosaccharides with a degree of polymerization (DP) ranging from 3 to 13 (26, 27). It is unclear whether this discrepancy is due to the use of different tissues or to other differences in the experimental procedures.

We have purified a fructosyltransferase from *A. sydowi* IAM 2455 conidia, obtained peptide sequences, and cloned the corresponding cDNA. Expression of this cDNA in various heterologous systems revealed that catalytic specificity is strongly dependent on experimental conditions.

MATERIALS AND METHODS

Strains and plasmids. *A. sydowi* IAM 2455 was obtained from the Institute of Applied Microbiology (Tokyo, Japan). *Saccharomyces cerevisiae* YSH 2.64-1A (12) and *Escherichia coli* XL1blue (Stratagene, Heidelberg, Germany) were used as microbial expression systems. *Solanum tuberosum* Var. Désirée (Saatzucht Lange, Bad Schwartau, Germany) served as a higher eucaryote for expression of the fructosyltransferase cDNA.

Vectors pBluescript SK (Stratagene), pUC 19 (New England Biolabs, Schwalbach, Germany), and pCR II (Invitrogen, Leek, The Netherlands) were used for transformation of *E. coli*. Plasmid p112A1NE (32) was used as the yeast expression vector. This vector contains the promoter of the alcohol dehydrogenase gene ADH1 (45) and the transcriptional terminator of the same gene. The phage vector λ ZAP II (Stratagene) was used for construction of the cDNA library.

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Culture of *A. sydowi*. Preparation of conidia was done as described by Harada et al. (14). Briefly, the fungus was grown on 2% malt extract (Merck, Darmstadt, Germany), 0.5% peptone (Difco, Detroit, Mich.), and 2% sucrose at 25°C. After drying of the medium, conidia were harvested by filtration through filter paper to remove mycelium and through a 0.42-μm-pore-size nylon membrane to obtain the conidia.

Protein purification. The fructosyltransferase was purified following a method described by Muramatsu and Nakakuki (27) with several modifications. Conidia were harvested from five agar plates (diameter, 13 cm), resuspended in 30 ml of 50 mM sodium phosphate buffer (pH 5.6), and homogenized by two passages through a French pressure cell (FA-030; SLM Aminco Instruments, Urbana, Ill.) at 40,000 lb/in^2. This homogenate, containing about 60 mg of protein, was loaded on a Q-Sepharose fast-flow column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with 50 mM sodium phosphate (pH 5.6), and protein was eluted with linear gradient ascending to 1 M KCl in 50 mM sodium phosphate (pH 5.6). Fractions with sucrolytic activity were identified between 0.5 and 0.7 M KCl. The pooled fractions were dialyzed against 100 volumes of 50 mM sodium phosphate buffer (pH 5.6) and were adjusted to 2 M ammonium sulfate before loading on a 10-ml Phenylsuperose column (Amersham Pharmacia Biotech). This column was eluted with a descending ammonium sulfate gradient. Fractions with sucrolytic activity started to elute when the ammonium sulfate concentration fell below 180 mM. The fractions were pooled and concentrated using Centricon 10 (Amicon, Beverly, Mass.). Protein (10 μg) was loaded on seminative polyacrylamide gels. From preparative gels, the band with sucrolytic activity was excised. Generation of proteolytic fragments of the protein by endopeptidases LysC and AspN, purification of the peptides by high-pressure liquid chromatography, and sequencing was performed at TopLab GmbH (Munich, Germany).

Detection of sucrolytic activity and seminative PAGE. Seminative polyacrylamide gels were prepared according to Laemmli (24) containing 0.1% sodium dodecyl sulfate (SDS) and 15% acrylamide-bisacrylamide (29:1). Samples were loaded in a buffer containing 0.1% SDS, 10% glycerol, and 50 mM Tris (pH 6.8) without prior heating. After polyacrylamide gel electrophoresis (PAGE), the gel was washed extensively with 50 mM sodium acetate (pH 5.6) containing 0.5% (vol/vol) Triton X-100 to remove SDS.

To detect sucrolytic activity, protein fractions, seminative gels, and protein extracts from *E. coli* or yeast cultures were incubated in 500 mM sucrose and 50 mM sodium acetate (pH 5.6). Incubation times were 30 min at room temperature for purified protein fractions or seminative gels or several days for protein extracts. Visualization of sucrolytic activity was performed by incubation with 1% (wt/vol) 2,3,5,-triphenyltetrazoliumchloride (TTC) in 0.25 M NaOH at 95°C. Sucrolytic activity resulted in formation of a red formazan dye due to the reaction of TTC with reducing sugars. The reaction was stopped with 5% (vol/vol) acetic acid.

Carbohydrate analysis. High-performance anion-exchange chromatography (HPAEC) analysis was performed as described by Hellwege et al. (15). Glucose release from sucrose was measured enzymatically (41). Inulinase and amyloglucosidase digestion was done in 50 mM sodium acetate (pH 5.2) at 55°C for 2 h.

RNA preparation and construction of cDNA library. *A. sydowi* was cultivated in liquid medium until conidia formation was visible. Fungal biomass was harvested by filtration through Miracloth (Calbiochem, La Jolla, Calif.), frozen in liquid nitrogen, and homogenized by grinding in a mortar. Plant and fungal RNA was isolated following the method of Logemann et al. (25), and polyadenylated RNA was enriched using the polyATract kit (Promega, Madison, Wis.). The cDNA, synthesized with the ZAP-cDNA synthesis kit (Stratagene), was ligated into λ Zap II phage vector arms and packaged using Gigapack II Gold Packaging Extract (Stratagene). *E. coli* RNA was prepared according to the method of Summers (42).

Cloning of *A. sydowi* SFT. From the sequence of proteolytic fragments of the purified fructosyltransferase, five degenerated PCR primers were designed: AspN19down (5′-GAYGAYYTNGTNACNTAYMG-3′), AspN19up (5′-CKRT ANGTNACNARRTCRTC-3′), AspN31down (5′-GTNTTYCARAAYCAYGA RG-3′), AspN31up (5′-TGRTTYTGRAANACRTANGG-3′), and Lys1up (5′-GCYTGNSWNGTNSWNGG-3′). PCR reactions with the whole cDNA library as template and *Taq* polymerase (Q Biogene, Heidelberg, Germany) were performed with 2 mM MgCl$_2$ and annealing temperatures calculated for the various primer combinations following the "2 plus 4" rule. The combination AspN19down-AspN31up yielded a fragment of 320 bp that was used for screening the cDNA library after radioactive labeling (Megaprime kit; Roche, Mannheim, Germany). Screening of the library followed standard protocols (36) and yielded a full-size clone termed as1. DNA sequencing was performed at Replicon GmbH (Berlin, Germany).

Heterologous expression of *A. sydowi* fructosyltransferase. For expression in *E. coli* XL1blue (Stratagene), the fructosyltransferase cDNA was cloned as a *lacZ* fusion in the vector pBluescript SK II by digesting *pas1* with restriction enzymes *Bam*HI and *Sma*I, filling in sticky ends and religating the plasmid. This placed the cDNA into the *lacZ* reading frame. Bacteria were grown in full medium until mid-log phase before adding 0.5 mM isopropyl-β-D-thiogalactopyranoside. After culture for an additional 4 h, the cells were harvested and homogenized in 50 mM sodium phosphate (pH 5.6).

The yeast strain YSH 2.64-1A (12), which is a mutant defective in the invertase gene *suc2*, was transformed according to the method of Dohmen et al. (6) with the construct p112-as1 containing the fructosyltransferase cDNA cloned into the vector p112A1NE. The cDNA was cut out of a *pas1* as a *Not*I-*Asp718* (blunted) fragment and inserted into the *Not*I-*Bam*HI (blunted) cut vector. Transformed cells were grown in minimal medium to an optical density at 600 nm of 0.7, harvested, and disrupted by vigorous agitation in the presence of glass beads.

Potato was transformed as described by Rocha-Sosa et al. (35) with a derivative of pBin19 (1). The construct p35-as1 contained the fructosyltransferase cDNA inserted between the constitutive cauliflower mosaic virus 35S RNA promoter (35) and the octopine synthase terminator sequence (11). For analysis of plants, soluble sugars were extracted from leaves after grinding the tissue under liquid N$_2$. The ground tissue was extracted twice with sodium phosphate buffer (pH 5.6) at 60°C for 30 min. After centrifugation, the combined supernatants were extracted with phenol-chloroform and chloroform before HPAEC analysis.

High-performance size-exclusion chromatography. The molecular mass distribution of high-molecular-weight inulin samples was determined by HPSEC with dimethyl sulfoxide as eluent. The HPSEC system (Waters) consisted of a 600MS pump module, 717 autoinjector, column compartment, RI-detector 410 and MALLS detector, a Dawn-F-DSP laser photometer (Wyatt Technology, Santa Barbara, Calif.) fitted with an S2 flow cell, and an Ar-ion laser operating at $\lambda_0 = 488$ nm and equipped with 18 detectors at angles ranging from 7.5 to 157°. The columns were Waters Styragel HMW 7, HMW 6E, and HT 3 with dimensions of 300 by 7.8 mm. The elution of samples was carried out with dimethyl sulfoxide containing 0.09 M NaNO$_3$ at a flow rate of 0.5 ml/min and a temperature of 60°C.

Nucleotide sequence accession number. The sequence of the complete insert of the clone termed pas1 was deposited in the EMBL database under accession no. AJ289046.

RESULTS

Cloning of *A. sydowi* fructosyltransferase. The fructosyltransferase of *A. sydowi* IAM 2455 was partially purified in a three-step procedure involving anion-exchange chromatography, hydrophobic interaction, and ultrafiltration as described in Materials and Methods. The initial homogenate of fungal conidia, containing 60 mg of protein, yielded a fraction with 99.8 μg of protein, which was enriched in fructosyltransferase activity. Activity was assayed as the release of glucose and fructose from sucrose, and a value of 7.53 μmol of glucose/mg · min was obtained. The glucose to fructose ratio was 6.4. Analysis by seminative polyacrylamide gel electrophoresis revealed that the preparation contained at least seven major proteins (Fig. 1, lane 1). When the gel was incubated in 500 mM sucrose and stained for sucrolytic activity, a band with an apparent molecular mass of approximately 55 kDa could be identified, which is marked with an arrow in the figure (Fig. 1, lane 2). This band was excised from a preparative gel and used for peptide sequencing after the generation of proteolytic fragments with the peptidases LysC and AspN. Three peptide sequences were obtained, one with LysC and two with AspN: LysC (VLPSTSQASEK), AspN19 (DDLVTYR), and AspN31 (DPYVFQNHEV).

Five degenerated DNA sequences were chosen to design primers for PCR reactions (see Material and Methods). The combination AspN19down-AspN31up yielded a fragment of
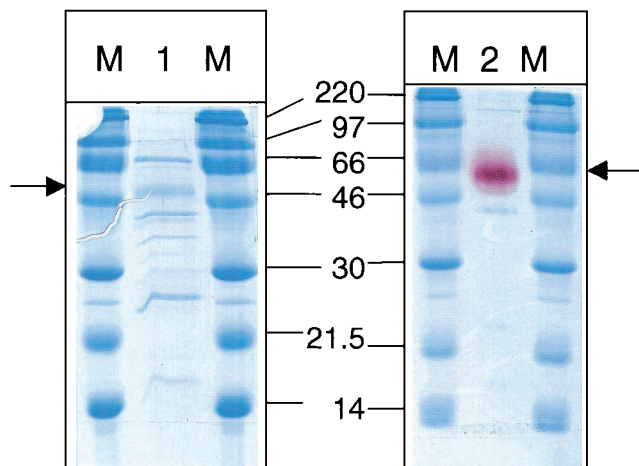
FIG. 1. PAGE of a protein fraction from conidia of *A. sydowi* IAM 2544 enriched in frucosyltransferase activity (lanes 1 and 2). Left: the gel was stained with Coomassie brilliant blue. The band with sucrolytic activity is marked by an arrow. Right: activity stain with 1,2,3 triphenyltetrazolium chloride. M, marker lane. The numbers between the two gels give the molecular mass of the marker proteins in kDa.

about 320 bp at an annealing temperature of 40°C. This fragment was used to screen an *A. sydowi* cDNA library.

Screening 7×10^5 PFU resulted in identification of 11 positive clones, which were obtained as plasmids by in vivo excision. Of these clones, 6 had an insert of 2.2 kb. Partial sequencing of these clones revealed that they were all derived from the same gene. The complete insert of one clone, termed pas1, was sequenced on both strands. The encoded gene was called *SFT* (sucrose-dependent fructosyltransferase). The deduced protein

sequence of *SFT* comprises 682 amino acids (Fig. 2) with a calculated molecular mass of 74,665 Da. The peptide sequences obtained from sequencing the proteolytic fragments are underlined in the figure. Because the N terminus of the isolated protein was blocked, we could not obtain an N-terminal peptide sequence. It is therefore not possible to judge whether the protein is posttranslationally processed. The discrepancy between the calculated molecular mass of 75 kDa and the apparent molecular mass in SDS-PAGE might be taken as an indication for proteolytic processing of the protein. Comparison of the deduced protein sequence with other fructofuranosidases revealed low homology to all known fructosyltransferases and invertases of bacterial or plant origin. The conserved domains of fructofuranosidases (13) were missing except one, which is marked in Fig. 2 by a dotted line. Only the invertase of *A. niger* showed significant identity (Table 1).

Expression in *E. coli* and *S. cerevisiae*. For heterologous expression of *SFT*, the *E. coli* strain XL1blue and the yeast YSH 2.64-1A were used. Both strains completely lack sucrolytic activity and therefore served an ideal expression system. Using the plasmid pas1, expression vectors for *E. coli* and yeast were constructed as described in Materials and Methods. The analysis by HPAEC of extracts of transformed cultures as well as fungal conidia incubated with sucrose for 1 week is shown in Fig. 3. Incubation of conidial protein extracts with sucrose led to the formation of fructooligosaccharides with a maximal DP of 8 hexose units (Fig. 3B). The peaks were split into doublets and triplets, and comparison with an inulin standard isolated from roots of globe artichoke demonstrated that structural types of fructans different from the inulin series (β2→1 linkages) were present. The same complex pattern of oligosaccharides was obtained from extracts of transformed yeast (Fig. 3C)

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  1   MKLPSSLDIL LARQAVGGTE VDYDSPPPDL TTLPENSLFE TWRPKIHVLP

 51   PNGQIGDPCA HYNDPATGLF HVGFLHNGTG ISSVYTDDLV TYRDINPNGG

101   YIIVAGGPND PEAVFDGSVI PSGIDDLPTL LYTSVTSLPI HWTLPYTPGS

151   ETQSLAVSDD GGHHFDKLDR GPVIPLPPDG LDVTAFRDPY VFQNHEVDEV

201   TGSDPDTWYA AISGGVHDVG PGIFLYRNQD SSFENWEYLG EWWQEPANST

251   WGDGTWAKRW GYNFETGNVF SLDREGYNVD GHTFMTIGVE GAYAPIQPSV

301   TSMHAMLWAA GNVSSENGEN VTFTPYMAGA LDWGMAAYAG AGKVLPSTSQ

351   ASEKSGAPDR FISWVWLTGD EFGAAAGFPA AQQGWQNTLL LPRELSIHTI

401   QNVVDNELIH ETASWRVAEH GGERRSGGVE LETLGINIAR ETYDAIVSSG

451   TSFEEPSRDI NESGTIPFER SPTSRFFALE AQISFPQSAR DSEVQSGFQI

501   LASELEWTTI YYQFSNESIV IDRNHTSAAS ETTPGLGTVT ESGRIRLFDI

551   AGGCDHDGHG GHDGGNDDDH NGDGDHSGDG DHNDDDDHNV DGDDKERARY

601   QKRDGPCDKD HDKVETLDLT IVVDNSVLEV YANSRFVVST WVRPWYTNST

651   EIRFFHNGEG EVSFDNIAVH DGLYDAYPDR DN
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FIG. 2. Amino acid sequence of the fructosyltransferase of *A. sydowi*. The sequences obtained from peptide sequencing of the purified protein are underlined. The dotted line indicates a conserved "fructofuranosidase domain."

TABLE 1. Sequence comparison of *A. sydowi* fructosyltransferase with invertases and fructosyltransferases of bacterial, fungal, and plant origin

Invertase or fructosyltransferase	% Identity of amino acid sequence with *A. sydowi* fructosyltransferase
A. niger invertase[a]	64
S. cerevisiae invertase[b]	24
A. foetidus fructosyltransferase[c]	23
Cynara scolymus sucrose-dependent fructosyltransferase[d]	21
C. scolymus fructan-dependent fructosyltransferase[e]	22
Bacillus subtilis levansucrase[f]	16
Streptococcus mutans fructosyltransferase[g]	18

[a] EMBL accession no. L06844 (2).
[b] EMBL accession no. K03294 (43).
[c] EMBL accession no. AJ000493 (31).
[d] EMBL accession no. Y09662 (15).
[e] EMBL accession no. AJ000481 (16).
[f] EMBL accession no. X02730 (40).
[g] EMBL accession no. M18954 (37).

and *E. coli* (Fig. 3D). The complete absence of polymerizing activity in *E. coli* strains transformed with the empty vector pBluescript SK II is demonstrated in Fig. 3E. The two small peaks preceding the sucrose peak indicate contamination of the sucrose solution with traces of glucose and fructose. The absence of sucrolytic activity was also demonstrated for YSH 2.64-1A (data not shown). Neither in the transgenic *E. coli* nor in yeast expressing the *SFT* gene could the production of a high-molecular-mass fructan be observed. In contrast, incubation of intact conidia with 20% sucrose according to the method described by Harada et al. (14) yielded an inulin of a molecular mass of more than 20×10^6, as judged from HPSEC (Fig. 4). Fig. 4 demonstrates the molecular mass distribution of the high-molecular-weight inulin that was obtained from a 3-day incubation of conidia with a 20% sucrose solution. The high-molecular-weight inulin detected by HPSEC showed a relatively broad size distribution, which resulted in a polydispersity index for the polymer of 1.7.

To prove that transformation with the *SFT* gene led to formation of a complete transcript, we performed Northern blot analysis of RNA isolated from different hosts. In transgenic potato plants (see below) and in an *E. coli* strain transformed with the *SFT* gene as a fusion to the *lacZ* gene, a transcript of the correct size was formed (Fig. 5, lanes 2 and 5, respectively). Because of the low expression level in yeast, we could not detect the *SFT* transcript in this system (data not shown). The plant as well as the *E. coli* transcript were slightly larger than the fungal mRNA due to fusion of a transcriptional terminator derived from the octopine synthase gene of *Agrobacterium tumefaciens* in the plant transformation construct and the 5′ sequence of the *lacZ* gene in the bacterial vector. In *E. coli* a second, even larger, transcript could be detected, indicating that transcriptional termination at the end of the fungal cDNA was not complete.

Expression in potato plants. To investigate in vivo synthesis of fructan, potato plants were transformed with the construct p35-as1 carrying the fructosyltransferase of *A. sydowi* under the

control of the constitutive cauliflower mosaic virus 35S promoter. Leaf tissue of 50 transgenic plants kept in tissue culture was analyzed for fructan content. Of six positive transgenic lines, five individual plants per line were transferred to the greenhouse for further analysis. All fructan-producing plants showed strong phenotypic alterations like growth retardation, wrinkled leaves, and leaf necrosis. Most plants did not produce tubers and died within 4 to 8 weeks in the greenhouse. The phenotype of plants of line 35-as1 no. 7 was less strong, and these plants were used for further analysis. A Northern blot of RNA isolated from leaf tissue revealed a low abundance of a
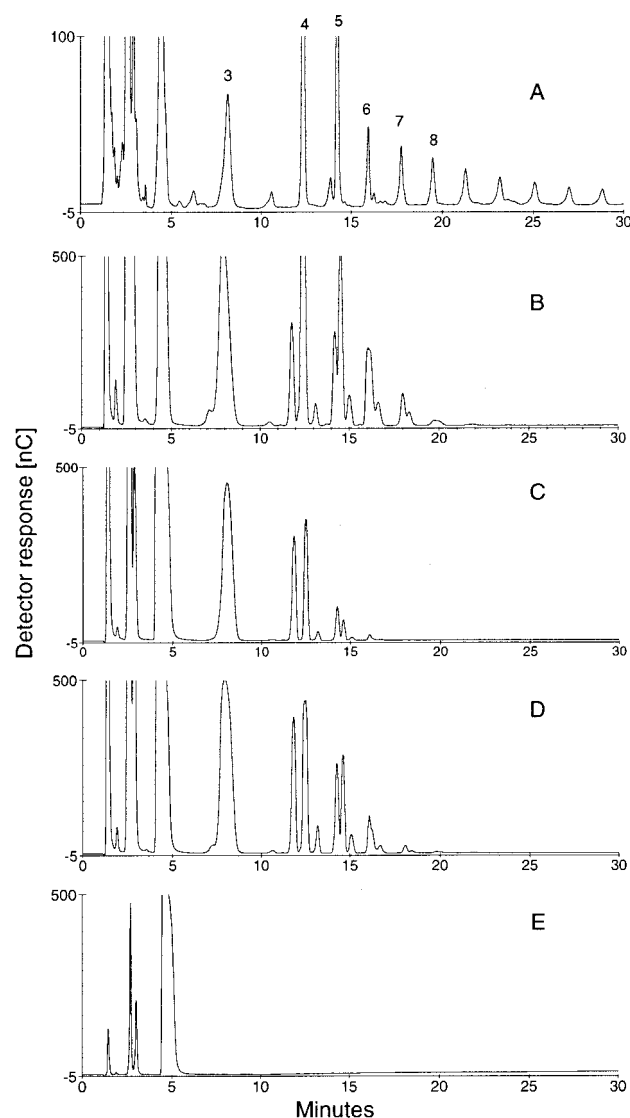

FIG. 3. HPAEC chromatogram of oligosaccharides produced from sucrose by *A. sydowi* fructosyltransferase. (A) Fructooligosaccharide standard of the inulin type (β2→1 linkages) purified from artichoke root. Numbers labeling the peaks represent the number of hexose units. (B) Protein extract of fungal conidia incubated with sucrose. (C) Protein extract of transgenic yeast expressing the fungal fructosyltransferase incubated with sucrose. (D) Protein extracts of transgenic *E. coli* expressing the fungal fructosyltransferase incubated with sucrose. (E) Protein extract of *E. coli* transformed with an empty vector incubated with sucrose.
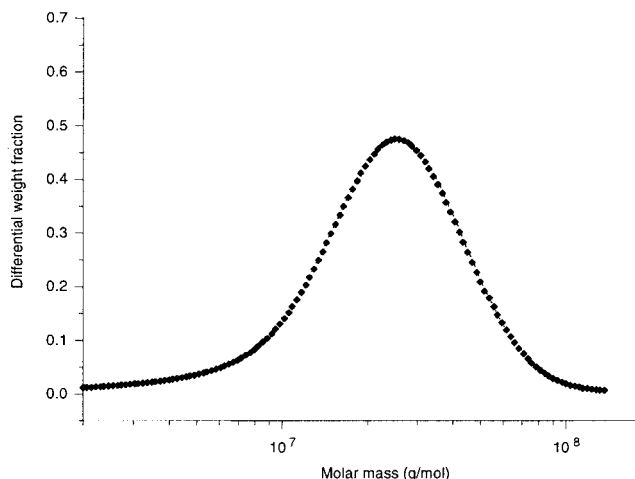
FIG. 4. HPSEC chromatogram of high-molecular-weight inulin isolated from an incubation of intact conidia of *A. sydowi* with a 20% sucrose solution. The graph shows the molecular mass distribution between 3×10^6 and 2×10^8 g/mol.

transcript of the expected size (Fig. 5, lane 2). HPAEC analysis of the soluble sugar extracts from leaves of plant 35-as1 no. 7 revealed production of polysaccharides of varying DP (Fig. 6B). To ensure that the polysaccharides detected in the column eluate were indeed fructans, the extracts were incubated with amyloglucosidase, which degrades $\alpha(1{\rightarrow}4)$ and $\alpha(1{\rightarrow}6)$ glucans, and with inulinase that degrades inulin-type fructans five times more efficiently than levans. The digests were analyzed by HPAEC and compared to the crude extracts of soluble
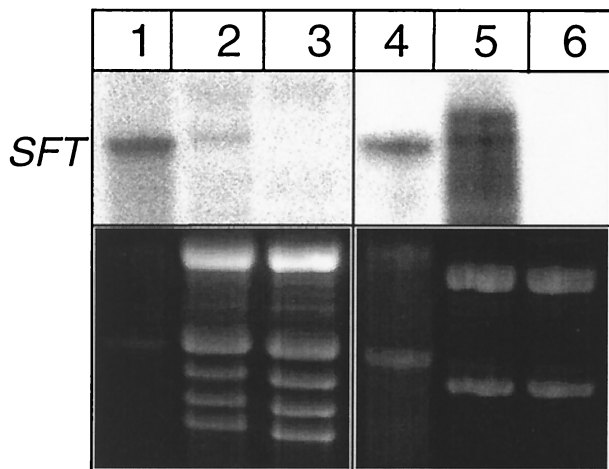

FIG. 5. Northern blot analysis of *SFT* expression in heterologous systems. Lane 1, 5 μg of RNA prepared from a 3-day culture of *A. sydowi* with visible formation of conidia; lane 2, 25 μg of RNA isolated from transgenic potato plant 35-as1 No 7; lane 3, 25 μg of RNA from untransformed potato plant cv. Désirée; Lane 4, 10 μg of RNA prepared from a 3-day culture of *A. sydowi* with visible formation of conidia; lane 5, 10 μg of RNA of *E. coli* expressing the *SFT* gene as a fusion to the *lacZ* gene; lane 6, 10 μg of RNA transformed with an empty vector. *SFT*, hybridization with the entire as1 insert. Lanes 1 to 3 were exposed to a phosphorimaging screen for 4.5 h and lanes 4 to 5 were exposed for 1 h. The lower panel shows the RNA gel stained with ethidium bromide.
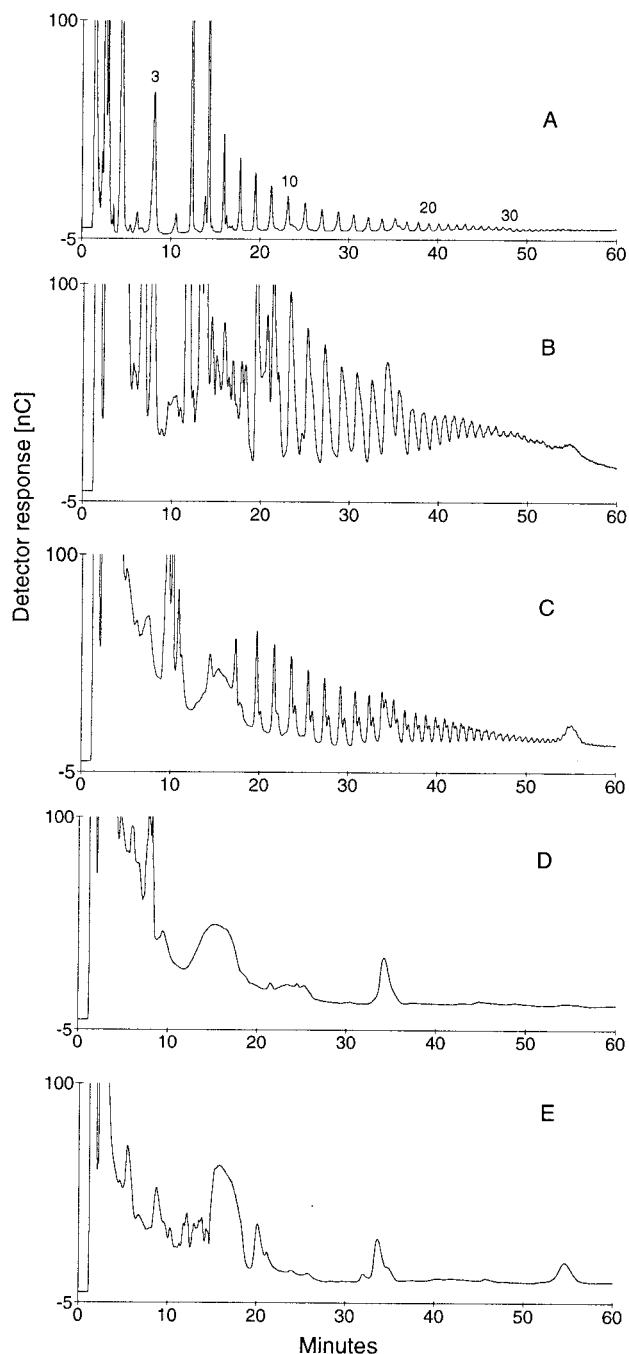

FIG. 6. HPAEC chromatogram of soluble sugar extracts. (A) Inulin standards isolated from artichoke roots. The peak labels indicate the DP of the polymer. (B) Extracts from leaves of transgenic potato plant 35-as1 No 7 expressing the *A. sydowi* fructosyltransferase. (C) Extract as in panel B treated with amyloglucosidase. (D) Extract from untransformed ancestral potato line treated as in panel C. (E) Extract as in panel B digested with inulinase.

sugars. Incubation with amyloglucosidase removed glucans and resulted in a more perspicuous chromatogram, allowing identification of fructans of a maximal DP of 39 (Fig. 6C). No fructan species could be detected in extracts from the untransformed ancestral line treated in the same way (Fig. 6D). Com-
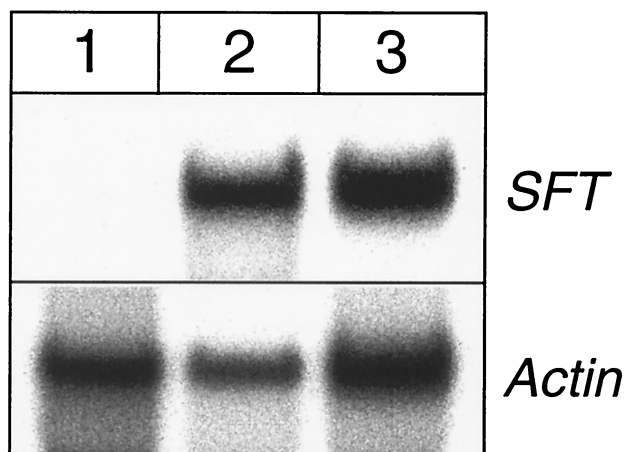
FIG. 7. Northern blot analysis of *SFT* expression in *A. sydowi*. RNA (25 µg) prepared from mycelium before visible formation of conidia (lane 1), 3-day culture (lane 2), and 5-day culture (lane 3) with visible formation of conidia. *SFT*, hybridization with the entire as1 insert. *Actin*, control hybridization with *Aspergillus nidulans* actin gene.

parison of the chromatogram of 35-as1 no. 7 with the inulin standards isolated from artichoke roots (Fig. 6A) identified the peaks as inulin-type fructans. This was further circumstantiated by a digestion of the preparation with inulinase, which completely degraded the polysaccharides of higher DP (Fig. 6E).

Pattern of *SFT* expression in *A. sydowi*. To investigate whether the cloned *SFT* gene corresponds to the fructosyltransferase activity described by Muramatsu and Nakakuki (27) for mycelia of *A. sydowi*, we studied the expression pattern of the *SFT* gene. Figure 6 shows a Northern blot analysis with RNA prepared from cultures before and after the formation of conidia. To obtain RNA from mycelium free of conidia, the fungus was grown for 2 days in liquid culture with constant shaking. No *SFT* expression was detected under this condition (Fig. 7, lane 1). Conidial RNA was prepared from cultures grown on filter paper that was placed on solidified medium. Formation of conidia was visible after 3 days at 25°C. RNA was prepared at day 3 and day 5. For both preparations (Fig. 7, lanes 2 and 3, respectively), a strong signal for *SFT* was obtained, indicating that *SFT* expression was restricted to conidia. This coincided with a lack of detectable sucrolytic activity in protein preparations of mycelium prepared as described above.

DISCUSSION

We have cloned the gene encoding a sucrolytic activity of *A. sydowi* IAM 2544. The gene encodes a sucrose-dependent fructosyltransferase, as demonstrated by heterologous expression in different systems. We therefore called the gene *SFT*. The coding region of *SFT* shows 64% identity to the invertase of *A. niger* and does not contain the conserved boxes of the "fructofuranosidase family" (13) except one. Together with *A. niger* invertase, the fructosyltransferase comprises a separate class of β-fructofuranosidase genes, which is clearly distinct from the genes identified in another *Aspergillus* species (31). The synthesis of fructooligosaccharides of a DP not higher than 10 by

A. niger invertase at high sucrose concentrations has been known for a long time (19), and this enzyme is used in industrial processes to produce fructans for human consumption (18). The fact that the highly homologous enzyme from *A. sydowi* is capable of synthesizing longer-chain fructans indicates that a clear discrimination of invertases and fructosyltransferases might not be possible.

Production of fructan by *A. sydowi* was first reported in 1920 by Kopeloff et al. (cited in reference 22). After initial classification as a levan, it could be shown that this fructan is of the inulin type (22), and recently a degree of branching of about 6% (48) could be demonstrated. Differing results have been obtained regarding the size of the fructan. Kawai et al. (22) obtained two types of products when they incubated conidia with sucrose, one comprising fructooligosaccharides of up to DP 5 and the other being a high-molecular-weight inulin. Harada et al. (14) describe the production of inulin of a molecular mass of over 10 million by conidia of *A. sydowi* IAM 2544. In contrast, working with the same strain Muramatsu and coworkers found only oligosaccharide production (26, 27). The experimental difference between the reports is twofold. First, for the production of high-molecular-weight inulin, conidia of the fungus were incubated with sucrose, whereas oligosaccharides were synthesized using mycelium or mixtures of both. Second, for polymer production, the conidia were left intact; i.e., polymer was obtained by in vivo fructan synthesis, whereas oligomers were produced in vitro with lyophilized tissue or purified protein.

When we incubated protein extracts of *A. sydowi* conidia with sucrose, we observed production of fructooligosaccharides, as reported for mycelial protein by Muramatsu and Nakakuki (27). The same was obtained for protein extracts of transgenic *E. coli* or yeast expressing the fungal fructosyltransferase. A slightly lower DP of oligosaccharides synthesized with yeast extracts most probably reflects the relatively low level of expression of the transgene under the control of the alcohol dehydrogenase promoter and, as a consequence, the low level of fructosyltransferase activity. In contrast, in vivo fructan synthesis in transgenic plants yielded a higher DP of inulin molecules of about 40 hexose units. Although this is clearly different from the results of the in vitro experiments, it is also not comparable to in vivo inulin synthesis with fungal conidia, which yields a product of a molecular weight of more than 2×10^7 as measured by HPSEC. A possible explanation is that an additional factor is present in the conidia and necessary for high-molecular-weight inulin formation. This factor might have been lost or inactivated during protein extraction and was not delivered to the heterologous hosts by single gene transfer. Alternatively, correct compartmentation of the fructosyltransferase might be essential for polymer production. The subcellular localization of the protein is controversial, as intracellular (27) as well as extracellular (22) localization has been reported. The possibility that the oligomer-producing and the polymer-synthesizing enzyme are different proteins located in the mycelium or the conidia seems unlikely since we observed oligomer as well as polymer production using the same conidial fructosyltransferase under different experimental conditions. By Northern blot analysis we demonstrated that the *SFT* gene is not expressed in mycelia and is induced as soon as the formation of conidia is visible. We therefore conclude that

the *SFT* gene product is different from the activity described by Muramatsu and Nakakuki (27), despite the fact that it catalyzed fructooligosaccharide production in vitro. While we could not detect a sucrolytic activity in our mycelium preparations, we cannot rule out the existence of two different enzymes in conidia, one being a soluble intracellular and the other a membrane-associated form. The latter would have been lost during extract preparation. The band with sucrolytic activity that was observed in zymograms would then correspond to the intracellular enzyme. Nevertheless, the gene encoding this enzyme mediated the production of inulin molecules of DP 40 in transgenic plants. It is therefore clearly distinct from invertases that produce short-chain fructans at high sucrose concentrations.

To our knowledge, functional expression of a eucaryotic fructosyltransferase in bacteria has so far not been demonstrated. Fungal as well as plant genes have successfully been expressed in yeast systems (20, 31), and several plant fructosyltransferases have been expressed in transformed plants or protoplasts (7, 16, 38, 39, 47). Because plant fructosyltransferases are vacuolar enzymes (9), it can be assumed that posttranslational modification is necessary for enzymatic function and thus prevents functional expression in bacterial systems. The fructosyltransferase activity of the *A. sydowi* enzyme expressed in *E. coli* demonstrates that at least the oligomer synthesizing activity is not dependent on posttranslational modification of the enzyme. We found no evidence that polymer production depends on a posttranslational modification such as prenylation that would anchor the protein in the membrane, since we could not detect a membrane-associated fructosyltransferase activity in transgenic yeast cells.

The strong phenotype of transgenic plants expressing the fructosyltransferase of *A. sydowi* resembles that of plants expressing bacterial levansucrases (3, 33). Levansucrases show only low specificity for fructosyl-acceptor molecules, allowing them to transfer fructose units to various hydroxyl-containing compounds (4). This is a possible reason for the tissue damage in transgenic plants, because levansucrase activity could interfere with protein glycosylation or other cellular processes. For the fructosyltransferase of *A. sydowi*, Muramatsu and Nakakuki showed a comparably low specificity, with fructosyl residues being transferred to sugars such as xylose, arabinose, and galactose (27).

From the results presented in this work, we conclude that fructooligosaccharides up to DP 10, which are used for human consumption, can efficiently be produced with bacterial cultures expressing the *A. sydowi* fructosyltransferase or with protein purified from these cultures. The production of high-molecular-weight inulin for industrial purposes, however, will require further research.

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