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Identification and characterization of starch and inulin modifying network of *Aspergillus niger* by functional genomics

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Chapter 6

Molecular and biochemical characterization of a novel intracellular invertase from *Aspergillus niger* with transfructosylating activity

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Abstract

A novel sub-family of putative intracellular invertase enzymes (glycoside hydrolase family 32) has previously been identified in fungal genomes. Here we report phylogenetic, molecular and biochemical characteristics of SucB, one of two novel intracellular invertases identified in *Aspergillus niger*. The *sucB* gene was expressed in *Escherichia coli* and an invertase negative strain of *Saccharomyces cerevisiae*. Enzyme purified from *E. coli* lysate displayed a molecular weight of 75 kDa, judging from SDS-PAGE analysis. Its optimum pH and temperature for sucrose hydrolysis were determined to be 5.0 and 37-40°C, respectively. In addition to sucrose, the enzyme hydrolyzed 1-kestose, nystose and raffinose, but not inulin and levan. SucB produced 1-kestose and nystose from sucrose and 1-kestose, respectively. With nystose as substrate, products up to a degree of polymerization (DP) of 4 were observed. SucB displayed typical Michaelis-Menten kinetics with substrate inhibition on sucrose (apparent K_m , K_i , and V_{max} of 2.0 (± 0.2) mM, 268.1 (± 18.1) mM and 6.6 (± 0.2) $\mu\text{mol min}^{-1} \text{mg}^{-1}$ of protein (total activity) respectively). At sucrose concentrations up to 400 mM, FTF activity contributed approximately 20-30% to total activity. At higher sucrose concentrations, FTF activity increased up to 50% of total activity. Disruption of *sucB* in *A. niger* resulted in earlier onset of sporulation on solid media containing various carbon sources, whereas no alteration of growth in liquid culture media was observed. SucB thus does not play an essential role in inulin or sucrose catabolism in *A. niger*, but may be needed for intracellular conversion of sucrose to fructose, glucose, and small oligosaccharides.

Introduction

Fructans and fructooligosaccharides (FOS) consist of a chain of fructose molecules linked to a terminal glucose residue. These fructose monomers are linked by either β -2,1- (inulin) or β -2,6- (levan) glycosidic bonds. Inulin and levan have several favorable properties which make them commercially interesting for applications in pharmaceutical- and food industries (Vijn & Smeekens, 1999). In the human digestive track, FOS are almost exclusively fermented by bifidobacteria and lactobacilli, which have beneficial health effects (May *et al.*, 1994; Tannock, 1997; Sghir *et al.*, 1998). Commercially, FOS are produced by the enzymatic hydrolysis of inulin isolated from plants, primarily chicory and Jerusalem artichoke (Vijn & Smeekens, 1999). Alternatively, sucrose can be converted into FOS, using a range of different transfructosylating enzymes, originating from plants, bacteria and fungi (Vijn & Smeekens, 1999). FTF and hydrolytic enzymes belong to glycoside hydrolase families (GH) 32 and 68 (Coutinho & Henrissat, 1999) constituting enzyme clan GH-J, based on shared conserved domains (Nagem *et al.*, 2004). These enzymes have been reported to be present in a variety of plants, bacteria and fungi (Vijn & Smeekens, 1999). FOS synthesis has been reported for the commercially important fungus *Aspergillus niger*, reflecting a side reaction of an invertase (EC. 3.2.1.26; (Somieri *et al.*, 1997; this chapter)) or as the result of the activity of a specific fructosyltransferase (EC. 2.4.1.9; (L'Hocine *et al.*, 2000)). Nguyen

et al. (1999) reported the presence of an intracellular invertase in *A. niger* IMI303386, grown on sucrose or inulin as sole carbon source. The purified enzyme produced free glucose and fructose from sucrose hydrolysis, as well as 1-kestose and nystose from sucrose FTF. However, the gene encoding this enzyme has not been identified and characterized. Yanai *et al.* (2001) reported characteristics of an extracellular β -fructofuranosidase from *A. niger* 20611. This enzyme displayed increased FTF activity compared to other known *Aspergillus* invertases. The strain was, however, later reclassified as *Aspergillus japonicus* ATCC 20611. The true identity, diversity, and characteristics of invertases and FTF enzymes present in *A. niger* thus remained to be determined.

Recently, the complete genome sequence of *A. niger* has become available (Pel *et al.*, 2007) and was analyzed for putative sucrose and fructan-modifying enzymes (Yuan *et al.*, 2006). In addition to *sucA*, encoding the previously characterized extracellular invertase (Boddy *et al.*, 1993), two novel putative invertase genes were identified (*sucB* and *sucC*) (Fig. 1). The *sucB* (but not the *sucC*) gene was (constitutively) expressed at a low level on starch and xylose, and up-regulated in the presence of sucrose and inulin (Yuan *et al.*, 2006). Orthologues of these genes have also been identified in other fungal genomes (see below). We have cloned and heterologously expressed the *A. niger sucB* gene, allowing a biochemical characterization of the purified enzyme. An *A. niger sucB* gene disruption strain was constructed to determine whether this novel intracellular invertase enzyme plays a significant role in growth on sucrose and inulin. This paper reports on the phylogenetic, molecular and biochemical characterization of SucB. The data show that in addition to invertase activity, SucB displays transfructosylating activity.

Materials and methods

Phylogenetic analysis

The complete amino acid sequence of SucB (DQ233219) (39) was blasted against the protein database at Swissprot (<http://www.ncbi.nlm.nih.gov/BLAST/>). Identified sequences containing family GH32 domains (<http://afmb.cnrs-mrs.fr/CAZY/>) were aligned with SucB (CLUSTALW interface in MEGA 3.1, <http://www.megasoftware.com>) followed by Bootstrap test of phylogeny (gap-opening, 10; extension penalties, 0.2; 1000 replicates). Sequence logos were created by SequenceLogo (<http://bio.cam.ac.uk/seqlogo/>).

Strains, plasmids, media and growth conditions

A. niger strains N402 (Bos *et al.*, 1988), NRRL3122 (Pel *et al.*, 2007) and AB4.1 (van Hartingsveldt *et al.*, 1987) and *Escherichia coli* strains XL1-Blue (Stratagene, La Jolla, Ca), TOP 10 and BL21 (DE3) STAR (Invitrogen, Carlsbad, Ca) were used in this study. The *A. niger* genome sequence derived from strain CBS513.88 (a natural derivative of strain NRRL3122). *A. niger* strains were grown in *Aspergillus* Minimal Medium (MM, (Bennet *et al.*, 1991)) or Complete medium (CM; MM supplemented with 0.5% (w/v) yeast extract and

0.1% (w/v) casamino acids). Conidiospores were obtained by harvesting spores from a CM plate containing 1% (w/v) glucose, after 4-6 days of growth at 30°C, using a 0.9% (w/v) NaCl solution. Transformation of *A. niger* AB4.1 was as described previously (Punt & van den Hondel, 1992). Cloning of *sucB* was performed using Gateway cloning technology (Invitrogen) and the integrity of constructs were verified by DNA sequencing (Baseclear, Leiden, the Netherlands). The Gateway expression vectors pDEST17 and pYES-DEST52 were used for expression in *E. coli* and the invertase negative strain of *S. cerevisiae* (BY4743Δ*suc2*).

S. cerevisiae strains were grown aerobically at 30°C in SC plus glucose media (1.7 g.l⁻¹ yeast nitrogen base, 5 g.l⁻¹ ammonium sulphate, 2.5 g.l⁻¹ sodium succinate, 5 g.l⁻¹ Casamino acids, 0.1 g.l⁻¹ tryptophan, 20 g.l⁻¹ glucose), followed by induction of expression in SC media plus 20 g.l⁻¹ galactose.

Cloning and purification of SucB

The coding sequence of *sucB* was amplified in a two step procedure. The first two exons were amplified using primers sets SucBGATEF, SucBDNAP1 and SucBDNAP2, SucBGATER, respectively, followed by joining of the two exons in a single PCR reaction together with outside primers SucBGATEF and SucBGATER (Table 1). Amplifications were performed in a GeneAmp PCR system 2700 thermal cycler (Applied Biosystems, Foster City, Ca) using the Expand High Fidelity PCR System (Roche Diagnostics Corporation, Indianapolis, IN.) under the following conditions: initial denaturation of 2 min at 94°C, 30 cycles of 15 s denaturation at 94°C, annealing at 55°C for 30 s and elongation at 72°C for 90 s, followed by a final elongation step of 7 min at 72°C. Gateway cloning of the fragments was performed according to the manufacturer's instructions (Invitrogen.) to create constructs pDEST17-*sucB* and pYES-DEST52-*sucB*, respectively.

Table 1. Primers used during this study

Name	Sequence
SucBGATEF	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATAATGGAGCGGCAAACTAGCCCTCAG
SucBGATER	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACTCGGCATCGACTTCTTCC
SucBcDNAP1	TGTTGGTGGATGGTGCCATGGAGGACTCTAT
SucBcDNAP2	CATGGCACCATCCACCAACACAACACAGCCCT
SucBP1f	TAGCGGGCCGCATCGCGACTGTCTCATACA
SucBP2r	CAGCGGCTTGGGTCTAGAATCATCCGAATATTCTCGATT
SucBP3f	ATCCTCTAGAGTCGACCTGCAGTCGATCGCGAGTGAGAT
SucBP4r	TGGAATTCTCATTTACGTCATCGTCGGCGA

Cultures of *E. coli* BL21 (DE3) STAR containing pDEST17-*sucB* were inoculated into fresh LB media containing 100 µg ml⁻¹ ampicillin. Soluble expression of SucB was achieved at 18°C (optical density at 600nm of approximately 0.4), followed by induction at 18°C for 6 h (1 mM isopropyl-β-D-thiogalactopyranoside [IPTG]). Cells were harvested by centrifugation (10 min, 4°C, 4000 x g) and cell pellets were resuspended in 5 ml 50 mM sodium phosphate buffer (pH 8) containing 250 mM NaCl, 10 mM imidazole and 5mM beta-mercaptoethanol. Following sonification (seven times for 15 sec at 8 µm with 30 s intervals), cell-free lysate was obtained by centrifugation (20 min at 4°C, 10,000 x g). SucB

was purified from the cell-free lysates using His-tag affinity chromatography according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, Mo.). Protein concentration, size, and purity were determined using the Bradford reagent (Bio-Rad, Hercules, Ca.), sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Biosafe Coomassie staining (Bio-Rad).

Cultures of *S. cerevisiae* BY4743 Δ suc2 cells containing the expression vector pYES-DEST52-sucB were used to inoculate 50 ml of fresh medium containing 2% galactose to induce protein expression. After 5 h of growth, cells were pelleted by centrifugation and washed with sterile demineralized water. Y-PER (Pierce Biotechnology, Rockford, IL) was used for cell lysis (according to the manufacturer's recommendations), followed by the preparation of cell-free lysate as described above.

Activity assays

Enzymatic activity was quantified spectrophotometrically by separate measurements of the released glucose and fructose from sucrose (D-glucose/D-fructose kit, Roche Diagnostics Corporation). The pH and temperature optima were determined by measuring initial rates in 50 mM phosphate-citrate buffer containing 100 mM sucrose using a pH range of 4.0 to 7.5 and a temperature range of 25 to 60°C. The effect of sucrose concentration on enzyme activity was determined using two independent experiments performed in triplicate by measuring initial rates over a substrate range of 12 sucrose concentrations (2.5 mM to 1 M) in 50 mM acetate buffer at 37°C, pH 5.0. Corrections for background glucose and fructose values at high substrate concentrations were made accordingly. Non-linear regression curve fitting was done using Sigma Plot (Systat Software, Richmond, Ca) applying the Michaelis-Menten formula for substrate inhibition ($y = V_{max} \cdot [S] / (K_m + [S] + ([S]^2 / K_i))$, where y equals the specific activity ($\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$).

Substrate specificity and product range

In order to identify substrate specificity of SucB, and the products made after incubation, 14 μg of purified SucB was incubated overnight in 50 mM acetate buffer (pH 5.0) at 37°C with a range of substrates. The effect of reducing agents dithiothreitol (DTT) and β -mercaptoethanol (BME) on SucB activity was also tested by incubating the enzyme at optimal conditions overnight in the presence of these agents at concentrations up to 100 mM. Substrate conversion and product formation were analyzed by thin layer- (TLC) (aluminum sheets silica gel 60 F 254, Merck and Co., Whitehouse station, NJ.) and high performance anion exchange chromatography (HPAEC) Dionex Corporation, Sunnyvale, Ca.). Sample separation on TLC was performed using a mixture of butanol, ethanol and water (3.8:3.8:2.4 [vol/vol/vol]) or ethyl acetate, 2-propanol and water (6:3:1 [vol/vol/vol]). Subsequently, plates were dried and sprayed with developer solution [95% methanol, 5% sulfuric acid and 3 g l⁻¹ 2-(1-naphthylamino) ethylamine dihydrochloride]. Product formation by SucB was confirmed by HPAEC as described previously (Ozimek *et al.*, 2006).

Construction of the *sucB::pyrG* gene deletion strain

A *sucB* deletion cassette was constructed by PCR amplification of 1.0 kb of 5' and 3' DNA flanking regions of the *sucB* gene using primers SucBP1-SucBP4 (Table 1). Both fragments were cloned into pBlue-ScriptII (Stratagene) using appropriate restriction enzymes (Table 1). The *A. oryzae pyrG* gene from pAO4-13 (de Ruiter-Jacobs *et al.*, 1989) was isolated as a 2.7 kb *Xba*I fragment and cloned between the 5' and 3' *sucB* flanking regions to give p Δ *sucB*.

Prior to transformation into AB4.1, p Δ *sucB* was linearized with *Eco*RI. Uridine prototrophic transformants were purified and screened for *sucB* deletion by Southern blot analysis (Sambrook *et al.*, 1989). Genomic DNA was isolated and digested with *Xho*I and the 3' region flanking the *sucB* gene was used as a probe. As predicted, a 2.2-kb hybridizing DNA fragment was observed in the wild type strain, whereas a 4.0-kb DNA fragment was detected in *sucB* deletion strains (data not shown). Several *sucB* deletions strains were independently obtained, and strain NC1.1 (Δ *sucB*) was used throughout this study.

Microtiter plate growth assay

Growth of *A. niger* strains N402 and NC1.1 was determined using a HTS7000 BioAssay Reader (Perkin Elmer Life and Analytical Sciences, Inc., Wellesley, MA.). Spores (1×10^4) were inoculated in each well of a 96-wells microtiter plate (Nalge Nunc International, Rochester, NY.) and incubated at 32°C for 56 h. Each well contained 200 μ l of MM with 1% (wt/vol) of one of the various carbon sources, supplemented with 0.1% (wt/vol) casamino acids to stimulate spore germination. Six replicates of each condition were made. Growth was monitored by measuring the optical density at 595 nm (OD₅₉₅) every 2 h.

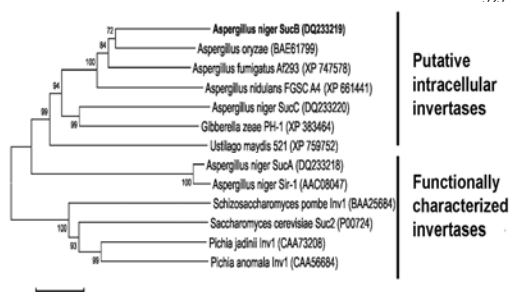


Fig. 1. Neighbor-joining tree of functionally characterized and putative fungal invertases. Bootstrap values are indicated on the node of each branch. The tree was created with MEGA 3.1 using default settings for gap and extension penalties. The bar indicates 10% amino acid sequence difference.

Results

Sequence analysis

Using the predicted amino acid sequence of SucB in phylogenetic analysis, we have identified SucB orthologues in various other fungal species. *In silico* analysis indicated that

these putative invertases also lack any recognizable signal peptide sequences, as has been reported for SucB (Yuan *et al.*, 2006). Multiple sequence alignment between these new group of putative intracellular invertases and known fungal invertases indicate that they cluster together, in a separate subfamily, clearly distinct from known extracellular fungal and yeast invertase proteins (Fig. 1). Table 2 depicts sequence logos (Crooks *et al.*, 2004) constructed from alignments of the SucB subfamily members, revealing the presence of all eight conserved domains characteristic for family GH32.

Table 2. Sequence logo depiction of conserved motifs identified in invertases with known function and SucB orthologues *

Domain	Functionally characterized invertases	Putative intracellular invertases
A		
B		
B1		
C		
D		
E		
F		
G		

* See Yuan *et al.*, 2006. Conserved motifs of glycoside hydrolase family 32 are indicated on the left. Sequences used to construct the logos are the same as depicted in Fig. 1.

Cloning and purification of SucB

Initial attempts to clone the *sucB* from a cDNA library constructed from an inulin growing *A. niger* strain N402 were unsuccessful, probably because of the relatively low level of expression of the *sucB* gene (Yuan *et al.*, 2006). Following amplification, the full coding region of *sucB* (1,854 bp) was obtained. The same procedure was followed to clone the

second putative intracellular invertase identified in *A. niger* (*sucC*); however, we failed to obtain functional expression in either *E. coli* or *S. cerevisiae*.

Purification of SucB from *E. coli* was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, where the protein size was estimated at 75 kDa (70 kDa, calculated). C-terminally His-tagged SucB expressed in *S. cerevisiae* strain BY4743 Δ *suc2* could not be sufficiently purified using Ni-nitrilotriacetic acid affinity chromatography. Therefore, the cell-free lysate was used for comparative studies. SucB expressed in both *E. coli* and *S. cerevisiae* displayed similar characteristics, whereas no activity could be detected in *S. cerevisiae* BY4743 Δ *suc2* containing the empty expression vector. SucB in the cell-free extract of *E. coli* or *S. cerevisiae*, as well as the affinity purified SucB from *E. coli*, only displayed activity for a maximum storage time of three days (4°C or -20°C in 20% glycerol). Thus, for all subsequent analysis, the enzyme was used directly after purification.

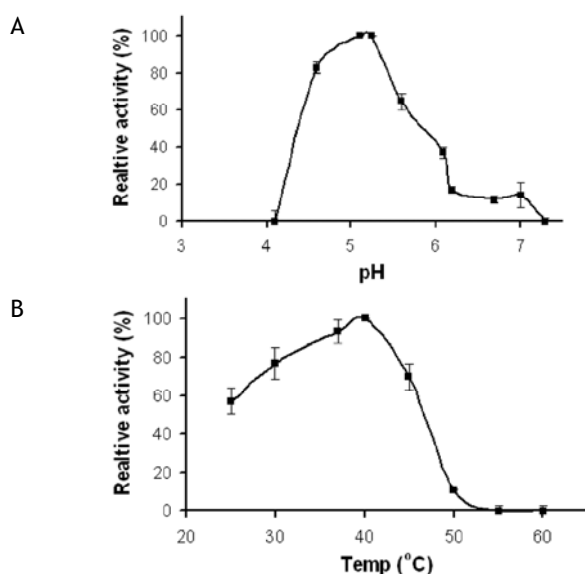


Fig. 2. Effect of pH (A) and temperature (B) on SucB activity. The enzymatic activity was determined by measuring the amount of glucose released from the initial reaction of SucB (14 μ g) incubated with 200 μ l of 100 mM sucrose in citrate-acetate buffer at 37°C. Values depicted are the mean of duplicates (\pm SEM), based on at least two independent experiments.

Influence of pH and temperature on SucB enzyme activity

The optimal pH and temperature conditions for SucB activity with sucrose as substrate were determined by measuring the amount of released glucose enzymatically (total activity). SucB activity could be detected from pH 4.5 to 7, albeit at very low activity levels at pH values above 6.3. SucB displayed maximal activity at pH 5.0 (Fig. 2A). The optimal temperature for SucB total activity is in the range 37 to 40°C (Fig. 2B). At temperatures of

50°C or higher, no activity could be detected, whereas at lower temperatures, the SucB specific activity remained relatively high, with 50% activity remaining at 25°C.

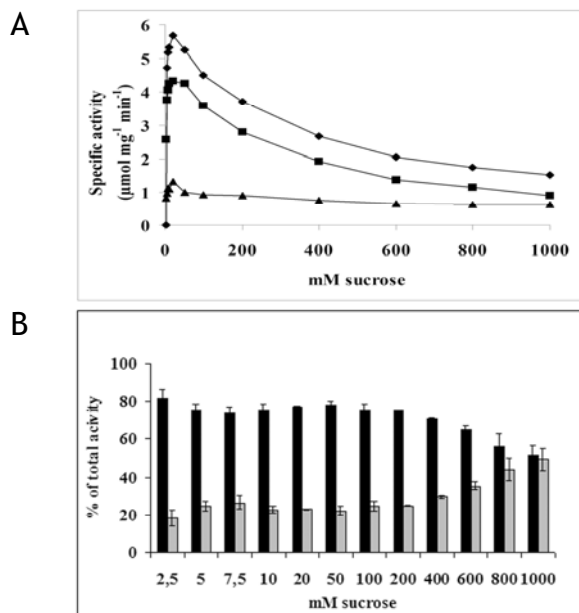


Fig. 3. Effect of sucrose concentration on SucB activity. (A) Total activity was determined by measuring the amount of glucose released from the initial reaction of SucB incubated with 12 sucrose concentrations ranging from 2.5 mM to 1 M in 50 mM acetate buffer pH 5.0 at 37°C. Values depicted were calculated from triplicate measurements. (♦) Total activity; (■) Invertase activity; (▲) FTF activity. (B) Percentage of either hydrolytic (black bars) or FTF (grey bars) activity compared to the total activity of SucB, displayed for a range of sucrose concentrations. Measurements are the mean of duplicates (\pm SEM), based on two independent initial measurements. Approximately 14 μ g of purified protein was used in each case.

Kinetic analysis of SucB activity

Incubation of SucB with increasing sucrose concentrations lead to a typical Michaelis Menten-type kinetics with substrate inhibition (apparent K_m , K_i and V_{max} of 2.0 ± 0.2 mM, 268.1 ± 18.1 mM and 6.6 ± 0.2 μ mol $\text{mg}^{-1} \text{min}^{-1}$ respectively). Hydrolysis and FTF reactions displayed similar patterns, with apparent K_m values of 2.5 ± 0.2 and 0.9 ± 0.5 mM, apparent V_{max} values of $5.5 (\pm 0.1)$ and $1.2 (\pm 0.1)$ μ mol $\text{mg}^{-1} \text{min}^{-1}$, and apparent K_i values of $206.3 (\pm 12.8)$ and $797.6 (\pm 196.5)$ mM, respectively. Increasing the sucrose concentration from 2.5 mM to 1 M resulted in a decreased hydrolysis activity and in an increased (20% to 50%) FTF activity in relation to total SucB activity (Fig. 3). These observations were confirmed by the TLC product analysis, showing increased 1-kestose and decreased free fructose synthesis at higher sucrose concentrations (Fig. 4). HPAEC analysis showed that apart from the formation of 1-kestose as the major FTF product, minor amounts of nystose were also produced (result not shown).

SucB substrate specificity and product formation

Substrate specificity analysis showed that SucB is able to hydrolyze sucrose, raffinose and the inulin-type oligosaccharides 1-kestose and nystose (releasing fructose in each case), as well as to perform oligomerization reactions (Fig. 5 A). Incubation of SucB with 100 mM of 1-kestose in the presence or absence of 100 mM of sucrose produced free fructose, sucrose and nystose, indicating that 1-kestose could be used as both donor and acceptor substrates.

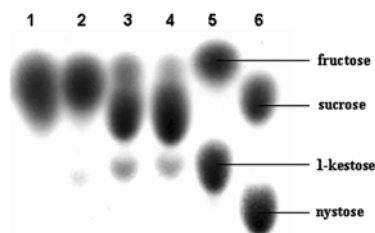


Fig. 4. TLC analysis of the reaction products after overnight incubation of SucB at 37°C with 50 mM (lane 1), 100 mM (lane 2), 500 mM (lane 3) and 1 M sucrose (lane 4). Lane 5 shows the standards fructose and kestose and lane 6 sucrose and nystose. Approximately 14 µg of purified protein was used in all incubations. All samples were equilibrated to the same concentration before spotting on the plate.

Alternatively, the sucrose formed subsequently was used as donor substrate for transfer of free fructose to 1-kestose. Incubations of SucB with nystose alone or nystose plus sucrose, yielded only free fructose, sucrose and 1-kestose (Fig. 5 A). HPAEC analysis confirmed that nystose was the largest product produced from incubation of SucB with kestose, and that a minor amount of pentakestose (degree of polymerization of 4) was produced after overnight incubation. SucB incubation in the presence of sucrose plus 1-kestose or nystose did not facilitate product diversification, but resulted in an increase in concentration of the observed products only. Furthermore, SucB incubation with galactose and sucrose did not yield any other products than observed for sucrose alone (Fig. 5 A).

SucB hydrolysed the sucrose moieties of the sugars raffinose [α -D-galactose-(1,6)- α -D-glucose-(1,2)- β -D-fructose] (Fig. 5 A) and stachyose [α -D-galactose-(1,6)- α -D-galactose-(1,6)- α -D-glucose-(1,2)- β -D-fructose] (Fig 5 B). No hydrolysis of any of the α -glycosidic linkage sugars {trehalose [α -D-glucose-(1,1)- α -D-glucose], turanose [α -D-glucose-(1,3)- β -D-fructose], palatinose [α -D-glucose-(1,6)- β -D-fructose] or melizitose [α -D-glucose-(1,2)- β -D-fructose-(1,3)- α -D-glucose]} was observed. Using trehalose alone or in combination with sucrose (ratios 5:1 to 1:5) as substrate, only 1-kestose formation from sucrose could be observed as FTF product (results not shown). None of these α -glycosidic bond substrates were used as donor/acceptor substrates in FTF reactions with sucrose (ratios 5:1 to 1:5). Hydrolysis of larger polysaccharides such as inulin or levan could not be detected, not even after overnight incubation (Fig. 5 A). Similar results were obtained when cell free extracts from the recombinant *S. cerevisiae* strain carrying the construct pYES-DEST52-*sucB* were used.

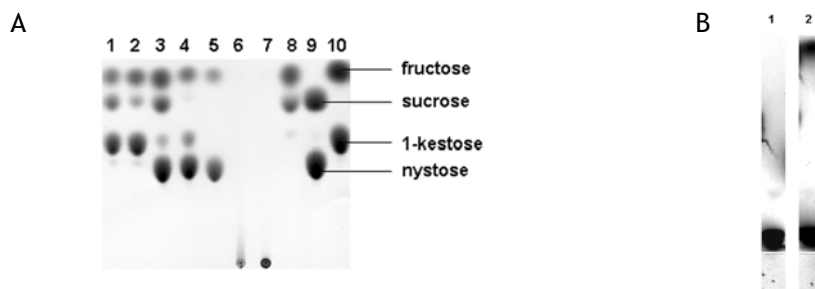


Fig. 5. TLC analysis of substrate specificity of SucB and reaction products after overnight incubation at pH 5.0 and 37°C. (A) 100 mM kestose with sucrose (lane 1), kestose (lane 2), nystose with sucrose (lane 3), nystose (lane 4), raffinose (lane 5), 1% inulin (chicory, see text, lane 6), levan (Bacillus subtilis produced, gift from Cosun Food Technology, the Netherlands, lane 7) and 100 mM galactose with sucrose (lane 8). Lanes 9 and 10 contain the standards fructose, sucrose, 1-kestose and nystose. (B) 100 mM stachyose incubated without (lane 1) and with (lane 2) SucB. Released fructose is indicated. Approximately 14 µg of purified protein was used in each case.

In silico translation of the SucB reading frame revealed the presence of 13 cystein residues, which can potentially form disulfide bridges. Addition of up to 100 mM of the reducing agents DTT or BME did not influence the sucrose hydrolysis or FTF activities of SucB indicating the absence of any structurally important disulfide bridges.

Disruption of the *sucB* gene

Disruption of *sucB* did not result in a significant change in growth rates and yields of *A. niger* in liquid media containing sucrose, inulin, glucose, fructose, xylose, maltose or starch (data not shown). Interestingly, growth of the *sucB* deletion mutant strains (e.g. strain NC1.1) on solid media containing these substrates resulted in an earlier onset (approx. 1 day) of sporulation compared to that of the wild type *A. niger*. The inclusion of additional uridine in the culture media to exclude suboptimal complementation by PyrG did not rescue the NC1.1 strain from this sporulation effect. No difference in colony diameter was observed between the NC1.1 and the wild type strains on the various carbon sources, indicating that the growth of the Δ *sucB* strain was not affected.

Discussion

The recent availability of the complete genome sequence of *A. niger* (Pel *et al.*, 2007) enabled identification of two novel putative intracellular invertases (*sucB* and *sucC*) (Yuan *et al.*, 2006). Although these genes share conserved amino acid residues with other family GH32 members, phylogenetically they cluster together with other putative intracellular invertases from fungal origin in a new distinct group (Fig. 1). These new putative invertases also contain all the conserved catalytic residues, as depicted in the sequence logos (Table 2). The only exception is Sir-1, which is missing the catalytic aspartate in domain A. This invertase was isolated from *A. niger* strain IBT10sb and displayed increased FTF properties (Somari *et al.*, 1997).

We have previously shown that SucB expression was upregulated by sucrose and inulin, whereas the enzyme was constitutively expressed at a low level with all other substrates used (Yuan *et al.*, 2006). The *sucB* gene also appears to be under catabolite repression control, evident from expression profiling in a *creA* deletion strain (Yuan *et al.*, 2006). With regards to *sucC*, no expression could be detected in mycelial mRNA under the same conditions. Using chromosomal DNA to construct the predicted open reading frame of SucC also failed to produce functional protein in both *E. coli* and *S. cerevisiae*.

In silico analysis of the SucB sequence revealed the absence of any recognizable signal peptide sequences for protein secretion, indicating that it may play a role in *A. niger* intracellular metabolism. In view of the low levels of expression observed for SucB in *A. niger*, and to avoid simultaneous separation with other invertases/fructosyltransferases present in *A. niger* (L'Hocine *et al.*, 2000), we overproduced the SucB enzyme in *E. coli* as well as in an invertase-negative strain of *S. cerevisiae* and subsequently determined its biochemical properties.

SucB clearly acts as an invertase, able to hydrolyze the glucose-fructose glycosidic linkage in the smaller fructose-containing oligosaccharides sucrose, kestose and nystose. Oligosaccharides larger than nystose, including polymeric inulin and levan could not be hydrolyzed. The enzyme was unable to hydrolyze α -glycosidic bonds in substrates or to use these compounds as donor/acceptor substrates in FTF reactions. Weak hydrolysis of the sucrose moiety of stachyose, but not melizitose, indicates that the fructose of the sucrose moiety should thus be positioned terminally to enable correct orientation and binding in the active site. A similar observation was made for the β -fructosidase from *Thermatoga maritima*, where it was shown that hydrolysis occur in a typical exo-fashion (Liebl *et al.*, 1998). Taking into account the diversity of substrates hydrolyzed, one could assume that the -1 subsite in the active site cleft (for numbering see Davies *et al.*, 1997) can accommodate fructose (Alberto *et al.*, 2004; Nagem *et al.*, 2004; Alberto *et al.*, 2006), and that the enzyme most probably does not contain multiple binding sites for fructose (as in the case for the exo-inulinase of *A. awamori*) (Kulminskaya *et al.*, 2003). In the three-dimensional structure of the *Cichorium intybus* fructan 1-exohydrolase, Verhaest *et al.* (2005) observed the presence of multiple glycerol molecules bound in the cavity between the N- and C-terminal domains of the protein. This forms an open cleft which is emerging from the active site, and is believed to be responsible for the binding of inulin or higher molecular weight fructans. Obstruction of this cleft could possibly influence the binding of high molecular weight inulins, limiting the enzyme to hydrolysis of small oligosaccharides only (Verhaest *et al.*, 2005; Alberto *et al.*, 2006). In view of the low amino acid similarity between SucB and other characterized invertases and the absence of structural data, we can only speculate that the same obstructing feature is present in SucB. This feature could explain the inability of the enzyme to bind and hydrolyze larger oligo- and polymeric fructans.

Apart from the hydrolytic activity observed for SucB, the enzyme was also able to perform FTF reactions. This activity was already detected at sucrose concentrations as low as 2.5 mM, with 1-kestose as the major oligomerization product (20-30% of total activity). At 1M, SucB displayed approximately 50% FTF activity, largely due to a decrease of hydrolytic

activity. Nystose was also produced as a minor product in the FTF reaction when 1-kestose was used a substrate. Also, the presence of a minor amount of pentakestose was observed after overnight incubation with nystose. Larger SucB products have never been observed. The data thus indicate that SucB is responsible for the intracellular production of small inulin-type oligosaccharides. In 1995, Muramatsu and Nakakuki previously described the purification and characterization of an intracellular beta-fructofuranosidase from *Aspergillus sydowi* that could transfer fructose from sucrose to trehalose, thus creating novel oligofructosyl trehaloses. However, when SucB was incubated with trehalose, no novel oligosaccharides were observed.

The SucB characteristics differ from the previously published data on the extracellular *A. niger* invertase Suc1/SucA/INV enzyme (Boddy *et al.*, 1993; Wallis *et al.*, 1997; L'Hocine *et al.*, 2000), and other invertases, in a number of aspects. SucB displayed an apparent K_m of 2.0 ± 0.2 mM, for sucrose, which is substantially lower than reported previously (30 and 160 mM for Suc1 and 35.67 mM for INV), but comparable to that of the extracellular acid invertase of *Fusarium solani* (3.57 mM) (Bhatti *et al.*, 2006). Extracellular invertases from both fungal as well as bacterial origin generally display lower affinity for sucrose than was observed for SucB (Gascon *et al.*, 1968; Reddy & Maley, 1996; Wallis *et al.*, 1997; Liebl *et al.*, 1998). However, Rubio and Maldonado (1995) described the purification and characterization of an invertase from an *A. niger* strain isolated from lemons. The invertase was purified from the mycelial lysate, and displayed a substrate affinity of 0.0625 mM for sucrose and a temperature optimum of 60°C. These characteristics clearly deviate from what has been observed for SucB, indicating that this protein might either be another intracellular invertase, or an isoform of SucB produced intracellularly in *A. niger* and not during recombinant expression in *E. coli* or yeast.

Using sucrose as substrate, SucB displays an apparent V_{max} (total activity) of 6.6 ± 0.2 $\mu\text{mol mg}^{-1} \text{min}^{-1}$ (this study). This figure is more than a thousand fold lower than the V_{max} for the extracellular invertase in *A. niger* AS0023 (7,758.3 $\mu\text{mol mg}^{-1} \text{min}^{-1}$) (L'Hocine *et al.*, 2000). The purified extracellular *A. niger* invertase also did not display any detectable FTF activity, not even at a sucrose concentration as high as 2.2 M (L'Hocine *et al.*, 2000), suggesting that the FTF described previously in literature could have derived from contaminating fructosyltransferases (Hirayama *et al.*, 2006).

Compared to the extracellular invertase of *A. niger* (Suc1), SucB displayed a lower optimum temperature value (37 to 40°C versus 50°C, respectively), whereas a comparable pH optimum was determined (Boddy *et al.*, 1993; Wallis *et al.*, 1997). However, SucB was only active in a narrow pH range (above pH 4, below pH 7 (Fig. 2A) compared to that observed for Suc1 (above pH 3, below pH10) (Boddy *et al.*, 1993; Wallis *et al.*, 1997). In the extracellular environment, Suc1 should be able to function in fluctuating pH conditions, where the extracellular pH could vary between 1.5 and 7.0 (Hesse *et al.*, 2002). In the intracellular environment, however, the cytoplasmic- and vacuolar pHs of *A. niger* is kept constant at 7.6 and 6.2, respectively. This balance is maintained in order to control pH sensitive processes such as DNA transcription and protein synthesis (Hesse *et al.*, 2002). Taking these facts into consideration, and in the absence of any detectable sequence for

protein export, we conclude that SucB functions sub-optimally in the intracellular environment.

Although intracellular invertases have been identified in fungi before (Gascon *et al.*, 1968; Muramatsu & Nakakuki, 1995; Nguyen *et al.*, 1999), little is known about the role they play in the intracellular environment. To determine whether SucB plays a crucial role in the metabolism of *A. niger*, a SucB disruption mutant strain was constructed. No difference in growth rate, yield, and morphology was observed between the *sucB* disruptant and the wild type *A. niger* N402 using liquid minimal media with sucrose or inulin as carbon sources. When the NC1.1 *sucB* disruptant strain was grown on solid minimal media containing various substrates, an earlier onset of sporulation was observed compared to the wild type. However, as observed in liquid media, no difference in growth was observed since the colony diameter was equal to that of wild type *A. niger*. Supplementing the culture media with uridine to minimize suboptimal complementation by the inserted *pyrG* gene did not alleviate the observed effect, suggesting that SucB (in)directly plays a role in the sporulation of *A. niger*.

Intracellular proteins usually do not contain disulfide bridges, which play a crucial role in structure and function of extracellular proteins (Raina & Missiakas, 1997). These bridges could be disrupted by the addition of reducing agents, e.g. DTT and BME, which in turn may cause loss of activity, or decreased enzyme stability. The inability of high concentrations of either DTT or BME to disrupt SucB activity gives a further indication that no disulfide bridges crucial to activity or structural integrity are present. This further supports the view that SucB is functioning in the intracellular environment in *A. niger*.

Considering the high affinity for sucrose, the narrow functional pH range, and the absence of an export signal sequence and functionally important disulfide bridges, we speculate that SucB plays an intracellular role in salvaging low concentrations of sucrose, kestose or nystose into fructose and glucose as energy sources. SucB may also function in transfer of fructose units from sucrose to fructan or unknown acceptor molecules. These molecules may be responsible for energy storage, the regulation of osmolarity or play a role in the induction of other proteins involved in the modification of fructans.

Further analysis and complementation studies should be conducted to determine the effect of *sucB* gene disruption on the expression of other fructan modifying enzymes, and the possible role it could play in initiating the earlier onset of sporulation.

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