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Sorbitol dehydrogenase of *Aspergillus niger*, SdhA, is part of the oxido-reductive D-galactose pathway and essential for D-sorbitol catabolism

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ABSTRACT

In filamentous fungi D-galactose can be catabolised through the oxido-reductive and/or the Leloir pathway. In the oxido-reductive pathway D-galactose is converted to D-fructose in a series of steps where the last step is the oxidation of D-sorbitol by an NAD-dependent dehydrogenase. We identified a sorbitol dehydrogenase gene, *sdhA* (JGI53356), in *Aspergillus niger* encoding a medium chain dehydrogenase which is involved in D-galactose and D-sorbitol catabolism. The gene is upregulated in the presence of D-galactose, galactitol and D-sorbitol. An *sdhA* deletion strain showed reduced growth on galactitol and growth on D-sorbitol was completely abolished. The purified enzyme converted D-sorbitol to D-fructose with K_m of 50 ± 5 mM and v_{max} of 80 ± 10 U/mg.

1. Introduction

For microbial D-galactose catabolism several pathways have been described. The most studied pathway is the Leloir pathway that is present in prokaryotic and eukaryotic microbes [1]. Another microbial pathway that was only described in prokaryotic microbes is the De Ley-Doudoroff pathway. Here D-galactose is first oxidised, followed by a dehydration, a phosphorylation and an aldolase reaction resulting in pyruvate and D-glyceraldehyde-3-phosphate [2]. A non-phosphorylating variant of this pathway has been described in the eukaryotic microorganism *Aspergillus niger* [3]. Yet another eukaryotic pathway for D-galactose catabolism was suggested to be active in filamentous fungi. It is an oxido-reductive pathway with similarities to the fungal L-arabinose pathway [4]. In *Trichoderma reesei* a deletion of the galactokinase gene of the Leloir pathway was not sufficient to abolish D-galactose catabolism [5]. Since galactitol was accumulating in the galactokinase mutant, it was suggested that the first step in the oxido-reductive D-galactose pathway is a reduction and galactitol the first intermediate. This reaction turned out to be catalysed by the D-xylose reductase, Xyl1, which is a part of the pentose catabolic pathway [6]. Galactitol is then oxidised to L-xylo-3-hexulose by the L-arabitol dehydrogenase, Lad1 [7], which is also the main enzyme for L-arabitol oxidation [8]. A *lad1* deletion in combination with a galactokinase deletion resulted in no growth on D-galactose [5]. In *A. niger* the reduction of D-galactose is also catalysed by the D-xylose reductase, XyrA [9], but oxidation of galactitol is catalysed by a specific galactitol dehydrogenase, LadB, which is not involved in pentose metabolism [9]. The following steps in the pathway are still uncertain. It was suggested that in *Aspergillus nidulans* the pathway is via L-sorbose [10], but the enzymes synthesizing L-sorbose from L-xylo-3-hexulose are still elusive. Another hypothesis is that L-xylo-3-hexulose is reduced to D-sorbitol which is subsequently oxidised to D-fructose. This would make the pathway very similar to the fungal L-arabinose pathway in which L-arabinose goes through a similar sequence of two oxidations and two reductions to form D-xylulose [4]. Fig. 1 shows the suggested oxido-reductive D-galactose pathway.

For D-sorbitol catabolism at least three different pathways are described. In bacteria the most known pathway is that in *Escherichia coli* where D-sorbitol is phosphorylated to D-sorbitol-6-phosphate which is then oxidised by an NAD-dependent dehydrogenase to produce D-fructose-6-phosphate [11]. In *Gluconobacter oxydans* the pathway is different and D-sorbitol is converted to L-sorbose and the sorbitol dehydrogenase responsible for the reaction is cytochrome c-dependent [12]. In eukaryotic organisms an NAD-dependent sorbitol dehydrogenase oxidises D-sorbitol to D-fructose which is then phosphorylated to D-fructose-6-phosphate. In *A. niger* it has been demonstrated that sorbitol dehydrogenase and fructokinase activity are induced on D-sorbitol [13], however the corresponding genes have not been identified.

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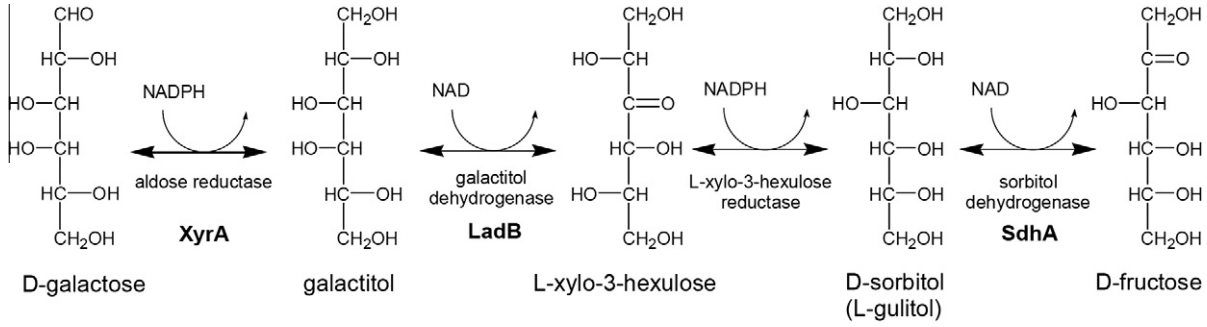

Fig. 1. Oxido-reductive D-galactose catabolic pathway of *A. niger*. The enzymes identified as part of the pathway have the abbreviation of their name shown. Conversion of L-xylo-3-hexulose to D-sorbitol is a hypothetic step, likely to be catalysed by an enzyme related to L-xylulose reductase.

In this communication we identified a sorbitol dehydrogenase that is responsible for the last step of the oxido-reductive pathway for D-galactose catabolism and an essential part of D-sorbitol catabolism.

2. Materials and methods

2.1. Strains and plasmids

A. niger, strain ATCC 1015 was obtained from CBS (CBS113.46). For constructing a deletion cassette for *A. niger* the pRSET-A plasmid (Invitrogen) was used. *Saccharomyces cerevisiae* CEN.PK2-1D transformed with the pYX212 vector (R&D Systems) expressing the *sdhA* gene was used for heterologous expression and purification of SdhA for enzymatic characterisation.

2.2. Media and cultivations

A. niger was grown overnight in YPG medium containing 10 g/l of yeast extract, 20 g/l of Bacto peptone, and 3% Difco™ gelatine (Becton, Dickinson and Company; Sparks; USA) to produce mycelia.

For the qPCR analyses, the overnight cultures were transferred to fresh YP medium containing 10 g/l of yeast extract, 20 g/l of Bacto peptone and 20 g/l of L-arabinose, D-galactose, galactitol, D-sorbitol or D-glucose. The mycelia were incubated in this medium for up to 8 h at 28 °C. Samples for RNA isolation and transcription analysis were taken at different time intervals.

For comparison of growth of *A. niger* strains on agar plates, the spores were inoculated on agar plates containing 6.7 g/l of yeast nitrogen base (YNB, Becton, Dickinson and Company), synthetic complete amino acid mixture without uracil, 20 g/l of agar, and 20 g/l of carbon source (for details see Section 3).

For the sorbitol dehydrogenase assays with purified enzyme, *S. cerevisiae* was grown overnight in 6.7 g/l of YNB (Becton, Dickinson and Company), synthetic complete medium without uracil [14] and 20 g/l of glucose.

2.3. Transcription analysis

To quantify the transcription of the selected genes in *A. niger* in different conditions, mycelia were grown as described above. Mycelia were removed by filtration at 0, 1, 2, 4, 6 and 8 h and washed with water. The total RNA was isolated with RNeasy®Plant Mini Kit (Qiagen) and subsequently used in the reverse transcription reaction (SuperScript; Invitrogen).

cDNA produced in the reverse transcription reaction was analysed by qPCR in a LightCycler 480 Instrument II (Roche, Switzerland) and the analysis was performed with the accompanying software (Advance Relative Quantification tool). The level of transcription of each gene in each time-point was normalised to the level of the actin (*actA*) transcription. The primers used for the analysis are listed in Table 1.

2.4. Deletion of the sdhA in A. niger ATCC 1015 ΔpyrG

ATCC 1015 *ΔpyrG* strain, which was generated as described previously [15], was transformed with the *sdhA* (JGI53356) deletion cassette. The deletion cassette contained 1460 bp upstream and 1525 bp downstream of the *sdhA* gene and a 1928 bp fragment containing the *pyrG* gene with its native promoter and terminator. These fragments were obtained by PCR of *A. niger* ATCC 1015 genomic DNA using primers sdhA-5-F, sdhA-5-R, sdhA-3-F, sdhA-3-R, pyrG-del-F_n, and pyrG-del-R_n (Table 1), and the proofreading DNA polymerase Phusion (Finnzymes). The downstream fragment (*sdhA*-3) digested with *Eco*RI (NEB) was inserted into the plasmid pRSET-A (Invitrogen), which was digested with *Eco*RI and *Pvu*II (both NEB). This intermediary construct was digested with *Sma*I and *Bam*HI (both NEB). The resulting fragment was ligated to the *Sma*I and *Bam*HI digested upstream fragment (*sdhA*-5). The resulting vector was digested with *Sma*I (NEB). The *pyrG* DNA fragment, after digestion with *Sma*I, was inserted between the two *sdhA* flanking regions. The resulting plasmid was verified by restriction analysis and sequencing. The deletion cassette, 4920 bp, containing the *sdhA* flanking regions and the *pyrG* gene, was released by *Mlu*I (NEB) digestion and transformed into *A. niger* ATCC 1015 *ΔpyrG*

Table 1
Primers used for PCR.

Primer	Sequence
sdhA_5_F	ATTAGGATCCACGCGTATGATGAATGGACCTTGCCA
sdhA_5_R	ATATCCCGGGCAACAAGATCTCAGGGATTGG
sdhA_3_F	ATATCCCGGGAATCCTCCTATCGGTCCGTTA
sdhA_3_R	ATTAGAATTCACGCGTTGAGGAGATTGAGGAACGGAA
sdhA_ORF_F	ATATGAATTCACAATGGCTCCTTCAGGAATCAATC
sdhA_ORF_R	ATATGGATCCTTAGGTCAAGGTTGCTTTCGCTG
sdhA_C-HIS_R	TAATGGATCCTCAGTGATGGTGATGGTGATGCCCGGT CAAGGTTGCTTTCGCTG
pyrG-del-F_n	TATACCCGGGTGATTGAGGTGATTGGCGAT
pyrG-del-R_n	TATACCCGGGTTATCACGCGACGGACAT
sdhA_qPCR_F	GGTTGTTGACATTAACTCCGA
sdhA_qPCR_R	CCACAGTTCATACAAGGCTC
xdhA-qPCR_F	GTGGTAGTGATGTTCATTATTGGG
xdhA-qPCR_R	TTTAGACTGGTAACGGCAGAG
act_qPCR_F	CAACATTGTCATGTCTGGTGG
act_qPCR_R	GGAGGAGCAATGATCTTGAC

strain. Transformants were selected by their ability to grow in the absence of uracil. Strains with successful deletions were verified by PCR. The primers for testing the presence/absence of the ORF are listed in Table 1.

2.5. Expression of sdhA in S. cerevisiae for purification of SdhA

For heterologous expression in *S. cerevisiae*, the ORF of s*dhA* (JGI53356) was amplified with C-terminal 6xHIS-tag from the cDNA of *A. niger* grown in the presence of galactitol with the primers sdhA-ORF_F and sdhA-C-HIS_R (Table 1). The fragment was inserted into the pYX212 plasmid between *Eco*RI and *Bam*HI sites allowing expression under the control of the *TPI1* promoter. All constructs were verified by sequencing. *S. cerevisiae* strain CEN.PK2-1D was transformed with the constructed plasmid, transformants were selected by growth in the absence of uracil, and the expression of *sdhA* was verified by enzymatic activity measurements in the cell extracts.

2.6. Protein extraction and enzyme activity measurements

To measure the D-sorbitol dehydrogenase activity in the *A. niger* extracts, mycelia were collected after induction with galactitol, mixed with 800 µl of 100 mM Tris–HCl (pH 7.0) with a protease inhibitor cocktail (Complete, Roche) and 800 µl of acid-washed glass beads (Sigma). The cells were disrupted twice for 40 sec in Fast Prep (Bio 101). The cell extracts were centrifuged (30 min, 25000 g, 4 °C) and the supernatants were used in the assay. The enzymatic activity was measured at room temperature by monitoring the NADH/NADPH formation at 340 nm in microtiter plates (NUNC) using the Varioskan (Thermo Electron Corporation) spectrophotometer.

To measure the substrate specificity of SdhA, the histidine tagged *sdhA* was expressed in *S. cerevisiae*. The cells from an overnight culture were harvested, washed and subsequently resuspended in Tris–HCl (pH 7) buffer supplemented with a protease inhibitor cocktail (Complete, Roche). The yeast cells were disrupted by vigorous mixing with glass beads in Fast Prep (Bio 101). The extracts were centrifuged (30 min, 25000 g, 4 °C), and the histidine tagged SdhA was purified using Ni-NTA resin (Qiagen).

Sorbitol dehydrogenase activities were measured in reaction mixtures containing 1 mM NAD, various concentrations of galactitol, L-arabitol, xylitol, D-sorbitol, L-iditol and ribitol and the purified histidine-tagged protein in 100 mM Tris–HCl buffer (pH 8.5). The formation of NADH in the reactions was monitored by measuring absorbance at 340 nm. The enzyme assays were performed at 30 °C in an Arena 20XT (Thermo, Vantaa, Finland) automated analyser. The K_m and v_{max} were estimated from the Michaelis–Menten equation fitted to the measured data. Protein concentrations were determined by the Protein Assay Kit (BioRad Laboratories, Hercules, CA).

2.7. HPLC analysis

To confirm that the reaction product from D-sorbitol was D-fructose and not L-sorbose, the reaction products from the *in vitro* reactions with purified SdhA were analysed with HPLC. A Fast Acid Analysis Column (100 × 7.8 mm, BioRad Laboratories) was used, linked to an Aminex HPX-87H organic acid analysis column (300 × 7.8 mm, BioRad Laboratories). The eluent was 2.5 mM H_2SO_4 at a flow rate of 0.5 ml min^{-1}. The column was maintained at 55 °C. Peaks were detected using a Waters 410 differential refractometer and a Waters 2487 dual wavelength UV (210 nm) detector.

3. Results and discussion

3.1. Identification of the sdhA gene

In the last step of the oxido-reductive D-galactose pathway, D-sorbitol is oxidised to D-fructose by a sorbitol dehydrogenase (Fig. 1). Sorbitol and xylitol dehydrogenases are similar enzymes and have overlapping activities. It has also been suggested that in *T. reesei* the Xdh1 is the sorbitol dehydrogenase of the oxido-reductive D-galactose pathway [16]. The xylitol dehydrogenase would thus be a candidate in *A. niger* as well. In order to identify

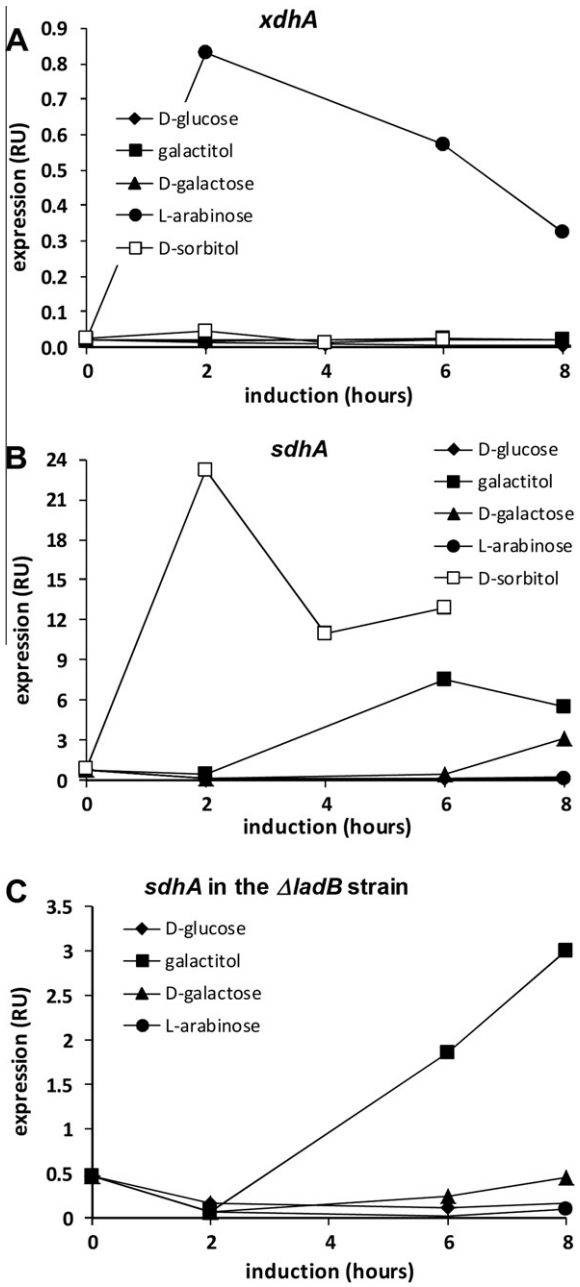

Fig. 2. Transcription analysis of selected *A. niger* genes on different carbon sources. (A) The expression profile of the *xdhA* gene reveals that it is not involved in the D-galactose pathway of *A. niger*. (B) The *sdhA* gene is specifically upregulated in the presence of D-sorbitol, galactitol, and D-galactose. (C) In the Δ*ladB* strain the upregulation of *sdhA* is less pronounced in comparison to the wild type. The level of transcription shown in the graphs is relative to the actin mRNA signal.

a sorbitol dehydrogenase that is active in the oxido-reductive D-galactose pathway in *A. niger* we monitored transcription of *xdhA* (xylitol dehydrogenase; JGI203198) and a number of its homologues in the medium chain dehydrogenase (MCD) family on various carbon sources. The *xdhA* was only upregulated on L-arabinose, as described previously [17], and not on D-galactose, galactitol or D-sorbitol (Fig. 2A), indicating that the product of this gene is not primarily involved in D-galactose or D-sorbitol catabolism. It should, however, be emphasized that in native environments of *A. niger*, where D-galactose and D-sorbitol are often accompanied by other carbon sources such as pentose sugars, the *xdhA* could be expressed. In addition, XdhA has previously been shown to be active with D-sorbitol [18] and therefore it is likely that it contributes to the D-galactose metabolism in a similar manner as XyrA and LadA [9]. On the other hand, two other MCD genes were identified to be upregulated on D-galactose and galactitol, but not on L-arabinose or D-glucose; the previously described galactitol dehydrogenase, *ladB* [9], and JGI53356 (An07g01290) which was also strongly upregulated on D-sorbitol (Fig. 2B). We called the gene *sdhA*. The other MCD genes which were analysed and showed no upregulation in the tested conditions were: An08g09380 (JGI37988), An05g02260 (JGI212968), An03g05190 (JGI50731), An14g03510 (JGI185262), and An09g03900 (JGI188914). The *ladA* gene was also included and showed upregulation only in the presence of L-arabinose (data not shown). The transcriptional profile of *sdhA* indicates that the encoded enzyme is involved in the oxido-reductive D-galactose pathway.

The *sdhA* gene was rapidly upregulated on D-sorbitol, suggesting that it also has a role in the D-sorbitol catabolism (Fig. 2B). A D-sorbitol-induced sorbitol dehydrogenase activity has been described previously [13]. A fructokinase was induced together with the dehydrogenase, suggesting that D-fructose-6-phosphate is produced. Since we observed upregulation of the *sdhA* gene in the presence of both galactitol and D-sorbitol, we speculated that galactitol might induce the expression indirectly after being metabolised to D-sorbitol. To test whether galactitol is a direct inducer of *sdhA* transcription, we monitored *sdhA* expression in the galactitol dehydrogenase deficient (Δ*ladB*) strain [9]. In this mutant, which is unable to grow on galactitol, *sdhA* is still induced, however not to the levels observed in the wild type strain (Fig. 2C). This suggests that indeed galactitol could be an inducer in addition to D-sorbitol. However, we cannot rule out the possibility that there is some residual galactitol dehydrogenase activity that is too low to support growth, but sufficient to convert some galactitol to L-xylo-3-hexulose that is then converted to D-sorbitol.

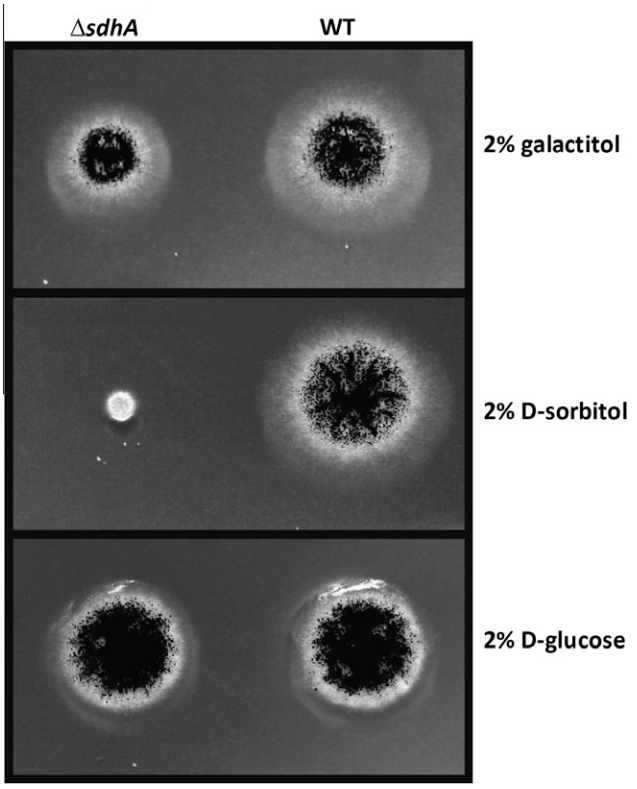

Fig. 3. Growth of the Δ*sdhA* and parent strains. Equal amounts of spores of the Δ*sdhA* and the parent strain were used for inoculation of each colony. The agar media containing the different carbon sources, as indicated, were incubated for 3 days at 28 °C.

3.2. Deletion of the sdhA and analyses of the resulting strain

In order to demonstrate the importance of the *sdhA* gene for D-galactose and D-sorbitol catabolism we constructed a deletion strain. The growth of the parental and the *ΔshdA* strain was compared on agar media with different carbon sources: galactitol, D-sorbitol, and D-glucose. Growth of the *ΔsdhA* strain was reduced on galactitol as compared to the wild type, but not completely abolished (Fig. 3). The ability to grow on D-sorbitol was, however, completely lost in the deletion strain. The growth on D-glucose was identical for both of the strains tested.

These observations suggest that *sdhA* encodes an essential enzyme for growth on D-sorbitol as a sole carbon source. In contract,

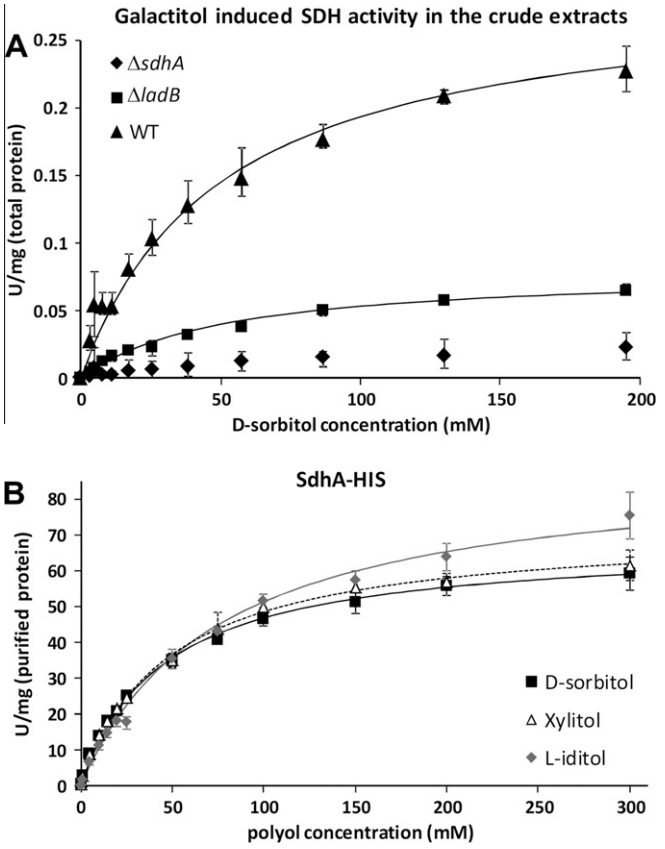

Fig. 4. *In vitro* enzymatic characterisation of SdhA. (A) Sorbitol dehydrogenase activity in the crude extracts of *A. niger* after transfer to galactitol for 6 h. The wild type, Δ*sdhA* and Δ*ladB* strains were compared and activity measured with a range of D-sorbitol concentrations. The reaction profile of the SDH activity in the Δ*sdhA* strain does not allow fitting the Michaelis–Menten kinetics. (B) Kinetic properties of the purified recombinant sorbitol dehydrogenase with different polyols. The drawn line is a fit of the data points to the Michaelis–Menten equation: solid line (D-sorbitol), dashed line (xylitol), grey line (L-iditol).

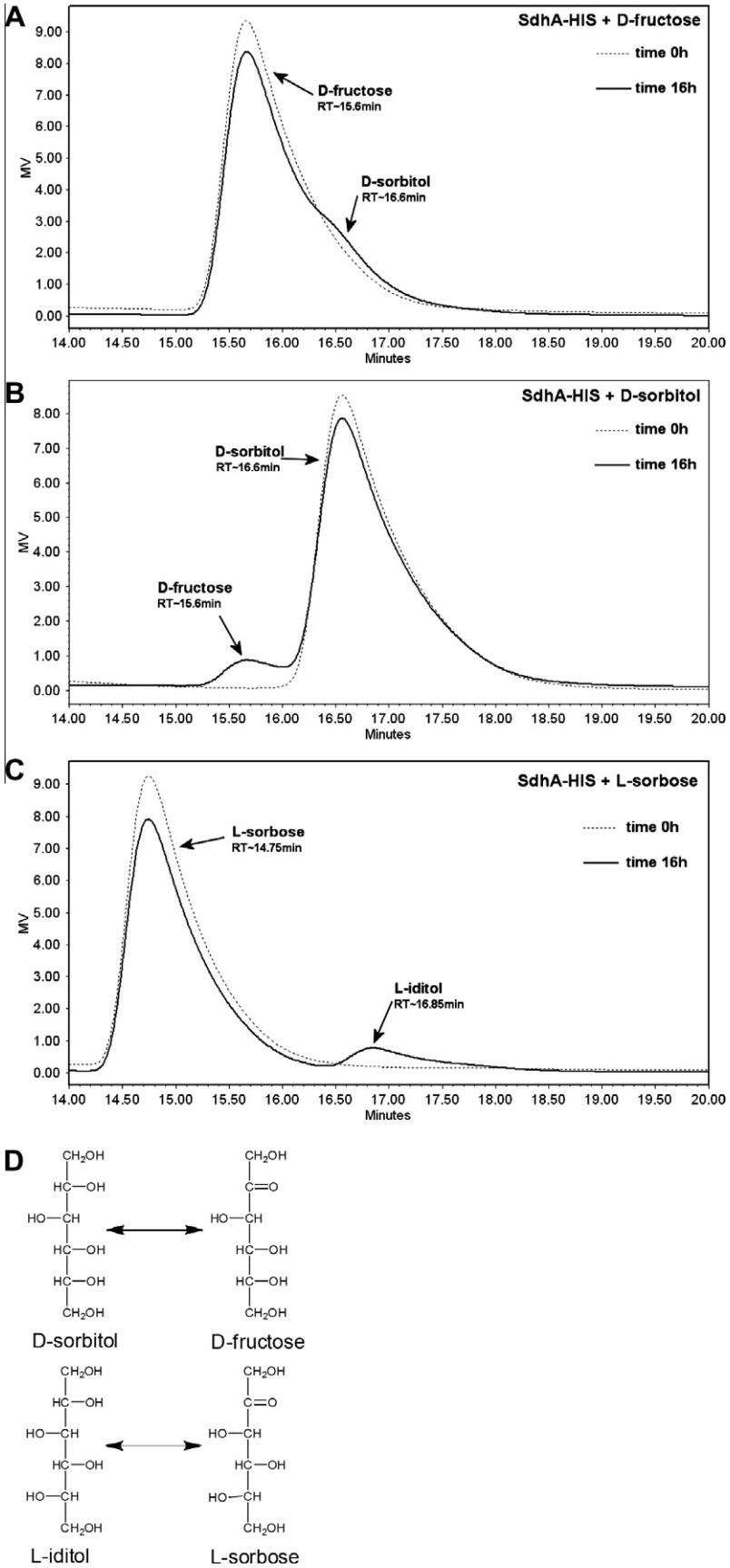

Fig. 5. HPLC analysis of SdhA reaction products. (A) D-fructose, (B) D-sorbitol, and (C) L-sorbose were used as substrates in the reactions with purified SdhA. The initial reaction mix (dotted lines) and the reaction mix after 16 h of incubation at room temperature (solid lines) were analysed by HPLC; the graphs show overlays of the two samples detected by refractive index (RI). The arrows and retention times (RT) show the individual sugars and polyols as identified based on HPLC analysis of the pure compounds. (D) Chemical structures of the compounds in question.

another enzyme activity can take over for growth on galactitol when *sdhA* is deleted. One obvious candidate for this galactitol-induced, sorbitol dehydrogenase activity is the galactitol dehydrogenase, LadB, which was shown to be expressed in the presence of galactitol and is active with D-sorbitol [9]. To test this we performed *in vitro* D-sorbitol dehydrogenase assays with crude cell extracts from the wild type, *ΔsdhA*, and *ΔladB* strains, which had been transferred to galactitol-containing medium for 6 h (Fig. 4A). The deletion of *sdhA* resulted in a strong reduction but not a complete elimination of D-sorbitol dehydrogenase activity. Deletion of *ladB* caused moderate decrease in the activity as compared to the wild type, which is consistent with the lower level of transcription of the *sdhA* gene in this strain on galactitol (Fig. 2C).

3.3. In vitro characterisation of SdhA

For the enzymatic characterisation of SdhA, the *sdhA* gene was C-terminally fused with 6xHIS-tag and the resulting construct expressed from a multicopy plasmid with a strong constitutive promoter in *S. cerevisiae*. The purified recombinant enzyme was then analysed. It showed activity with NAD and D-sorbitol, xylitol or L-iditol (Fig. 4B), but not with other polyols (mannitol, ribitol, galactitol, L-arabitol, or D-arabitol – data not shown) confirming that this enzyme is indeed a sorbitol dehydrogenase. The v_{max} were 80 ± 10, 85 ± 10 and 90 ± 5 U/mg and the K_m 50 ± 5, 50 ± 3 and 65 ± 10 mM for D-sorbitol, xylitol and L-iditol respectively (Fig. 4B). In the reverse reaction, the enzyme was active with NADH and D-fructose and L-sorbose (data not shown). The reaction products from D-sorbitol, D-fructose and L-sorbose were analysed by HPLC. From D-fructose, sorbitol was produced (Fig. 5A). Since D-fructose HPLC peak overlapped the sorbitol peak in our system, we also analysed the reaction product of the reverse reaction using D-sorbitol as substrate to produce D-fructose (Fig. 5B). From L-sorbose, iditol was produced (Fig. 5C). The HPLC method did not allow discrimination between the L- and D-form of the sugars or sugar alcohols, but if only the keto-group is reduced, the reaction products are D-sorbitol and L-iditol (Fig. 5D). In the case of D-sorbitol oxidation, the reaction product can be D-fructose or L-sorbose and the HPLC analysis clearly confirmed that it was D-fructose. It was earlier suggested that in *A. nidulans* galactitol is converted to L-sorbose [10]. Here we demonstrated that SdhA does not catalyse the conversion of sorbitol to L-sorbose.

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