

Pyrroloquinoline Quinone-Dependent Dehydrogenases from *Ketogulonicigenium vulgare* Catalyze the Direct Conversion of L-Sorbose to L-Ascorbic Acid†

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A novel enzyme, L-sorbose dehydrogenase 1 (SNDH1), which directly converts L-sorbose to L-ascorbic acid (L-AA), was isolated from *Ketogulonicigenium vulgare* DSM 4025 and characterized. This enzyme was a homooligomer of 75-kDa subunits containing pyrroloquinoline quinone (PQQ) and heme *c* as the prosthetic groups. Two isozymes of SNDH, SNDH2 consisting of 75-kDa and 55-kDa subunits and SNDH3 consisting of 55-kDa subunits, were also purified from the bacterium. All of the SNDHs produced L-AA, as well as 2-keto-L-gulonic acid (2KGA), from L-sorbose, suggesting that tautomerization of L-sorbose causes the dual conversion by SNDHs. The *sndH* gene coding for SNDH1 was isolated and analyzed. The N-terminal four-fifths of the SNDH amino acid sequence exhibited 40% identity to the sequence of a soluble quinoprotein glucose dehydrogenase from *Acinetobacter calcoaceticus*. The C-terminal one-fifth of the sequence exhibited similarity to a *c*-type cytochrome with a heme-binding motif. A lysate of *Escherichia coli* cells expressing *sndH* exhibited SNDH activity in the presence of PQQ and CaCl₂. Gene disruption analysis of *K. vulgare* indicated that all of the SNDH proteins are encoded by the *sndH* gene. The 55-kDa subunit was derived from the 75-kDa subunit, as indicated by cleavage of the C-terminal domain in the bacterial cells.

L-Ascorbic acid (L-AA) is an essential nutrient for humans. It is known that L-AA is directly converted from aldonolactones, such as L-gulono- γ -lactone and L-galactono- γ -lactone, by aldonolactone dehydrogenase/oxidases in plants and mammals (except some primates, including humans). The L-gulono- γ -lactone oxidase that catalyzes the conversion of L-gulono- γ -lactone to L-AA was isolated from rat and goat livers and characterized by Nishikimi et al. (19). An L-galactono- γ -lactone oxidase of yeast origin was described by Bleeg (4). In addition, L-sorbose is also known to be a potential precursor of L-AA in plants. An NADP-dependent L-sorbose dehydrogenase (SNDH) that converts L-sorbose to L-AA in spinach leaves has been reported (17), but the enzyme has not been purified or characterized in detail.

In current industrial L-AA production processes, as summarized by Hancock and Viola (13), 2-keto-L-gulonic acid (2KGA) is a key intermediate that is chemically converted to L-AA. All of the processes require a large amount of energy and organic solvent, and thus a cheaper and environmentally conscious substitute process, such as enzymatic conversion, is desirable. It has also been reported that L-sorbose is converted to 2KGA in bacteria. Many microbial L-sorbose dehydrogenases, including enzymes from *Acetobacter liquefaciens* (27),

Gluconobacter melanogenus UV10 (14), and *Gluconobacter oxydans* T-100 (23), have been isolated and characterized. Moreover, *Ketogulonicigenium vulgare* DSM 4025 has been reported to produce aldehyde dehydrogenase (ALDH) (15) and L-sorbose/L-sorbose dehydrogenases (SSDHs) (3), which are responsible for sequentially converting L-sorbose to 2KGA via L-sorbose.

The metabolic pathways from primary sugars in *K. vulgare* DSM 4025 have been studied in our laboratory. Recently, it was reported that *K. vulgare* DSM 4025 has the ability to produce L-AA from sugars such as D-sorbitol, L-sorbose, L-gulose, and L-sorbose (30). One L-AA-producing enzyme that catalyzes oxidation of L-gulono- γ -lactone was isolated previously (29), but the detailed pathway from primary sugars to L-AA was unclear. In this study, we report identification of novel SNDHs that directly convert L-sorbose to L-AA and their genes in *K. vulgare* DSM 4025.

MATERIALS AND METHODS

Chemicals. L-Sorbose, L-sorbose, D-glucosone, L-gulono- γ -lactone, and 2KGA were supplied by F. Hoffmann-La Roche, Switzerland. Pyrroloquinoline quinone (PQQ) was purchased from Mitsubishi Gas Chemical, Japan. Other chemicals used in this study were commercially available.

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Strain DSM 4025, formerly classified in the genus *Gluconobacter* (35) and recently proposed to be reclassified in the new genus *Ketogulonicigenium* (34), was used for preparation of the SNDHs and chromosomal DNA.

Media and culture conditions. *K. vulgare* DSM 4025 cells were grown at 27°C for 4 days on B8 agar containing 8% (wt/vol) L-sorbose, 0.25% MgSO₄ · 7H₂O, 1.75% corn steep liquor (Nissiyoku, Japan), 5% live baker's yeast (Oriental, Japan), 0.5% urea, 0.5% CaCO₃, and 2% agar. The cells were also cultivated in 2 liters of medium containing 8% L-sorbose, 0.05% glycerol, 0.25% MgSO₄ · 7H₂O, 3% corn steep liquor, 0.4% yeast extract, and 0.15% antifoam agent at 30°C and pH 7.0 using a 3-liter jar fermentor. After 48 h, 38.4 g (wet weight) of the cells was obtained from 6 liters of broth. For conjugal transfer of plasmids, DSM 4025 derivative recipients were cultivated in T broth consisting of 3% Trypticase soy broth (Difco, United States) and 0.3% yeast extract (Difco) at

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristic(s) ^a	Source or reference
<i>E. coli</i> strains		
VCS257	Derivative of DP50supF [<i>supE44 supF58 hsdS3</i> (r _B ⁻ m _B ⁻) <i>dapD8 lacY1 glnV44 Δ(gal-uvrB)</i> 47 <i>tyrT58 gyrA29 tonA53 Δ(thyA57)</i>]	Stratagene
JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)</i> /F' [<i>traD36 proAB⁺ lacI^q lacZΔM15</i>]	Takara Bio
DH5α	F ⁻ φ80d <i>lacZΔM15 Δ(lacZYA-argF)</i> U169 <i>deoR recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA supE44 λ⁻ thi-1 gyrA96 relA1</i>	Takara Bio
HB101	<i>leuB6 proA2 recA13 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL20 supE44 Δ(mcrC-mrr)</i>	Takara Bio
M15	Nal ^s Str ^s Rif ^s Thi ⁻ Lac ⁻ Ara ⁺ Gal ⁺ Mtl ⁻ F ⁻ RecA ⁺ Uvr ⁺ Lon ⁺	QIAGEN
DSM 4025 derivatives		
DSM 4025	<i>K. vulgare</i> strain	34
GOMTR1	Rif ^r , spontaneous mutant of DSM 4025	This study
GOMTR1SN::Km	Rif ^r Km ^r , <i>sndH</i> gene disruptant of GOMTR1	This study
Plasmids		
pVK100	ATCC 37156, RK2 (IncP) derivative, 23 kb, Km ^r Tc ^r , <i>cos</i>	ATCC ^b
pRK2013	RK2 (IncP) derivative, 45 kb, Km ^r , <i>mob</i>	ATCC
pSUP202	Ap ^r Tc ^r Cm ^r , <i>mob</i>	28
pSUPSN	pSUP202 carrying <i>sndH</i>	This study
pSUPSN::Km	pSUP202 carrying <i>sndH</i> disrupted with Km ^r gene cassette	This study
pQE30	Six-His tag, Ap ^r	QIAGEN
pUC18/19	Ap ^r	Takara Bio
pUC4K	Ap ^r Km ^r	TOYOBO

^a Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Rif^r, rifampin resistance.

^b ATCC, American Type Culture Collection.

30°C for 24 h. Transconjugants were cultivated on M agar consisting of 5% mannitol, 1.75% corn steep liquor, 5% live baker's yeast, 0.25% MgSO₄ · 7H₂O, 0.5% CaCO₃, 0.5% urea, and 2% agar (pH 7.0) at 27°C for 24 h. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium with vigorous shaking at 30°C. Antibiotics were used in the medium at the following concentrations: ampicillin, 100 µg/ml; kanamycin (Km), 50 µg/ml; rifampin, 100 µg/ml; and tetracycline, 2 µg/ml.

PAGE. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed with 12.5% polyacrylamide gels using the method of Laemmli (16), and proteins were stained with Coomassie brilliant blue R-250 (CBB). For activity staining, native PAGE (pH 9.4) was performed with a 10% polyacrylamide gel by using a method based on the method of Davis (7) at 4°C. The gel was immersed in a staining solution (100 ml) consisting of 100 mM potassium phosphate buffer (pH 7.0), 4 mg nitroblue tetrazolium, 14 mg phenazine methosulfate (PMS), and 200 mg L-sorbose with gentle shaking at 25°C for 20 min. The reaction was stopped by immersing the gel in 7% (vol/vol) acetic acid.

Enzyme assays. For the product assay, reaction mixtures consisting of 1.0 mM PMS, 25 mM potassium phosphate buffer (pH 7.0), 1.0 µM PQQ, 1.0 mM CaCl₂, 50 mM L-sorbose, and an enzyme solution were prepared. Each reaction mixture was incubated at 30°C for 30 to 60 min, and the reaction was stopped with metaphosphoric acid at a final concentration of 2%. The amount of L-AA produced was measured at a wavelength of 264 nm using a Tosoh high-performance liquid chromatography (HPLC) system-equipped YMC-Pack Polyamine-II column with a mobile phase consisting of 50 mM NH₄H₂PO₄ and acetonitrile (30:70). For measurement of 2KGA, the reaction product was treated with 1% *o*-phenylenediamine at 95°C for 15 min and measured at a wavelength of 340 nm using a YMC-Pack Pro C₁₈ column with a mobile phase consisting of H₂O and methanol (82:18). One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol of the product per min. The protein concentration was determined by using a CBB protein assay solution (Nacalai Tesque).

For a photometric assay of dehydrogenation activity, a basic reaction mixture containing 0.1 mM 2,6-dichlorophenolindophenol (DCIP), 1.0 mM PMS, 50 mM potassium phosphate buffer (pH 7.0), and 1.0 µM PQQ was prepared. For determination of the substrate specificity, 1.0 mM CaCl₂ and 2 to 100 mM (final concentration) substrate were added to the basic mixture immediately before the assay. For investigation of the effects of inhibitors, 2 mM L-sorbose and 5 mM (final concentration) inhibitor or 1.0 mM (final concentration) metal ion were added to the basic mixture. The reaction was started at 25°C, and the enzyme

activity was measured by determining the initial rate of reduction of DCIP at 600 nm using a Shimadzu UV-VIS recording spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the reduction of 1 µmol DCIP per min.

Purification of the enzymes. *K. vulgare* DSM 4025 cell paste (19.2 g) was suspended in 100 ml of 50 mM potassium phosphate (pH 7.0) and passed through a French pressure cell at 1,500 kg force/cm². The resulting cell extract (CFE) was centrifuged at 100,000 × g for 60 min to prepare the soluble fraction. The dialyzed soluble fraction was placed on a column (3 by 50 cm) of Whatman DE-52 DEAE-cellulose. SNDHs were eluted with a linear gradient (800 ml) of 0 to 0.8 M NaCl and concentrated with an ultrafiltration device (Centriprep-10; Amicon, United States). The dialyzed enzyme fraction was placed on a column (3 by 25 cm) of CL-6B DEAE-Sepharose. SNDHs were eluted with a linear gradient (600 ml) of 0.3 to 0.6 M NaCl. The eluted SNDH fractions were dialyzed and placed on a column (1.5 by 25 cm) of Q-Sepharose (Pharmacia). A linear gradient (375 ml) of 0.3 to 0.6 M NaCl was applied. SNDH1 and SNDH2 were eluted at 0.56 M NaCl (Q1 fraction SN), and SNDH3 was eluted at 0.45 M NaCl. The enzyme fractions were desalted and concentrated, and 1.5 M (final concentration) ammonium sulfate was added. After centrifugation (15,000 × g), the supernatant was placed on a hydrophobic Resource ISO column (1.0 ml; Pharmacia) equilibrated with buffer containing 1.5 M ammonium sulfate. SNDH1 and SNDH2 were eluted with 1.12 to 1.10 M and 1.15 to 1.13 M ammonium sulfate, respectively. SNDH3 was eluted with 1.04 to 1.00 M ammonium sulfate. The enzyme fractions were dialyzed and stored at -20°C.

Alternatively, SNDH1 was purified by native PAGE from Q1 fraction SN. The SNDH1 band was excised from a slab gel and electrically eluted using Max-Yield (Atto, Japan) in a running buffer (pH 8.3) containing 5 mM Tris and 3.8 mM glycine. The eluted enzyme solution was ultrafiltered in order to replace the buffer.

Measurement of molecular mass. The molecular masses of the native enzymes were estimated using HPLC with a size exclusion gel column (TSK gel G3000_{SWXL}; Tosoh). Cyanocobalamin (1,350 Da), myoglobin (16,950 Da), ovalbumin (45,000 Da), bovine gamma globulin (158,000 Da), and thyroglobulin (670,000 Da) were used as molecular mass standards. The molecular mass of the 55-kDa subunit was determined by liquid chromatography-electric spray injection mass spectrometry (Finnigan LCO; Thermo Finnigan, California).

Analysis of prosthetic groups. The PQQ groups of the SNDHs were identified by the method of Duine et al. (10). Prosthetic groups of the enzymes were extracted with 0.5 M NaH₂PO₄ (pH 1.0) and 67% (wt/vol) methanol. Each

extract was subjected to PQQ analysis by reverse-phase HPLC using a YMC-Pack Pro C₁₈ AS-312 column at a wavelength of 313 nm.

Heme *c* was identified by the reduced-minus-oxidized difference spectrum. Reduced and oxidized enzyme solutions at a concentration of 50 µg/ml in 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM sodium hydrosulfite and 10 mM ammonium persulfate, respectively, were prepared for measurement of the difference spectrum with a Shimadzu UV-2200.

Determination of the N-terminal sequences of the proteins. Purified SNDH1 or SNDH2 (10 µg each) was subjected to SDS-PAGE, and the protein band was electroblotted on a polyvinylidene difluoride membrane (Bio-Rad, California). To remove the blocked glutamyl residue at the N terminus, the protein blotted on the membrane was incubated with pyroglutamate aminopeptidase (Sigma) at 30°C for 24 h. The treated membrane was subjected to N-terminal amino acid sequencing with an automated amino acid sequencer (ABI model 490; Perkin-Elmer Corp., Connecticut).

Construction and screening of the gene library. *K. vulgare* chromosomal DNA was digested partially with EcoRI, ligated to cosmid vector pVK100, and used for in vitro packaging to construct the gene library. For preparation of the gene probe, PCR amplification was performed with the chromosomal DNA and degenerate oligonucleotide DNA primers 5'-CARGGYAACCCSGTBGA-3' and 5'-GTYTCGTTCGRCVAC-3', which were synthesized according to the N-terminal sequence of the 75-kDa subunit using *Taq* polymerase (Ex Taq; Takara Bio, Japan). A 41-bp DNA fragment encoding the N-terminal amino acids of SNDH was specifically amplified and cloned into vector pCR-TOPO to obtain plasmid pMTSN2. The DNA probe for hybridization was prepared using a PCR digoxigenin (DIG) labeling kit (Roche Diagnostics GmbH, Germany), pMTSN2 DNA as the template, and primers 5'-CAGGGTAACCCGGTC-3' and 5'-GAC TCGTTTGC GCCC-3'. *E. coli* colonies carrying the gene library were blotted on a Biotrans A membrane (1.2 µm; Pall Biosupport Corp., New York). The membrane filter was incubated with a DIG-labeled DNA probe at 30°C for 12 h, and this was followed by washing with 2× SSC (0.3 M NaCl plus 30 mM sodium citrate, pH 7.0) containing 0.1% SDS. The dried membrane was subjected to DIG chemiluminescent detection (Roche Diagnostics).

Subcloning and DNA sequence analysis. Plasmid DNA was prepared by the alkaline SDS method (24) or by using an automatic DNA isolation system (PI-50; Kurabo, Japan). The 8.0-kb PstI fragment, which contained the intact *sndH* gene, was subcloned into the pUC18 vector to obtain pUCSNP4. The 2.1-kb EcoRI-HindIII fragment containing the 1.0-kb N-terminal region of the *sndH* gene was cut out from pUCSNP4 and cloned into pUC19 to obtain pUCSN2001. Deletion plasmids containing the truncated *sndH* gene were prepared using a kilobase deletion kit (Takara Bio). Dideoxy chain termination with Cy5-dATP (Amersham Pharmacia Biotech) was used to determine the DNA sequence.

Disruption of the *sndH* gene in *K. vulgare* DSM 4025. pSUPSN was constructed by inserting the 8.0-kb PstI fragment containing the *sndH* gene into suicide vector pSUP202 (28). A kanamycin resistance gene cassette (Km cassette) from pUC4K was then inserted into the EcoRI site of the *sndH* gene in pSUPSN. The resulting plasmid, pSUPSN::Km, was introduced into GOMTR1 by conjugal transfer (26) with helper strain *E. coli* HB101 harboring pRK2013 and donor strain *E. coli* DH5α harboring pSUPSN::Km. The *sndH* null mutant was selected as Km^r, Rif^r, and Tc^r.

Preparation of anti-SNDH antiserum and immunological analysis. The *sndH* coding sequence was cloned into pQE30, yielding pQESN8, which encoded a recombinant SNDH protein with a six-His tag at the N terminus. The recombinant protein was expressed in *E. coli* M15 with isopropyl-β-D-thiogalactopyranoside (IPTG) induction, purified with an Ni-nitrilotriacetic acid agarose column (QIAGEN), and used to immunize rabbits.

After SDS-PAGE of the cell extracts, the protein bands on the gel were electroblotted on a nitrocellulose filter by using a semidry electroblotting apparatus (Trans-blot SD; Bio-Rad, United States). The subunits of SNDHs were detected using anti-SNDH rabbit antiserum and an alkaline phosphatase conjugate substrate kit (Bio-Rad).

Nucleotide sequence accession number. The nucleotide sequence data have been deposited in the DDBJ, EMBL, and GenBank databases under accession number AB205130.

RESULTS

Purification of three SNDHs. Five major activity bands that reduced dyes using L-sorbose as the substrate were obtained by native PAGE of the cell extract of *K. vulgare* DSM 4025 (Fig. 1A). Two of these bands, bands for SSDH and ALDH,

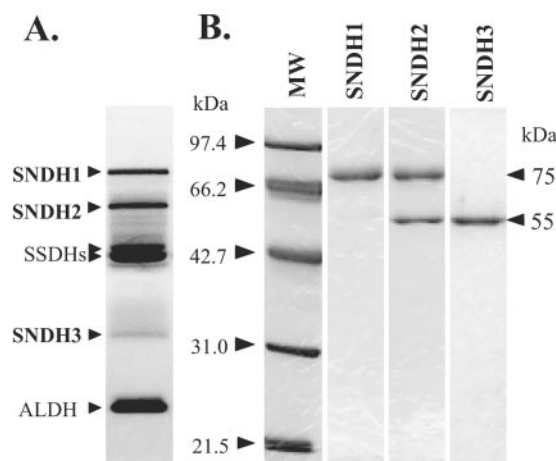


FIG. 1. PAGE analysis of SNDHs. (A) Activity staining of the L-sorbose-dehydrogenating enzymes of *K. vulgare* DSM 4025 cell extract on a native 10% polyacrylamide gel. The locations of SNDH, SSDH, and ALDH proteins are indicated on the left. (B) SDS-PAGE (12.5% polyacrylamide gel) analysis of purified SNDHs. Lane MW contained a mixture of molecular mass markers. Purified SNDH1 (1.0 µg), SNDH2 (1.3 µg), and SNDH3 (0.75 µg) were loaded into the lanes. The proteins were stained with CBB.

have been characterized previously in our laboratory. SSDHs comprising four types of isozymes are common PQQ-dependent enzymes in *K. vulgare* cells and catalyze in three steps: D-sorbitol to L-sorbose, L-sorbose to L-sorbose, and L-sorbose to 2KGA (3). ALDH converts L-sorbose to 2KGA (15). However, the other three enzyme bands (SNDH1, SNDH2, and SNDH3) have not been characterized previously. Recently, it was found that *K. vulgare* DSM 4025 could produce L-AA from L-sorbose (30). Therefore, we analyzed these bands for activity that converts L-sorbose to L-AA.

We purified the SNDHs by conventional chromatography. The dialyzed soluble fraction (specific activity for L-AA production, 16.3 mU/mg protein from L-sorbose) of *K. vulgare* cells was used for purification. All three SNDHs eluted at almost the same NaCl concentration in DEAE anion-exchange chromatography. SNDH3 was isolated from SNDH1 and SNDH2 using Q-Sepharose anion-exchange chromatography. Finally, SNDH1 and SNDH2 were separated by hydrophobic chromatography. Thus, SNDH1, SNDH2, and SNDH3 were purified almost to homogeneity and appeared slightly reddish. The purified SNDH1, SNDH2, and SNDH3 migrated to their original positions on a native polyacrylamide gel (Fig. 1A). It was found that the purified SNDHs simultaneously converted L-sorbose to L-AA and to 2KGA. SNDH1 had specific activities at pH 7.0 for production of L-AA and 2KGA from L-sorbose of 3.01 and 0.60 U/mg protein, respectively; the corresponding values for SNDH2 were 3.26 and 0.74 U/mg protein, respectively, and the corresponding values for SNDH3 were 4.63 and 1.16 U/mg protein, respectively. SDS-PAGE revealed that SNDH1 and SNDH3 were composed of 75-kDa and 55-kDa subunits, respectively (Fig. 1B). However, SNDH2 was a heterooligomer of 75-kDa and 55-kDa subunits. Gel filtration chromatography analysis showed that the molecular masses of SNDH1, SNDH2, and SNDH3 were 150,000 to 230,000, 190,000 to 250,000, and 100,000 to 150,000 Da, re-

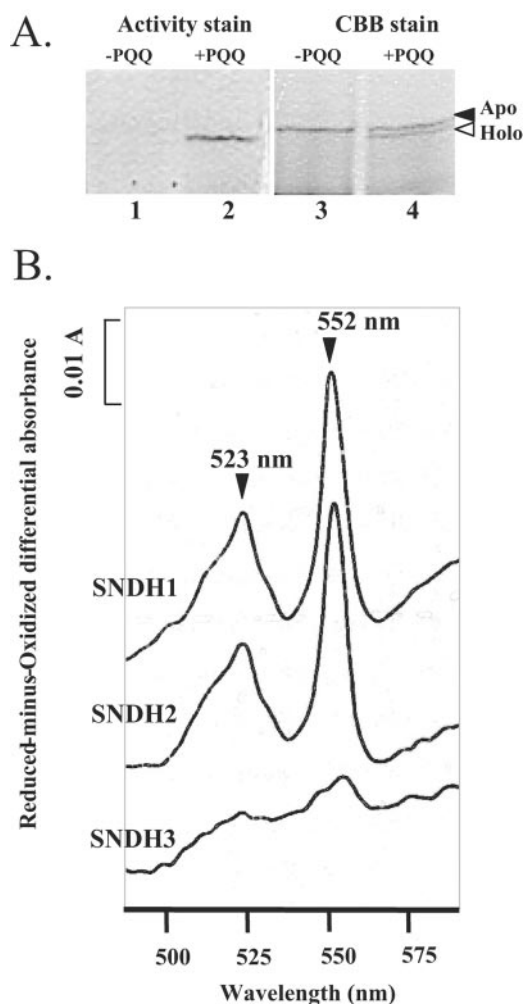


FIG. 2. (A) Reconstruction of holo-SNDH from apo-SNDH prepared by electroelution from a native polyacrylamide gel. In each lane, 0.75 μ g of the protein was used. Lanes 1 and 2, activity staining of L-sorbose-dehydrogenating enzymes; lanes 3 and 4, protein staining with CBB. Lanes 1 and 3 contained apo-SNDH, and lanes 2 and 4 contained SNDH treated with 1 μ M PQQ and 1 mM CaCl_2 . The solid arrowhead indicates the position of the apoenzyme. The open arrowhead indicates the position of the holoenzyme in which PQQ binds to the apoenzyme. (B) Reduced-minus-oxidized difference spectra of the purified SNDHs.

spectively (data not shown). Gel filtration chromatography with SDS revealed that SNDH2 contained equal amounts of the two subunits. The molecular mass of the 55-kDa subunit was determined to be 49,601 Da by mass spectrum analysis.

Prosthetic groups of SNDHs. HPLC analysis of the acidified methanol extract of SNDH1 produced a peak with detection at 313 nm at the same retention time as an authentic PQQ sample (data not shown), indicating that SNDH1 has PQQ as a prosthetic group. When SNDH1 was recovered from a native polyacrylamide gel by electroelution, apo-SNDH protein was obtained. Apo-SNDH protein did not exhibit activity and migrated as a single band on a native polyacrylamide gel (Fig. 2A, lanes 1 and 3). When apo-SNDH was treated with 1 μ M PQQ and 1 mM CaCl_2 at 25°C, approximately one-half of the apo-SNDH was converted to holo-SNDH within 10 min (Fig.

2A, lane 4). The reconstituted holoenzyme showed activity (lane 2). Holo-SNDH migrated slightly faster than apo-SNDH.

The reduced-minus-oxidized difference spectra of SNDH1 and SNDH2 showed a typical pattern for *c*-type cytochromes, which had two major peaks at 523 nm and 552 nm (Fig. 2B). This suggests that these enzymes contain heme *c*. In contrast, SNDH3 had no apparent peaks around 550 nm, suggesting a lack of heme *c* in this enzyme.

Characterization of the three SNDHs. To investigate the effects of pH on the enzyme activity, 100 mM sodium citrate (pH 4.5 to 6.5), potassium phosphate (pH 6.5 to 8.5), and Tris-HCl (pH 7.5 to 9.0) were used. For conversion of L-sorbose to L-AA, all three SNDHs had optimum reaction rates at pH 7.0 to 8.0. In contrast, for conversion to 2KGA, they exhibited less activity around neutral pHs but high activity at pHs greater than pH 8.5 (data not shown). A reaction mixture (pH 7.0) containing 50 mM L-sorbose was incubated for 60 min at 20 to 60°C. For both production of L-AA and production of 2KGA, the optimum temperatures for the reaction were rather broad; the three enzymes exhibited similar high activities at 20 to 40°C (data not shown). The effects of metal ions [CoCl_2 , CuSO_4 , $\text{Fe}(\text{SO}_4)_3$, NiSO_4 , ZnCl_2 , MgCl_2 , TiCl_4 , and CaCl_2] at a concentration of 1 mM and inhibitors (EDTA, NaN_3 , and sodium monoiodoacetate) at a concentration of 5 mM on the enzyme activity for L-sorbose were examined by a photometric assay using DCIP. The effects of most metal ions and inhibitors on the activities of the three SNDHs were similar (data not shown). Monoiodoacetate commonly inactivates SNDHs. Among the metal ions tested, Cu^{2+} almost completely inhibited the activity of the SNDHs. No enhancement of activity by the metal ions tested was observed.

The substrate specificities of the purified SNDHs were examined using several aldehyde, alcohol, or lactone compounds at two pHs, pH 7.5 for the activity to produce L-AA and pH 9.0 for the activity to produce 2KGA (see Table S1 in the supplemental material). For this photometric assay with DCIP, a concentration of the unstable osones L-sorbose and D-glucosone of 2 mM was used; in contrast, a concentration of the stable sugars D-glucose, L-sorbose, D-sorbitol, D-xylose, and L-gulonono- γ -lactone of 100 mM was used. The three SNDHs exhibited almost no activity with L-sorbose, D-sorbitol, or L-gulonono- γ -lactone, although they exhibited relatively high activities with aldoses, especially D-glucose and D-xylose.

Analysis of the *sndH* gene. The N-terminal amino acid sequences of the 75-kDa and 55-kDa SNDH subunits were analyzed with a sequencer. Since the N-terminal sequences of the proteins could not be read due to probable blocked residues, the proteins were treated with pyroglutamate aminopeptidase. It was found that both the 75-kDa and 55-kDa subunits had the same N-terminal sequence from pyroglutamate (pyroQ), which was pyroQXNPVEVPVGANET. To clone the *sndH* gene of *K. vulgare* DSM 4025, forward and reverse degenerate primers corresponding to the N-terminal amino acid sequence of SNDH were synthesized and used to obtain a DNA probe. pVSN5 carrying the *sndH* gene was identified from the *K. vulgare* DSM 4025 gene library using the DNA probe. Southern blot analysis of the genome DNA digested with EcoRI, EcoRV, PstI, SmaI, BamHI, and HindIII using the *sndH* probe showed hybridization to a single fragment (data not shown),

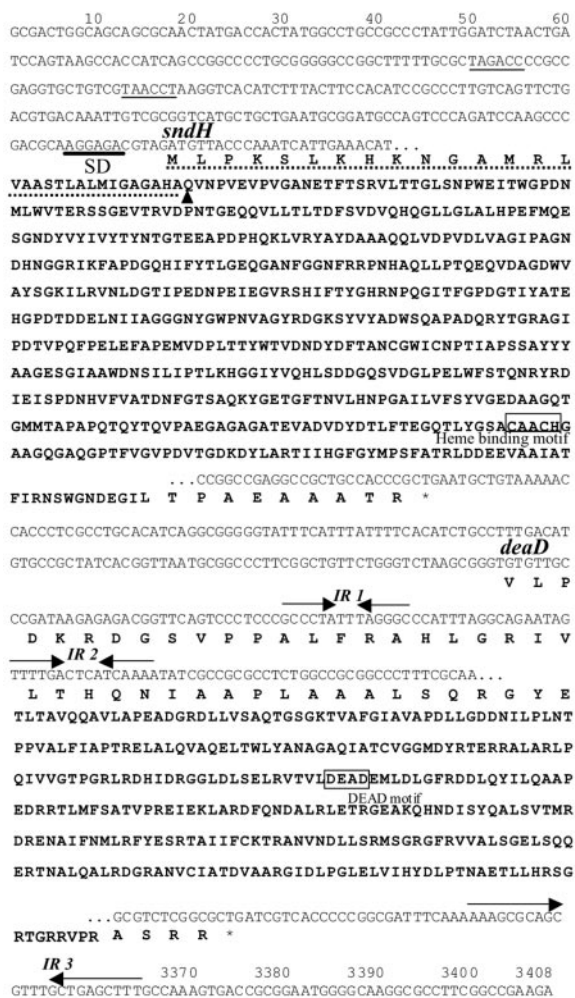


FIG. 3. Nucleotide and deduced amino acid sequences for the *sndH* region in *K. vulgare*. Thin lines indicate putative promoter sequences (TAGACC and TAACCT). The putative Shine-Dalgarno (SD) sequence is indicated by a boldface line. Inverted repeat sequences are indicated by pairs of inverted arrows (*IR 1*, *IR 2*, and *IR 3*). Stop codons are indicated by asterisks. For *sndH*, the signal sequence is indicated by a dashed line, the N terminus of the mature SNDH polypeptide is indicated by an arrowhead, and the heme-binding motif is enclosed in a box. For *dead*, the DEAD motif is enclosed in a box.

indicating the presence of only one copy of *sndH* in the *K. vulgare* genome.

Analysis of the 3,408-bp nucleotide sequence that was determined revealed the presence of two open reading frames, which corresponded to the *sndH* gene encoding the SNDH protein and another gene designated *dead* located downstream of the *sndH* gene (Fig. 3). It is likely that *sndH* and *dead* compose an operon, since no putative promoter regions or ribosome-binding sites were found just upstream of *dead*. The *sndH* gene consisted of 1,830 bp and encoded a 609-amino-acid polypeptide containing a putative 31-residue signal peptide. The *sndH*-encoded signal peptide contained (i) many hydrophobic residues, (ii) positively charged residues close to the N terminus, and (iii) an AXA motif as a signal cleavage sequence (Fig. 3). After the signal peptide, there was a QVN PVEVP sequence identical to the N-terminal amino acid se-

quences of the SNDHs. The putative Shine-Dalgarno sequence, AGGAGA, for the *sndH* gene was located 6 bp upstream of the initiation codon. There were three inverted repeat sequences, two short inverted repeat sequences (IR1 and IR2) in the N-terminal region of the *dead* gene and a long inverted repeat sequence (IR3) immediately downstream of the *dead* gene. Since IR3 contains AAAAA and TTT at both ends, it may function as a transcription terminator from both directions. Since SNDH has a heme-binding site in the C-terminal region (see below), the 75-kDa subunit is most likely a heme-bound mature SNDH protein. The 55-kDa subunit contained pyroQXNPVEVP as the N-terminal sequence and had a molecular mass of 49,601 Da. Since the molecular mass of a polypeptide from Gln¹ to Gly⁴⁵³ of SNDH has been calculated to be 49,592 Da, the 55-kDa subunit is thought to be SNDH(1-453), a C-terminal truncation. The absence of a heme-binding site in SNDH(1-453) is consistent with the difference spectrum (Fig. 2B).

A homology search of the deduced amino acid sequence encoded by the *sndH* gene was performed with the protein database. The SNDH amino acid sequence from residue Gln¹ to residue Glu⁴⁴⁶ exhibited 40% identity with the sequence of soluble quinoprotein glucose dehydrogenase (s-GDH) from *Acinetobacter calcoaceticus* (6, 9) (see Fig. S1 in the supplemental material). In the aligned region, 75% of the residues involved in PQQ, calcium, and glucose binding in the s-GDH reported by Oubrie (21) were conserved in SNDH. On the other hand, the remaining C-terminal region of the SNDH protein exhibited similarity (28 to 32% identity) to heme-containing proteins, such as *c*-type cytochrome, and cytochrome *f* from the cyanobacterium *Plectonema boryanum* (1) and the *cd*₁-type nitrite reductase NirS from a *Paracoccus denitrificans* strain (8). In the same region, a heme *c* binding motif (CAACH) was found at positions 530 to 534 of SNDH.

An additional open reading frame (1,101 bp) designated *dead* was found downstream of *sndH* (Fig. 3). The *dead* gene encodes a 367-amino-acid protein. The deduced DeaD protein exhibited 39 to 42% identity to ATP-dependent RNA helicases (5, 11, 22, 31) from bacteria.

Expression of the *sndH* gene. To express the *sndH* gene, we grew *E. coli* JM109 cells harboring pUCSNP4. The cell extract exhibited activity which converted L-sorbosone to L-AA and 2KGA when PQQ and CaCl₂ were added to the reaction mixture (Table 2). Without PQQ and CaCl₂, no activity was observed. This finding is consistent with the previous observation that *E. coli* is unable to synthesize PQQ in LB medium (18). Cell extract of *E. coli* JM109 harboring pUCSNP4 showed an enzyme activity of 29.3 mU/mg protein for L-AA production with the cofactors, while cell extract of JM109 cells harboring pUC18 did not show this enzyme activity. In contrast, cell extract of JM109 cells harboring pUC18 produced 2KGA (35.5 mU/mg protein), most likely due to an unidentified dehydrogenase(s) in *E. coli* (Table 2). The specific activity for 2KGA production in cell extract of JM109 cells harboring pUCSNP4 was 57.5 mU/mg protein.

Plasmids pUCSN2001, pUCSN2002, pUCSN2003, and pUCSN2004 (Fig. 4), which contained intact *sndH* genes or *sndH* genes with C-terminal deletions, were constructed and introduced into *E. coli* JM109 cells to analyze *sndH* expression. As shown in lane 2 of the upper gel in Fig. 5A, two activity

TABLE 2. Expression of intact and truncated *sndH* genes in *E. coli* and *K. vulgare* cells harboring various *sndH* segments

Plasmid	L-Sorbose-converting activity (mU/mg CFE protein) ^a		
	<i>E. coli</i> host ^b	<i>K. vulgare</i> GOMTR1SN::Km ^c	GOMTR1 ^c
pVSN109	9.80 ± 0.18 (3)	ND ^d	395.6 ± 22.3 (2)
pVSN114	7.23 ± 0.18 (3)	176.4 ± 57.7 (2)	251.8 ± 51.4 (2)
pVSN106	3.50 ± 0.28 (3)	135.6 ± 0.1 (2)	114.3 (1)
pVSN117	1.18 ± 0.08 (3)	29.8 ± 6.4 (2)	52.1 (1)
pVK100	0.00 ± 0.00 (3)	0.0 ± 0.0 (2)	75.0 (1)
pUCSN2004	137.2 (1)		
pUCSN2003	63.9 (1)		
pUCSN2002	0.0 (1)		
pUCSN2001	0.0 (1)		
pUCSNP4	29.3 (1)		
pUC18	0.0 (1)		

^a The values indicate the activities for conversion to L-AA and are means ± standard deviations. The numbers in parentheses are the numbers of experiments. For *E. coli* harboring pUCSNP4 and *E. coli* harboring pUC18 the activities for conversion to 2KGA were 57.5 and 35.5 mU/mg CFE protein, as determined by one experiment in each case.

^b *E. coli* DH5 α and JM109 cells were the hosts for pVSN and pUC plasmids, respectively. Each reaction mixture (100 μ l) contained 100 to 275 μ g CFE protein, 50 mM L-sorbose, 1.0 mM PMS, 1 μ M PQQ, 1 mM CaCl₂, and 25 mM potassium phosphate (pH 7.0) in a microtube (2 ml; Eppendorf, Germany).

^c Each reaction mixture (30 μ l) contained 5 to 20 μ g CFE protein, 50 mM L-sorbose, 0.05 mM PMS, and 25 mM potassium phosphate (pH 7.0) in a 96-well microplate (round-bottom type; Nalge Nunc International K.K., Japan).

^d ND, no data.

bands were observed in JM109 harboring pUCSN2004; the lower band corresponded to SNDH3, and the upper band did not correspond to any of the three SNDHs. A fourth activity band was also found in cell extract of *K. vulgare* DSM 4025 (Fig. 5A, lane 1). Although the fourth SNDH is expected to contain the 75-kDa subunit (see below), its exact nature is not known at this time. JM109 harboring pUCSN2003, which carried the truncated *sndH* gene to the position corresponding to Gly⁵³⁵, expressed only SNDH3 (lane 3). When more of the *sndH* coding sequence was deleted, no activity band was observed (lanes 4 and 5). In addition, JM109 harboring pUCSN2001 or pUCSN2002 showed no L-AA production from L-sorbose (Table 2). The cell extracts were subjected to Western blot analysis with polyclonal anti-SNDH antiserum

(Fig. 5A, lower gel). JM109 cells harboring pUCSN2004 produced an immunoreactive 75-kDa subunit as well as a 55-kDa subunit, while JM109 cells harboring pUCSN2003 produced only the 55-kDa subunit.

The *sndH* null mutation was introduced into *K. vulgare* GOMTR1, a spontaneous rifampin-resistant mutant of DSM 4025. pSUPS::Km was constructed by insertion of a Km cassette into the EcoRI site in *sndH* in suicide vector pSUP202. *sndH*::Km was introduced into the GOMTR1 genome by conjugal transfer of pSUPS::Km, followed by homologous recombination with wild-type *sndH*. Replacement of the *sndH* allele by *sndH*::Km in GOMTR1SN::Km was confirmed by PCR using *sndH*- and Km-specific primers. Analysis by native PAGE and activity staining of GOMTR1SN::Km cell extract showed that the *sndH* disruptant simultaneously lost the three SNDH bands (Fig. 5B, lane 2). The SNDH deficiency was also confirmed by an in vitro assay for activity converting L-sorbose to L-AA (GOMTR1SN::Km harboring pVK100) (Table 2). pVSN series plasmids (Fig. 4) that had either an intact *sndH* gene or an *sndH* gene with a C-terminal deletion were constructed. They were introduced into *sndH* disruptant GOMTR1SN::Km cells by conjugal transfer. The cell extracts of the recombinants were analyzed by native PAGE to clarify complementation of the deficiency of the SNDHs. The disruptants complemented with the intact *sndH* gene had the phenotype of the parent strain (Fig. 5B, lanes 3 and 4). The recombinant with pVSN117 encoding the truncated *sndH* gene to the position corresponding to Gly⁵³⁵ expressed only SNDH3 (Fig. 5B, lane 5). These results confirmed that the three SNDH proteins are products of the *sndH* gene and that the 55-kDa subunit has a C-terminal truncation.

We measured the production of L-AA from L-sorbose in cell extracts from *E. coli* and *K. vulgare* DSM 4025 *sndH* null and wild-type strains carrying intact or truncated *sndH* genes on the pVSN plasmids (Fig. 4). Extracts from the recombinant bacteria showed increasing activity for conversion of L-sorbose to L-AA in parallel with longer *sndH* DNA of the plasmid (Table 2). The presence of three inverted repeat sequences (IR1, IR2, and IR3) may have affected the stability of *sndH* mRNA in both *E. coli* and *K. vulgare*. In

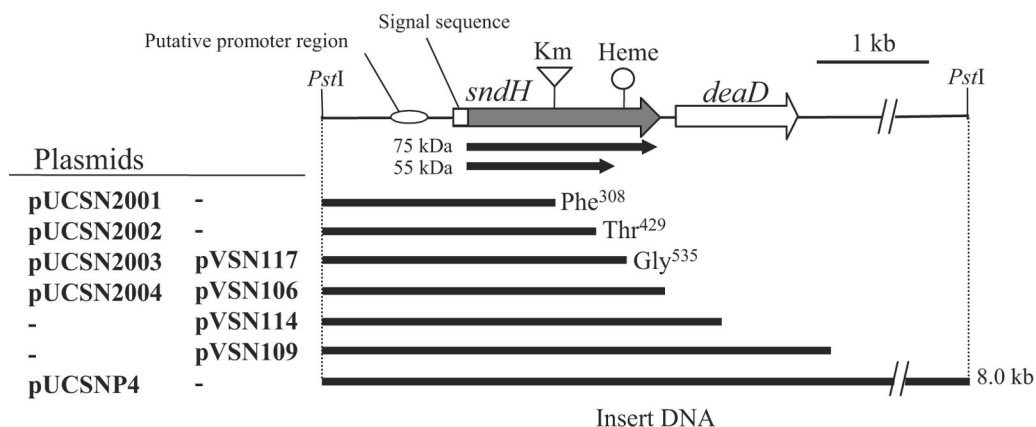


FIG. 4. Gene organization of *sndH* region in *K. vulgare*. Km, insertion site of the kanamycin resistance gene in GOMTR1SN::Km; Heme, heme c binding motif; solid arrows, coding regions of 55-kDa and 75-kDa subunits. Phe³⁰⁸, Thr⁴²⁹, and Gly⁵³⁵ indicate the C termini of the truncated SNDH molecules encoded in deletion plasmids.

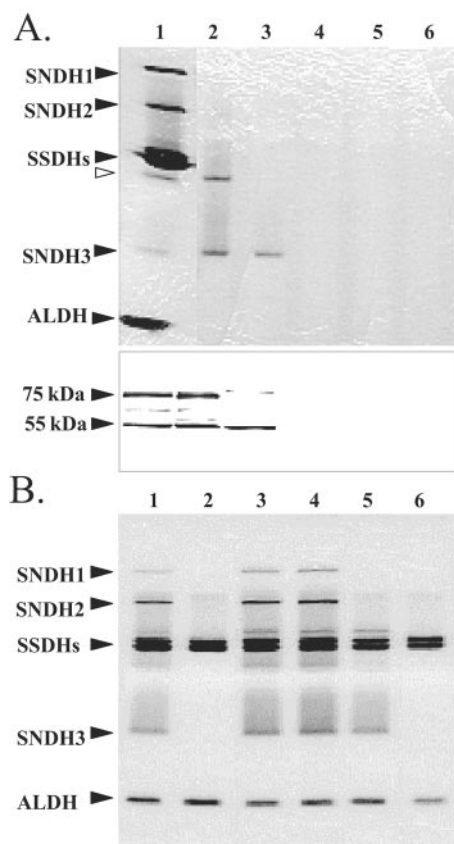


FIG. 5. Expression of intact and truncated *sndH* genes in *E. coli* (A) and *K. vulgare* (B). (A) *E. coli* cells harboring pUCSN series plasmids: activity staining (upper gel) and Western blot analysis (lower gel) of CFE proteins. For the upper gel, CFE proteins (15 μ g) were incubated with PQQ and CaCl_2 for 10 min at 25°C and subjected to native PAGE (10% polyacrylamide gel), followed by activity staining using L-sorbose. The locations of SNDH, SSDH, and ALDH proteins are indicated on the left. The open arrowhead indicates the position of the fourth SNDH band. For the lower gel, CFE proteins (5 μ g) were subjected to SDS-PAGE (12.5% polyacrylamide gel) and blotted onto a polyvinylidene difluoride membrane. The protein bands were detected using rabbit polyclonal anti-SNDH antiserum. The locations of 75-kDa and 55-kDa subunits are indicated on the left. Lane 1, *K. vulgare* DSM 4025; lanes 2 to 6, *E. coli* JM109 harboring pUCSN2004 (lane 2), pUCSN2003 (lane 3), pUCSN2002 (lane 4), pUCSN2001 (lane 5), and pUC18 (lane 6). (B) *K. vulgare sndH::Km* cells harboring pVSN series plasmids. CFE proteins (16 μ g) were subjected to native PAGE (10% polyacrylamide gel), followed by activity staining using L-sorbose. Lane 1, *K. vulgare* GOMTR1; lane 2, GOMTR1SN::Km; lanes 3 to 6, GOMTR1SN::Km harboring pVSN117 (lane 3), pVSN106 (lane 4), pVSN114 (lane 5), and pVK100 (lane 6).

K. vulgare sndH null or wild-type strains, introduction of *sndH* genes on pVK100 resulted in high SNDH activity.

DISCUSSION

Novel enzymes, SNDHs, were isolated from *K. vulgare* DSM 4025 and characterized. We found that the SNDHs could convert L-sorbose to both L-AA and 2KGA using artificial electron acceptors, such as PMS and DCIP, in vitro. Since 2KGA cannot be a substrate for these enzymes, L-AA is directly pro-

duced from L-sorbose enzymatically. This is the first report of a purified enzyme that forms L-AA from L-sorbose. In addition, the *sndH* gene encoding SNDH was cloned and characterized.

Three SNDH isozymes (SNDH1, SNDH2, and SNDH3) were purified. SNDH1 was proven to be quinohemoprotein and a homooligomer of 75-kDa subunits, SNDH2 was found to be a heterooligomeric complex of 75-kDa and 55-kDa subunits, and SNDH3 was found to be a homooligomer of 55-kDa subunits. The 75-kDa subunit was shown to be the product of *sndH*, which lacked the signal peptide and had a bound heme. The heme was thought to be a noncovalent prosthetic group because the 75-kDa subunit was not stained with 3,3',5,5'-tetramethylbenzidine when the protein was treated with SDS (data not shown). The 55-kDa subunit, lacking the C-terminal domain, was found to be derived from the 75-kDa subunit. All three SNDHs were detected on a native polyacrylamide gel when *K. vulgare* DSM 4025 cells were pretreated with a protease inhibitor cocktail (P1860; Sigma). The 55-kDa subunit was also produced in *E. coli* cells. These results suggest that production of the 55-kDa subunit has biological significance.

SNDHs catalyze the conversion of L-sorbose to L-AA at neutral pH and to 2KGA at alkaline pH. How is L-sorbose converted to two different products? These reactions may be explained by the tautomerization of L-sorbose. Seok et al. (25) described the tautomerization of L-xylosone, a five-carbon analogue of L-sorbose. L-Xylosone was shown to have several tautomeric interconversions by gas-liquid chromatography-mass spectrometry. Seok et al. proposed a furanose form of 1,4-lactone for L-xylosone in their scheme. It is thought that L-sorbose also forms a furanose structure, as well as a pyranose structure from intramolecular cyclization. We propose the hypothetical metabolic pathways for L-sorbose shown in Fig. 6. In this scheme, L-sorbose is converted to L-AA by SNDHs with dehydrogenation at the C-1 position (reaction 3a) when the substrate is the furanose form (1,4-lactone form). On the other hand, when L-sorbose is the pyranose form (2,6-lactone form), it is converted to 2KGA by SNDHs (reaction 3b) with dehydrogenation at the C-1 position after hydration of the aldehyde group in water.

The tertiary structure of s-GDH of *A. calcoaceticus* was solved previously (20), and the monomer was found to have a super barrel structure consisting of six four-strand antiparallel β -sheets (see Fig. S1 in the supplemental material). It is possible that the N-terminal domain of SNDH has a structure similar to that of s-GDH, as indicated by the high sequence identity. For the most part, the residues involved in cofactor and substrate binding proposed for s-GDH (21) are conserved in SNDH. In particular, the PQQ ligands Arg²²⁸, Asn²²⁹, Arg⁴⁰⁶, and Arg⁴⁰⁸ in s-GDH are invariant in SNDH (see Fig. S1 in the supplemental material). Since SNDH has an extended cytochrome domain, this enzyme has a composition similar to that of the quinohemoprotein type II alcohol dehydrogenases (type II ADH) from *Comamonas testosteroni* (12) and *Pseudomonas putida* (32). However, both the dehydrogenase domain (Gln¹ to Glu⁴⁴⁶) and the cytochrome domain (Asp⁴⁴⁷ to Arg⁵⁷⁸) of SNDH have lower levels of similarity (<13% identity) with type II ADH from *P. putida*. A putative PQQ binding motif proposed for membrane-bound quinoproteins and soluble methanol dehydrogenases (2) was not found

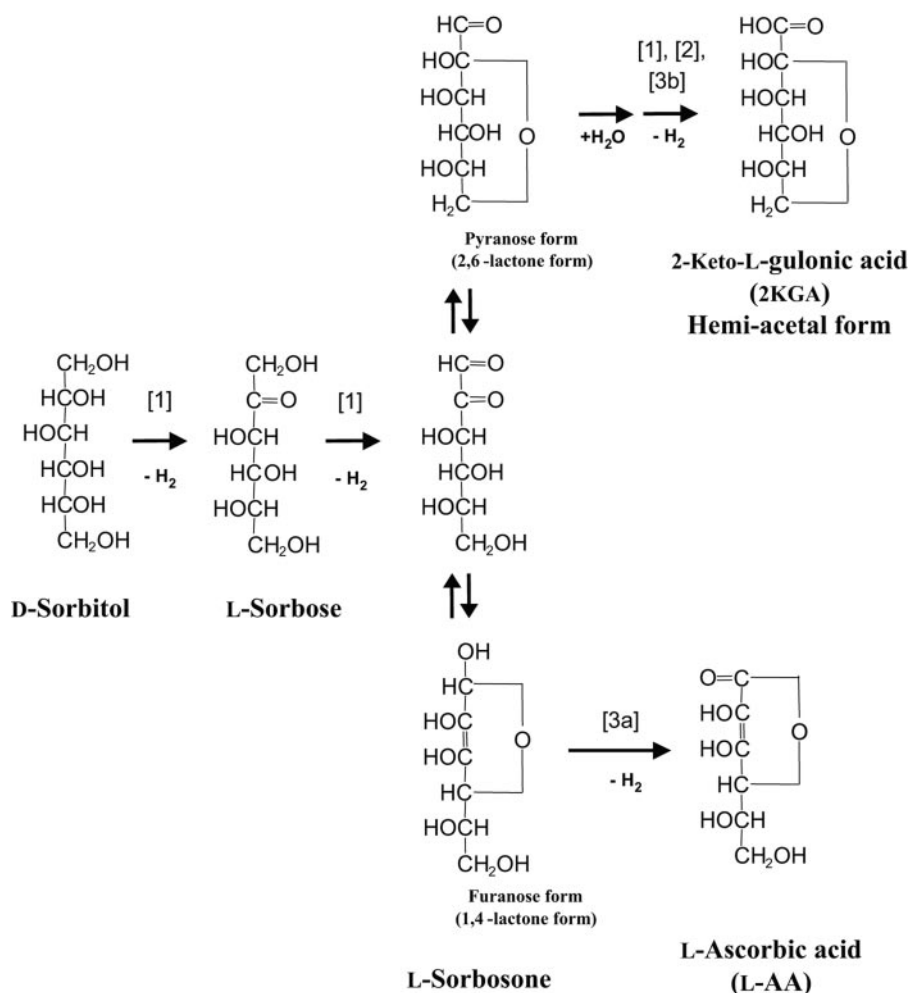


FIG. 6. Proposed metabolic pathways from D-sorbitol to L-AA and 2KGA in *K. vulgare* DSM 4025. Reaction 1 is catalyzed by SSDH (3); reaction 2 is catalyzed by ALDH (15); and reactions 3a and 3b are catalyzed by SNDHs.

in SNDH. We concluded that SNDH consists of an N-terminal dehydrogenase domain similar to s-GDH and the C-terminal cytochrome *c* domain, which is a novel quinohemoprotein.

The presence of a signal peptide in SNDHs suggests that the proteins are located in the periplasmic space. Several related enzymes, including s-GDH and type II ADH, are also known to have a signal peptide. In bacterial cells expressing high potential sugar-oxidizing activities, location of the enzymes responsible for oxidation in the periplasmic space would be advantageous. These systems may provide effective hydride transfer from the substrates to the respiratory system deployed on membranes, like ADH and GDH; the SNDH proteins may also be involved in this electron transfer. Although the natural electron acceptor for SNDH in the cell was not identified, it is expected that the enzymes transfer electrons from the substrate via PQQ and heme *c* eventually to the membrane-bound cytochrome *c* oxidase, as reported previously for type II ADHs (33).

In this study, valuable enzymes, SNDHs that connect the pathway from primary sugars to L-AA in *K. vulgare*, were identified. We expect that further analysis of the SNDHs will con-

tribute to the development of new economical L-AA production processes.

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