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2,5-Diketo-gluconic acid reductase from *Corynebacterium glutamicum*: Characterization of stability, catalytic properties and inhibition mechanism for use in vitamin C synthesis

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ABSTRACT

2,5-Diketo-D-gluconic acid (2,5-DKG) reductase is an NADPH-dependent, monomeric aldo-keto reductase (AKR) which catalyzes the reduction of 2,5-DKG to 2-keto-L-gulonic acid (2-KLG) – the immediate precursor of vitamin C. The reaction catalyzed by 2,5-DKG reductase is attractive to bypass several chemical steps and produce vitamin C biocatalytically. In a screening of 22 bacterial strains, nine 2,5-DKG reductase producing bacterial strains were found. The gene of *Corynebacterium glutamicum* 2,5-DKG reductase was cloned and overexpressed in *Escherichia coli*. By batch fermentation 409 mg L^{-1} of 2,5-DKG reductase with a C-terminal His_6-tag were obtained. The purified 2,5-DKG reductase was characterized in detail. The enzyme is most active in a pH range from 5.0 to 8.0 and its stability is high at temperatures below 35 °C. Catalytic constants for 2,5-DKG and NADPH were determined and a weak inhibition by the product 2-KLG was found. 2,5-DKG reductase activity is strongly inhibited by the common process ions Mg^{2+}, Ca^{2+}, SO_4^{3-} and Cl^-, which suggests that these should be avoided in the process. The inhibition mechanism for Cl^- was elucidated. It is a competitive inhibitor with respect to NADPH and a noncompetitive inhibitior with respect to 2,5-DKG.

1. Introduction

The bacterial enzyme 2,5-diketo-D-gluconic acid reductase (2,5-DKG reductase; 2,5-didehydrogluconate reductase; EC 1.1.1.274) is one of more than 140 members of the aldo-keto reductases (AKRs), an enzyme superfamily of NAD(P)(H)-dependent oxidoreductases [1,2]. This enzyme catalyses the stereo specific reduction of 2,5-diketo-D-gluconic acid (2,5-DKG) at position C-5 to 2-keto-L-gulonic acid (2-KLG) [3], which is an intermediate that can be transformed into L-ascorbic acid (vitamin C) by a single chemical step [4].

The first microorganisms available for conversion of 2,5-DKG to 2-KLG were isolated from soil and sewage by Sonoyama and colleagues in the 1970s [5,6]. These 2-KLG producing strains belong to the *Brevibacterium*, *Arthrobacter*, *Micrococcus*, *Staphylococcus*, *Pseudomonas*, *Bacillus* and *Corynebacterium* genera. In 1987, the conversion of 2,5-DKG to 2-KLG by *Corynebacterium* sp. was identified as a single catalytic step of 2,5-DKG reductase in the cytosol [7]. To date, only a few enzymes with 2,5-DKG reductase activity have been biochemically characterized. Those include two native DKGRs from a species of *Corynebacterium* (2,5-DKG reductase A; AKR5C and 2,5-DKG reductase B; AKR5D) [7,8], two homologous expressed 2,5-DKG reductase from *Escherichia coli* (YqhE and YafB) [9,10] and two heterologous expressed 2,5-DKG reductases from uncultured microbes. Two heterologous expressed 2,5-DKG reductases from uncultured microbes have been found by screening environmental DNA expression libraries [11]. Structurally, only 2,5-DKG reductase A from *Corynebacterium* sp. [12–14] and its quadruple mutant [15] have been studied. Native 2,5-DKG reductase A from *Corynebacterium* sp. is a monomeric enzyme (about 34 kDa) composed of eight α-helices and eight parallel β-strands (TIM barrel; $(\alpha/\beta)_8$), similar to most microbial AKRs [1,12]. The reduced form of pyridine nucleotide NADP(H) is bound to the C-terminal face of the barrel. The absence of a canonical Rossman fold in active site set AKRs apart from numerous dehydrogenases [16,17]. Mutation studies of residues in the coenzyme binding site and substrate binding pocket in the apo and coenzyme-bound form of 2,5-DKG reductase show that binding of NADPH causes communicated and coordinated structural changes into these regions [14].

2,5-DKG reductase is of high interest for the biocatalytic production of the key intermediate 2-KLG by the 2,5-diketo-D-gluconic acid pathway from D-glucose via D-gluconate, 2-keto-D-gluconate and 2,5-diketo-D-gluconate. Vitamin C can be obtained through transformation and refining of 2-KLG [18]. Sonoyama et al. [19]

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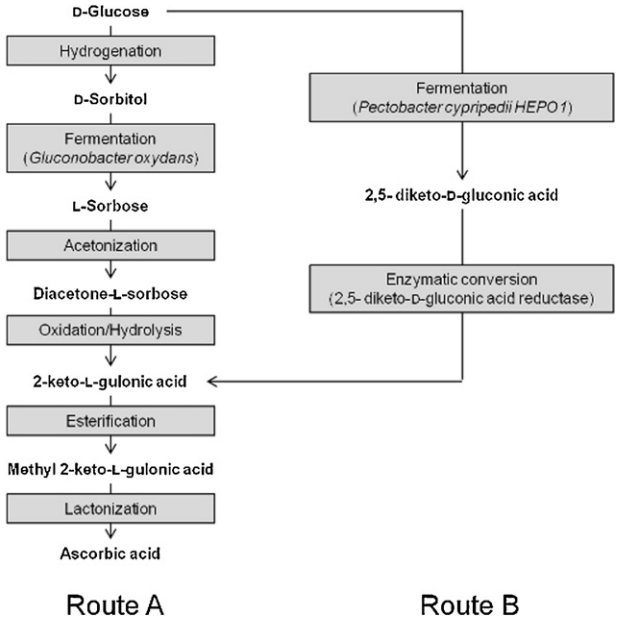

Fig. 1. Synthetic routes to ascorbic acid. Route A depicts the classical Reichstein process [26,27], Route B shows the process for utilizing the 2-KLG intermediate produced by 2,5-DKG-reductase [28].

invented a two-stage fermentation process for 2-KLG production where glucose is oxidized to 2,5-DKG by a mutated *Erwinia* sp. and is then reduced to 2-KLG by a mutant strain of *Corynebacterium* sp. The second step reaction is catalyzed by NADPH dependent 2,5-DKG reductase. A tandem fermentation process to produce 2-KLG from gluconic acid by using co-immobilized cells of *Gluconobacter oxydans* and *Corynebacterium* sp. has also been suggested [20]. Also the genetically engineered *Erwinia* strains; *Erwinia herbicola* [3] and *E. citreus* [21], which naturally accumulate 2,5-DKG from D-glucose have been employed. The gene encoding for 2,5-DKG reductase was cloned from *Corynebacterium* sp. into the above mentioned *Erwinia* strains, allowing an elegant one-organism fermentation of 2-KLG directly from D-glucose. According to Powers [22], the transport of 2,5-DKG into, and the diffusion of 2-KLG out of the 2-KLG synthesizing cells, appear to be the rate-limiting steps. Based on this, Genencor established an in vitro biocatalytic four steps method to produce 2-KLG from D-glucose [23,24]. Four enzymes: $NADP^+$ dependent glucose dehydrogenase (GDH) from *Thermoplasm acidophilum*, NADPH dependent 2,5-DKG reductase from *Corynebacterium* sp., gluconate dehydrogenase and 2-keto-D-gluconate dehydrogenase (both from permeabilized, modified *Pantoea citrea* cells with glucose dehydrogenase activity) are involved in the continuous conversion of D-glucose. The first two, soluble enzymes are exogenously added and regenerate the coenzyme in situ.

Nowadays a remarkable part of vitamin C industrial production is performed in a two-step fermentation process [4,25,26], but the traditional Reichstein process [27] which involves several environmentally hazardous chemical and energy consuming steps is still utilized for vitamin C synthesis (Fig. 1, process route A). In our previous published work the synthesis of 2-KLG from D-glucose was established (process route B) [28]. First, D-glucose is converted by *Pectobacter cypripedii* strain HEPO1 (DSMZ 12393) into 2,5-DKG, which is then enzymatically converted with NADPH-dependent 2,5-DKG reductase from *Corynebacterium glutamicum* to 2-KLG. NADPH is regenerated in situ by GDH from *Bacillus* sp. and D-glucose in the second biocatalytic step. Using this bi-enzymatic system, 2,5-DKG is completely reduced to 2-KLG. Here, we describe the screening and cloning of a 2,5-DKG reductase from *C. glutamicum* DSM 20301, its expression in *E. coli* and biochemical characterization in regard to process relevant properties of the recombinant enzyme. A detailed kinetic study of the catalytic mechanism of 2,5-DKG reductase and its inhibition by cations and anions provides mechanistic insights for further enzyme and process engineering.

2. Materials and methods

2.1. Materials

Salts, acids and bases for enzyme assays and media preparation were purchased from commercial suppliers at the highest level of purity possible. Media components were obtained from Sigma–Aldrich, Roth and Merck. 2,5-Diketo-D-gluconic acid (2,5-DKG) was produced by fermentation of *P. cypripedii* as previously described [28] and isolated from the culture broth by methanol precipitation. It was further purified by liquid chromatography, using isocratic elution with ultrapure water on Amberlite CG120-II (Sigma–Aldrich) in the calcium form. The purity and concentration of 2,5-DKG was determined by HPLC. Anhydrous 2-KLG was purchased from Hofmann-La Roche.

2.2. Microorganisms, plasmids and media

Bacterial strains used for screening experiments are given in Table 1 and were obtained from DSMZ (Braunschweig, Germany). The freeze-dried cultures were revived following the suppliers recommendations and periodically subcultured on recommended media containing 15 g L^{-1} agar. The media used and their composition are given in the supplemental information (Tables S1 and S2). The *E. coli* strains TOP10 and BL21/DE3 (Invitrogen, Carlsbad, CA) were used for cloning and expression. The construction of the pET-21d/*dkr* vector, which expresses the His-tagged 2,5-DKG reductase gene from *C. glutamicum* under the control of the T7 promoter, has been described recently [28]. The integrity of the construct was confirmed by DNA sequence analysis (VBC-Biotec, Vienna, Austria). The sequence of *dkr* was deposited in the GenBank database; accession number: JQ407590. To optimize the production of recombinant 2,5-DKG reductase by *E. coli* BL21 (DE3), the following media were screened: Luria broth [29], M9-medium (8.5 g L^{-1} $Na_2HPO_4{\cdot}2H_2O$, 3.0 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NaCl and 1.0 g L^{-1} NH_4Cl supplemented with 20 g L^{-1} glucose, 10 mL of a 1 M $MgSO_4$ (final concentration = 10 mM) and 0.5 mL of a 1 M $CaCl_2$ solution (final concentration = 0.5 mM)) and MCHGly-medium (M9-medium supplemented with 10 g L^{-1} casein and 10 g L^{-1} glycerol instead of glucose). For M9- and MCHGly-media the M9-salt solution was prepared in a 10 times concentrated solution and autoclaved separately.

2.3. Screening for 2,5-DKG reductase producing organisms

For shaking flasks cultivation experiments, 25 mL of the appropriate medium was inoculated with a single colony from agar-plates and cultivated overnight under the recommended conditions on an orbital shaker (eccentricity = 2.5 cm, rotational frequency = 140 rpm). One mL of this starter culture was used to inoculate 100 mL of the production stage medium supplemented with 10 g L^{-1} 2,5-DKG by using a 0.2 μm filter for sterilization. Samples were taken aseptically at 24, 48 and 72 h after inoculation for measuring enzymatic activity and protein concentration. After reaching the maximum cell density (measured by OD_{600}) the biomass was harvested by centrifugation at 10,000 × *g* for 10 min. The cells were resuspended by adding 3 mL of 50 mM Bis–Tris buffer, pH 6.4, containing 1 mM phenylmethylsulfonylfluoride (PMSF) to the centrifuged cells for homogenization by a French Press. Using the spectrophotometric assay (see Section 2.7) with 50 mM Bis–Tris buffer, pH 6.4, 2,5-DKG reductase activity was determined from this lysate (measurements were performed in triplicates and are reported as mean values and their standard deviation) and 2,5-DKG reductase activity was verified by small-scale conversion experiments of 2,5-DKG to 2-KLG. To that purpose, the lysate containing approx. 1 U of 2,5-DKG reductase activity was supplemented with 25 mM 2,5-DKG and an equimolar amount of NADPH as coenzyme in 1 mL 50 mM Bis–Tris buffer, pH 6.4, for 24 h at 25 °C. These samples were analyzed with HPLC and 2-KLG production was determined by comparison to standards. To further confirm the identity of the accumulated product, the formed 2-KLG was converted to the final product L-ascorbic acid. Therefore, a reaction mixture containing 0.5 mL of 2-KLG solution and 0.5 mL H_2SO_4 (98%) was incubated for 30 min at 100 °C. The reaction was stopped by adding 10 mL of cold, distilled water and the amount of L-ascorbic acid formed by chemical conversion was determined using a colorimetric test kit from R-Biopharm (Darmstadt, Germany) following the manufacturer's recommendations.

2.4. Expression of 2,5-DKG reductase in E. coli

Media optimization experiments were carried out in baffled shaking flasks with Luria broth, M9-medium and MCHGly-medium containing 0.1 mL L^{-1} antifoam and 0.1 g L^{-1} ampicillin. To induce *dkr* gene expression, isopropyl-β-D-thiogalactopyranoside (IPTG) or lactose in different concentrations were added: IPTG: 0.01 mM, 0.1 mM, 1 mM; lactose: 1 g L^{-1}, 5 g L^{-1}, 10 g L^{-1}. Induction was started at an OD_{600} of 0.5–0.7. Cultures were grown at temperatures ranging from 25 °C

Table 1
Bacteria screened for 2,5-DKG reductase activity.

Strain	Strain identifier	Cultivation time (hours)	Maximum OD_{600}	Activity[a] ($U\,L^{-1}$)	2-KLG ($mg\,mL^{-1}$)[b]	L-Ascorbic acid[c]
Acetobacter hansenii	DSMZ 5602	42	9.65	32 ± 2	1.5	+
Acetobacter lovaniensis	DSMZ 4491	47	4.62	750 ± 311	ND	
Arthrobacter agilis	DSMZ 20550	47	1.54	31 ± 18	ND	
Arthrobacter roseus	DSMZ 14508	47	1.02	19 ± 1	ND	
Bacillus alvei	DSMZ 29	42	3.76	8 ± 1	ND	
Bacillus funiculus	DSMZ 15141	42	7.22	64 ± 8	0.9	+
Brevibacterium imperiale	DSMZ 20530	42	1.1	19 ± 1	0.4	+
Brevibacterium testaceum	DSMZ 20166	24	4.88	30 ± 17	0.8	+
Corynebacterium barkeri	DSMZ 20145	24	4.81	48 ± 31	1.0	+
Corynebacterium glutamicum	DSMZ 20301	24	8.65	186 ± 34	2.0	+
Corynebacterium ilicis	DSMZ 20138	24	8.84	360 ± 85	ND	
Gluconobacter asaii	DSMZ 7148	47	2.03	830 ± 127	0.5	+
Kocuria kristinae	DSMZ 20032	24	7.11	53 ± 4	ND	
Kocuria varians	DSMZ 20033	24	12.88	26 ± 2	0.2	+
Micrococcus lylae	DSMZ 20315	24	13.45	12 ± 4	ND	
Pantoea stewarti subsp. stewartii	DSMZ 30176	42	6.12	22 ± 3	ND	
Pectobacterium chrysanthemi	DSMZ 30177	42	7.99	84 ± 25	ND	
Pimelobacter jenseii	DSMZ 20641	42	5.24	10 ± 6	ND	
Pseudomonas cichorii	DSMZ 50259	42	8.6868	235 ± 7	0.7	+
Pseudomonas syringae	DSMZ 1241	42	8.85	220 ± 14	ND	
Staphylococcus muscae	DSMZ 7068	24	8.43	14 ± 4	ND	
Staphylococcus pulvereri	DSMZ 9930	24	5.74	17 ± 4	ND	

[a] Activity was measured with spectrophotometric assay.
[b] ND. . .2-KLG peak not detected by HPLC.
[c] Detection of L-ascorbic acid colorimetric test kit from R-Biopharm (Darmstadt; Germany).

to 37 °C. After harvest and homogenization of the biomass, the 2,5-DKG reductase activity and protein concentration were measured in the supernatant. A large-scale batch cultivation of *E. coli* BL21 Star (DE3) transformants bearing the *dkr* gene was carried out in a 42-L stirred-tank bioreactor (Applikon, Schiedam, Netherlands) filled with 30 L of MCHGly medium containing 0.1 $g\,L^{-1}$ ampicillin. The cultivation was started by inoculation with shaking flask cultures (5% v/v, OD_{600} ~0.5). For induction lactose was added to a final concentration of 5 $g\,L^{-1}$ after an OD_{600} of 0.8 was reached. The pH was maintained at pH 6.5 by automatic titration with sterile NaOH and the concentration of dissolved oxygen (DO) was kept at 20% air saturation by sparging with filtered air (0–20 $L\,min^{-1}$) and adjusting the stirrer velocity (200–800 rpm). The cells were cultivated at 37 °C. Shortly before induction of *dkr* gene expression the temperature was reduced to 25 °C. All setpoint parameters were controlled by the Bio Controller ADI 1030 (Applikon). Samples were taken at 1-h interval to measure OD_{600}, dry cell weight (DCW), glycerol and lactose concentration, protein concentration and 2,5-DKG reductase activity.

2.5. Protein purification

An ÄKTA Explorer 100 (GE Healthcare) was used for 2,5-DKG reductase purification from *E. coli* cell-free lysate. An XK 26/20 column packed with 60 mL Ni-charged Chelating Sepharose Fast Flow (particle size 45–165 μm, GE Healthcare) was pre-equilibrated with 10 column volumes of loading buffer (0.02 M sodium phosphate, pH 7.4 containing 0.25 M NaCl and 0.01 M imidazole). Then the crude extract was applied to the column at a linear flow rate of 1.06 cm min^{-1}. Unbound proteins were washed out with loading buffer until a constant UV (280 nm) and conductivity baseline were obtained. The enzyme was eluted by a linear gradient from 0% to 100% of elution buffer containing 0.5 M NaCl and 0.5 M imidazole over 10 column volumes and the eluate was collected in fractions of 2.5 mL. Fractions with the highest specific activity were pooled, concentrated and diafiltrated with Amicon Ultra Centrifugal Filter Device with a 10 kDa cut-off membrane (Millipore; Billerica, MA, USA). The concentrated, imidazole free enzyme solution in 50 mM Bis–Tris buffer, pH 6.4 was diluted to a protein concentration of 20 $mg\,mL^{-1}$ and stored at 4 °C until further use.

2.6. Protein analysis

The protein concentration was determined by the dye-binding method of Bradford [30], using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. SDS-PAGE as well as isoelectric focusing was carried out with a PhastSystem unit (Pharmacia LKB/GE Healthcare Life Sciences, Uppsala, Sweden) according to the manufacturer's instructions. SDS-PAGE was performed with precast gels (PhastGel Gradient) with continuous acrylamide gradients in the ranges 8–25%, using the High Precision Dual Color (10–250 kDa, Bio-Rad) as molecular weight marker. Isoelectric focusing (IEF) was performed with precast homogeneous (5% T, 3% C) polyacrylamide PhastGel IEF gels with immobilized pH gradient (pH 3.0–9.0). Marker-proteins used to determine *pI* values were in a range of 3.5–10.7 (Serva Electrophoresis, Heidelberg, Germany). Bands on the SDS-PAGE and IEF Gels were visualized by silver staining according to the manufacturer's instructions.

2.7. Measurement of enzymatic activity

2,5-DKG reductase activity was determined spectrophotometrically by following the decrease of NADPH absorbance at 340 nm (ε_{340} = 6.22 $mM^{-1}\,cm^{-1}$) and 25 °C for 3 min. The assay mixture contained buffer (50 mM ammonium acetate buffer pH 6.4, unless otherwise stated), 8.256 mM 2,5-DKG, 0.2 mM NADPH and a suitable activity of 2,5-DKG reductase in a final volume of 1 mL in cuvettes of 1 cm optical path length. The substrate and co-substrate stock solutions were freshly prepared each day. A volumetric activity of 6.4 $U\,L^{-1}$ was determined to be the lower limit of reliable quantification. One unit of 2,5-DKG reductase activity was defined as the amount of enzyme necessary for reduction of 1 μmol min^{-1} of 2,5-DKG, which corresponds to the production of 1 μmol of $NADP^+$ per min under assay conditions. At high substrate concentrations the measurement of the enzymatic activity by absorbance was unreliable by spectral interference and was therefore verified by a fluorometric assay. This assay, which is not interfered by high concentrations of 2,5-DKG, is based on the oxidation of the fluorescent coenzyme species NADPH (Ex = 380 nm, Em = 480 nm). The optimal excitation and emission wavelengths were determined in ammonium acetate buffer pH 6.4 and in the presence of 2,5-DKG. The kinetics of purified enzyme was assayed in 50 mM ammonium acetate buffer at pH 6.4 and at 25 °C in a final volume of 200 μL. Assays were carried out in a VARIAN Cary Eclipse instrument and 3 mm path length quartz cuvettes. To determine steady-state kinetic constants the 2,5-DKG concentration and the NADPH concentration were varied within 2–100 mM and 0.002–0.3 mM, respectively. The data obtained for the forward reaction in the absence of product were fitted to the kinetic rate equation for an irreversible, ordered bi-bi mechanism [31]. Product inhibition by 2-KLG and the oxidized coenzyme $NADP^+$ were also determined. For these experiments one substrate concentration was varied at several fixed concentrations of a single product, the other substrate concentration was kept constant at a non-saturated concentration according to Segel [31]. All measurements were performed in triplicates and nonlinear least squares regression of unweighted averages of data was calculated with SigmaPlot 12 (Systat Software, San Jose, and CF, USA). Experimental data are shown as double-reciprocal Linewaever–Burk plots. Global fits of the data to various single-substrate single-inhibitor models were performed using the Enzyme Kinetics Wizard of SigmaPlot 12. All available inhibition models, including full competitive, full noncompetitive and hyperbolic mixed-type inhibition [31] were evaluated and ranked according to R^2 values and the Akaike Information Criteria (AIC).

2.8. pH dependence of 2,5-DKG reductase activity

The effect of pH on 2,5-DKG reductase activity was determined in the range of pH 4.5–9.5, using the following 50 mM buffers: sodium acetate (pH 4.5–6.5), ammonium acetate (pH 4.5–6.5), sodium citrate (pH 4.5–6.5), Bis–Tris (pH 6.0–7.5), MES (5.7–6.7), MOPS (6.7–7.9), tricine (7.7–8.7) and borate (pH 7.5–9.5). Measurements were performed in triplicates using the spectrophotometric assay.

2.9. Effect of ions on enzymatic activity

To detect inhibitors or activators of 2,5-DKG reductase activity, enzyme samples were assayed in the presence of various salts. The enzymatic activity was measured by the photometric assay at 25 °C and varying amounts of monovalent and divalent cations in the chloride, sulphate or acetate form. Fitting curves were calculated by nonlinear least square regression to a single exponential function (SigmaPlot 12). The inhibition kinetics of NaCl was examined in detail. Therefore, to assess the mode of inhibition with respect to NADPH, NaCl concentrations were varied (0–100 mM) in the presence of sub-saturating concentration of 2,5-DKG (23 mM) and increasing concentration of NADPH (0.002–0.12 mM). The effect of NaCl on 2,5-DKG turnover was measured in the presence of 0.2 mM NADPH and varying amounts of salts (0–300 mM) and 2,5-DKG (1.5–60 mM), respectively. As explained above, by fitting the initial reaction rates to the to the corresponding equations of single-substrate single-inhibitor models using the SigmaPlot software the observed data were analyzed using nonlinear least-square regression.

2.10. Temperature dependence of activity and stability

The temperature dependence of enzyme activity was determined by incubating the reaction mixture (1 mL) that consisted of 50 mM ammonium acetate buffer, pH 6.4 and 8.256 mM 2,5-DKG at temperatures from 20 °C to 70 °C (5 °C intervals) by starting the reaction with an aliquot of the enzyme and 20 μL of NADPH stock solution. For the determination of the energy of activation (E_a) Arrhenius plots was prepared from the initial rates obtained in the temperature experiments. For thermal stability experiments, enzyme solution (2 U mL^{-1}) in 50 mM ammonium acetate buffer, pH 6.4 was incubated at various temperatures from 4 °C to 50 °C. The enzyme activity was measured at specified intervals using the standard spectrophotometric assay. The inactivation of enzyme was analyzed assuming first-order kinetics, $A = A_0 \times e^{kt}$, where A and A_0 stand for enzyme activity at various time points (t) and initial enzyme activity, respectively. The inactivation is represented by k_{in}. The half-life of the enzyme activity $t_{1/2}$, defined as the time needed for the activity of enzyme to drop to one-half of the original value, was determined as $t_{1/2} = \ln 2/k_{in}$.

2.11. High performance liquid chromatography

2,5-DKG and 2-KLG were separated and quantified by HPLC with UV detection at a wavelength of 210 nm (PDA-100 Photodiode Array Detector), using a Dionex DX 500 system (Dionex; Sunnyvale, CA, USA) and a Luna Amino column (Phenomenex, Torrance, California) at 25 °C with 15 mM $NH_4H_2PO_4$ as eluent (1 mL min^{-1}).

3. Results and discussion

3.1. Screening

Twenty-two bacterial strains were screened in shaking flask cultures for the ability to reduce 2,5-DKG to 2-KLG (Table 1). Different optimal growth conditions (22–37 °C, 6 different media, pH 6.0–7.3, for detailed information see: Supplementary Tables S1 and S2) resulted in different specific growth rates. Therefore, the cultivation time to reach maximum cell densities was determined in preliminary experiments (20–50 h) and three classes of organisms were defined, which were harvested after 24, 42 or 47 h of cultivation. Still, the obtained biomass differed vastly between OD_{600} = 13.5 after 24 h and OD_{600} = 1.0 after 47 h. The enzymatic activity was measured in the lysates of the harvested cells and no correlation between biomass and activity was found. All tested strains showed at least minor activity, but overlapping substrate specificities of reductases and dehydrogenases in microbial cells [1] rendered these low measured activities ambiguous. To rescreen the obtained results, unambiguous conversion experiments of 2,5-DKG to 2-KLG and further to L-ascorbic acid were performed with the crude extracts. HPLC analysis shows that only 9 of the 22 strains actually formed the desired product (2-KLG). It was found that even some strains with high enzymatic activities did not produce 2-KLG in the small-scale batch conversions, which verifies the assumption of interfering reductase/dehydrogenase activities. The presence of a 2,5-DKG reductase was confirmed for the genera *Acetobacter*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Gluconobacter*, *Kocuria* and *Pseudomonas*. The highest amount of 2-KLG was detected in the crude extract of *C. glutamicum* (2 mg mL^{-1}), which was the reason for its overexpression and characterization. Good results were also obtained with *C. barkeri* (1 mg mL^{-1}), *Acetobacter hansenii* (1.5 mg mL^{-1}) and *Bacillus funiculus* (0.9 mg mL^{-1}). In addition to HPLC, the identity of accumulated 2-KLG was confirmed by conversion to L-ascorbic acid.

3.2. Heterologous overexpression of dkr from C. glutamicum

The 2,5-DKG reductase coding gene from *C. glutamicum* was amplified by a single PCR, cloned and expressed in *E. coli* BL21 (DE3), the full procedure is described in a patent [28]. The nucleotide sequence of full length *dkr* (GenBank accession number: JQ407590) contains an open reading frame of 846 bp, encoding 281 amino acids residues and a stop codon, and has 51.4% content of G + C base pairs. The amino acid sequence of *C. glutamicum* 2,5-DKG reductase shares a sequence identity of 74% with putative 2,5-DKG reductase from *C. efficiens* YS-314 (GenBank accession number NP_738878). 62% with 2,5-DKG reductase A from *Corynebacterium* sp. ATCC 31090 (GenBank accession number P06632), 40% with 2,5-DKG reductase B from the mutant strain *Corynebacterium* sp. SHS752001 (P15339), 51% with 2,5-DKG reductase A from *E. coli* (YghZ; AKR14A1; Q46857) and 42% with 2,5-DKG reductase B from *E. coli* (YafB; P30863). The low sequence identities even within one genus suggest that 2,5-DKG reductases from *Corynebacterium* spp. might differ in their physical and catalytic properties.

For overexpression in *E. coli* an expression vector containing *dkr* under the control of a lactose- or IPTG-inducible T7 promoter was fused in frame with a C-terminal His_6-tag. The resulting vector was transformed into the production strain BL21 Star (DE3), and ampicillin-resistant clones were tested for the presence of 2,5-DKG reductase activity by spectrophotometric assays and batch conversion of 2,5-DKG using the raw extract after small-scale cultivation. The best clone was selected for enzyme production. To maximize the heterologous expression, different media, temperatures and inducer concentrations were evaluated in shaking flasks. The maximum volumetric activity for all three media was obtained at 25 °C and the highest 2,5-DKG reductase activity (0.1 U mg^{-1}) produced by *E. coli* BL21 Star (DE3) carrying pET-21d/*dkr* was obtained after 26 h of induction with 1 g L^{-1} lactose in MCHGly medium at 25 °C. The production of 2,5-DKG reductase under strictly controlled growth conditions, was performed in a 30-L stirred bioreactor (Supplementary Fig. S1). The recombinant enzyme production was performed in MCHGly medium at 25 °C and at an oxygen concentration of 20% air saturation. Induction with 5 g L^{-1} lactose was started at the beginning of the exponential growth phase of the inoculum (OD_{600} = 0.5). The specific growth rate was 0.2 h^{-1} during the exponential growth phase and it is noteworthy that the enzyme production stopped within the growth phase with 70% of the glycerol left. Also, the lactose concentration after 21 h (~2 g L^{-1}) was still sufficient for induction. The time-course of the enzymatic activity showed a plateau after 16 h of cultivation with a maximal volumetric activity of 180 U L^{-1} after 21 h. By using the specific activity of the homogeneous enzyme a calculated concentration of 409 mg L^{-1} 2,5-DKG reductase was as active, soluble protein (no inclusion bodies were observed). Compared to other reported results, e.g. 50 U L^{-1} for 2,5-DKG reductase from *Corynebacterium* sp. in *E. coli* (pQE-82L-*dkr*) on modified Luria broth (with 3% glucose) with 0.1 mM IPTG as inducer [32], the obtained expression yield is quite high.

3.3. Enzyme purification and physical properties

The purification of His_6-tagged 2,5-DKG reductase consisted of a Ni-immobilized metal affinity chromatographic step followed by diafiltration, which resulted in an apparently homogeneous preparation by means of SDS-PAGE (Supplementary Fig. S2A). The enzyme was purified 5.5-fold from the lysate with a yield of 68.2%

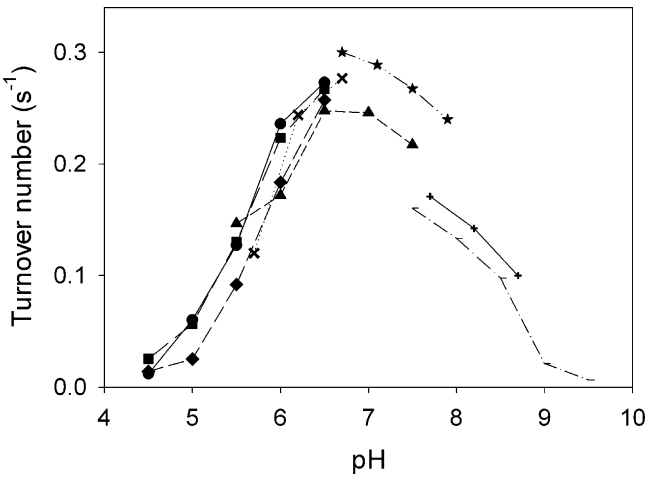

Fig. 2. Effect of pH on recombinant 2,5-DKG reductase activity. The buffers used were 50 mM sodium acetate (circles), 50 mM ammonium acetate (squares), 50 mM sodium citrate (diamonds), 50 mM Bis–Tris (triangles up), 50 mM MES (crosses), 50 mM MOPS (stars), 50 mM tricine (crosshairs) and 50 mM borate buffer (horizontal marks).

and a specific activity of 0.44 U mg^{-1} was obtained. SDS-PAGE of recombinant 2,5-DKG reductase resulted in a single band at 30 kDa, which compares well to the calculated molecular mass of the enzyme (31.033 kDa). Native-PAGE (data not shown) gave also a molecular mass of approx. 30 kDa, which indicates a monomeric structure of the active enzyme. This is in agreement with other reported 2,5-DKG reductases from various microorganisms from *Corynebacterium* spp. [7,8], *E. coli* [9] and an uncultured organism [11]. The isoelectric point of recombinant 2,5-DKG reductase was determined by isoelectric focusing under native conditions and one compact band was observed at pH 5.3 (Supplementary Fig. S2B), which is noticeably higher than for 2,5-DKG reductase A from *Corynebacterium* sp. (4.4) [7]. The *pI* of *C. glutamicum* 2,5-DKG reductase calculated from its amino acid sequence including the His_6-tag is 5.36, which matches the experimental result very well.

3.4. Effects of pH and temperature on 2,5-DKG reductase activity

Recombinant 2,5-DKG reductase exhibited a bell shaped pH profile with pH optima between 6.2 and 7.0 depending on the buffer used (Fig. 2). The enzymatic activity is little influenced by different buffers. The enzyme exerts more than 50% of its maximum activity over a pH range from 5.75 to 8.0, which favours the coenzyme regeneration in a biocatalytic process. The obtained values compare well with reported data for 2,5-DKG reductases from *Corynebacterium* sp. [7,8] whereas for the 2,5-DKG reductases from uncultured organisms [11] a preference for more acidic pH values was found. For the biocatalytic synthesis of 2-KLG ammonium acetate is considered to be a convenient buffer system, which was further used to determine the catalytic properties at pH 6.4. Its pH optimum favours coenzyme regeneration in a biocatalytic process.

The temperature optimum was only 35 °C in 50 mM ammonium acetate buffer, pH 6.4. The linear region of the Arrhenius plot was assumed between 293 K and 308 K from the initial rates of activity measurements at various temperatures. The activation energy (E_a) for the substrate was calculated to be 34 ± 1.1 kJ mol^{-1}. To investigate the thermal stability in more detail, the enzymatic activity was measured over time at 4, 20, 25, 30, 35 (Fig. 3A), 40 and 50 °C (Fig. 3B) in 50 mM ammonium acetate buffer, pH 6.4 without NADPH. Additionally, stability measurements in the presence of 200 μM NADPH were conducted at 25, 30 (Fig 3A, inset) and 40 °C (Fig 3B, inset). No difference in the time-dependent

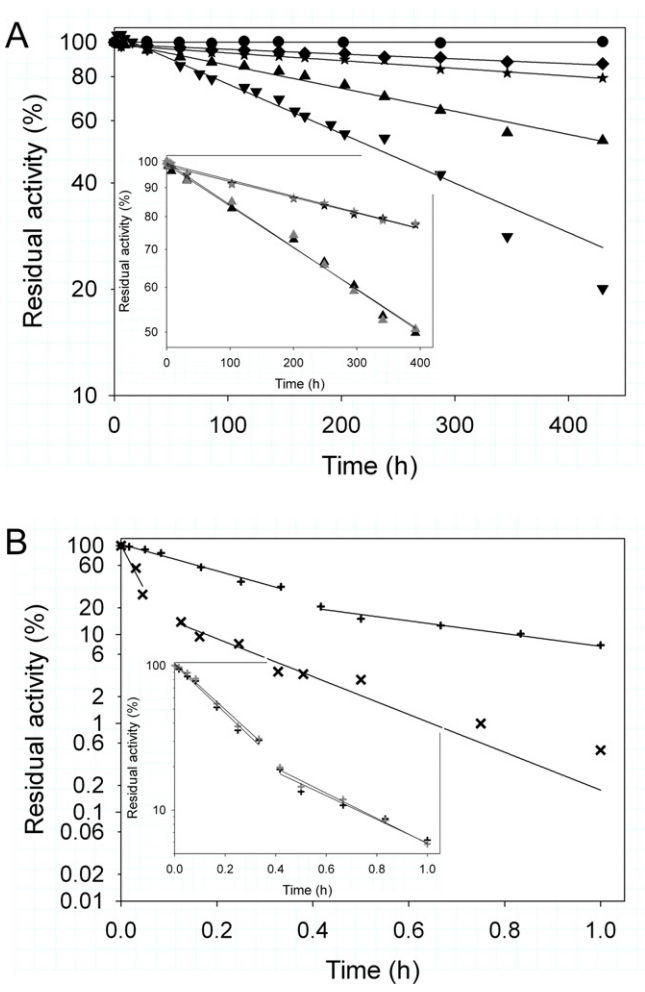

Fig. 3. Temperature-dependent deactivation of recombinant 2,5-DKG reductase. (A) In absence of NADPH at 4 °C (circles), 20 °C (diamonds), 25 °C (stars), 30 °C (triangles up) and 35 °C (triangles down). The inset shows the deactivation in presence (grey symbols) and absence (black symbols) of NADPH at 25 (stars) and 30 °C (triangles up). (B) In absence of NADPH at 40 °C (crosshairs) and 50 °C (crosses). The inset shows the deactivation in presence (grey symbols) and absence (black symbols) of NADPH at 40 °C (crosshairs).

deactivation of the enzyme was observed in the presence of NADPH. 2,5-DKG reductase showed first-order inactivation kinetics below 35 °C. The enzyme is very stable at 4 °C and 25 °C. At 30 °C and 35 °C, 40% of enzymatic activity was lost after 15 and 7 days, respectively. The half-life of the enzymatic activity ($t_{1/2}$) was calculated to be 462 h at 30 °C (k_{in} = 0.0015 h^{-1}), and 217 h at 35 °C (k_{in} = 0.0032 h^{-1}). The inactivation at 40 °C and 50 °C showed two distinct phases (both of first-order): a first phase of relatively rapid inactivation (k_{in} = 3.44 h^{-1} at 40 °C and k_{in} = 23.56 h^{-1} at 50 °C) and a slower second phase for the remaining 20% of enzymatic activity (k_{in} = 1.65 h^{-1} at 40 °C and k_{in} = 4.91 h^{-1} at 50 °C). These data are in agreement with thermal denaturation studies by differential scanning calorimetry (DSC, Fig. S3A), which found a thermal transition midpoint temperature (T_m) of 39 °C, at which both the folded and unfolded states are equally populated. This compares well to 2,5-DKG reductase A from *Corynebacterium* sp. (T_m = 38 °C) and 2,5-DKG reductase B (T_m = 32 °C) from *Corynebacterium* sp. [22]. In contrast to the above described time-dependent inactivation studies, a higher T_m (44 °C, Fig. S3B) was observed for *C. glutamicum* 2,5-DKG reductase in the presence of NADPH. This shows that the enzyme is stabilized against thermal unfolding by the bound coenzyme.

3.5. Effect of ions on 2,5-DKG reductase activity

Very few studies with respect to the effect of various ions have been done. Two reports indicate that metal ions Cu^{2+} and Ni^{2+} inhibit 2,5-DKG reductases strongly from *Corynebacterium* sp., whereas Ca^{2+}, Mg^{2+} and Mn^{2+} have little or no noticeable effect. However, the tested concentrations were below 1 mM [7,8]. We measured the effects of various ions on 2,5-DKG reductase activity to avoid inhibition in enzymatic or bioconversion experiments. The enzymatic activity was not influenced by the monovalent cations Na^+ and K^+, while bivalent Mg^{2+} and Ca^{2+} cations inhibit the enzyme severely (I_{50} Mg^{2+} = 97 mM, I_{50} Ca^{2+} = 141 mM). The acetate anion used in these experiments (Fig. 4A) exerts no inhibiting effect. Contrary to the acetates, for all sulphate- (Fig. 4B) and chloride salts (Fig. 4C) a strong inhibition was found. By inspection of the curves and I_{50} values (Na_2SO_4 = 215 mM, K_2SO_4 = 124.5 mM, $MgSO_4$ = 46 mM, NaCl = 119 mM, KCl = 119 mM, $MgCl_2$ = 20 mM, $CaCl_2$ = 32.5 mM (all data fit a very well to a single exponential equation, $R^2 > 0.98$)) the following conclusions can be drawn: (1) the acetate anion and monovalent Na^+ and K^+ cations have no effect on the activity of 2,5-DKG reductase, (2) bivalent Mg^{2+} and Ca^{2+} cations show a strong inhibition on 2,5-DKG reductase activity, (3) chloride is a more potent inhibitor than sulphate, the first has the same inhibitory effect as bivalent cations, the second a slightly lower, (4) the inhibitory effect of cations and anions is additive.

The I_{50} values show that extremely negative effects can be expected in a bioconversion process with the wrong buffer. To the best of our knowledge, inhibition of 2,5-DKG reductase activity by bivalent cations and monovalent and trivalent anions is a new fact has not been reported so far, but is of high importance for its biocatalytic application in the production of L-ascorbic acid via 2-KLG. To investigate the inhibition mechanism in more detail, chloride, the most severe inhibitor was chosen for further studies. The inhibition pattern (by using NaCl) was obtained for NADPH and 2,5-DKG turnover. The analysis of the Lineweaver–Burk plot for NADPH kinetics shows an increased apparent K_m, while V_{max} is not altered. A series of plots with the same y intercept ($1/V_{max}$) and the x intercepts ($-1/K_m$) closer and closer to the origin as NaCl increases, indicates that NaCl acts as full competitive inhibitor, which affects the binding of NADPH (Fig. 5A). The dissociation constant for NaCl (K_i) estimated from this and the Dixon plot is 7.4 ± 0.7 mM. With respect to 2,5-DKG a full noncompetitive inhibition was observed (Fig. 5B), which implies that the chloride ion directly interferes with the binding of the coenzyme but not with the substrate binding. The inhibitor dissociation constant (K_{ic}) and the inhibitor dissociation constant of the enzyme–substrate–inhibitor complex (K_{iu}) are equal (263 ± 23 mM). These inhibition patterns are indicative of a bi–bi ordered mechanism with NADPH binding first [31,33]. The inhibitory effect of other ions is likely to follow a similar pattern. By sequence alignment and homology modeling with the deposited structure of 2,5-DKGR-A (accession number 1A80) it was found that Asp45, Lys232, Arg238 and Glu241 (numbering according to the template 1A80) are involved in the binding of the adenine moiety, the ribose, the phosphate group on the 2′ position of the ribose ring and the pyrophosphate. We propose that these amino acids will be targeted by the investigated ions.

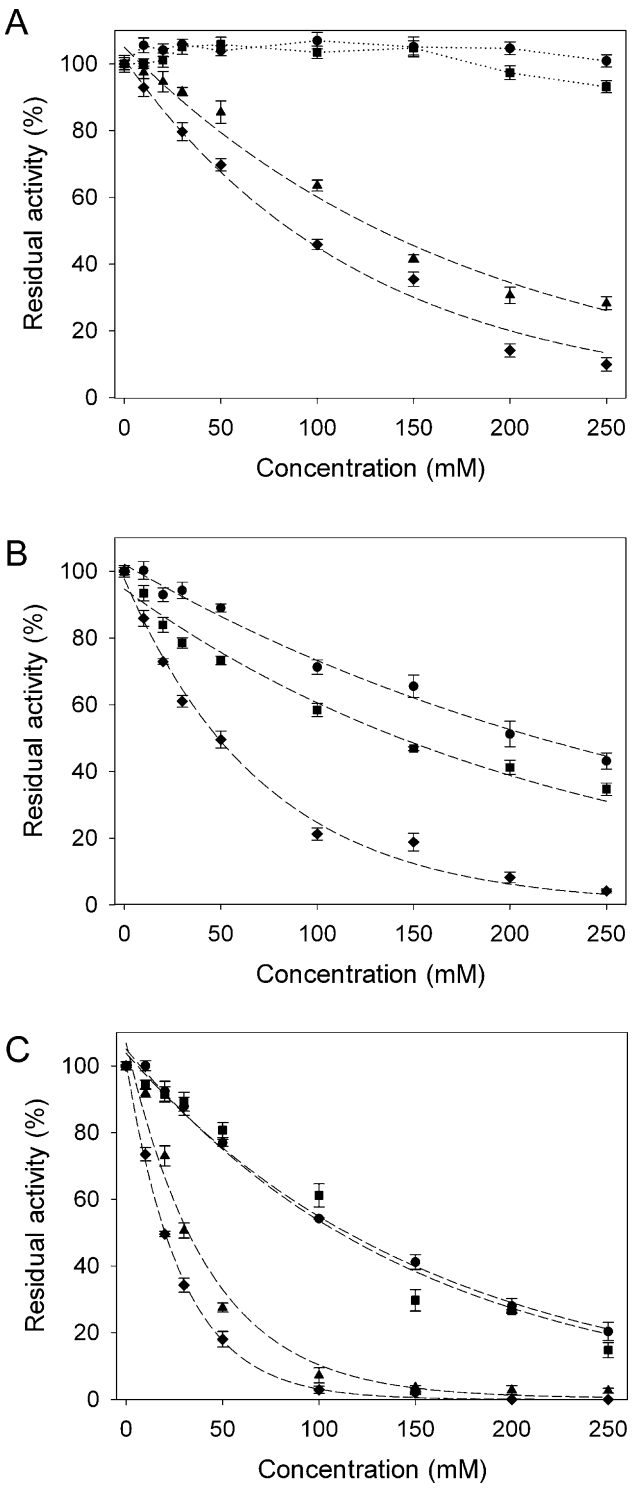

Fig. 4. Effect of different ions on recombinant 2,5-DKG reductase activity. The effect of the salts of acetic acid (A), sulfuric acid (B) and hydrochloric acid (C) were evaluated. A dotted line connects non-fitted data sets, short dashes indicate a single exponential fit. Sodium (circles), potassium (squares), calcium (triangles up) and magnesium (diamonds).

3.6. Catalytic properties

To evaluate the enzyme's capacity for 2,5-DKG conversion under suitable for industrial process conditions, the catalytic constants were measured in 50 mM ammonium acetate buffer at pH 6.4. Neither ammonium nor acetate ions inhibit the enzymatic activity. The kinetic measurements had to be performed with a fluorometric enzyme assay (in triplicates) because of the significant absorption of the substrate 2,5-DKG at 340 nm. At lower 2,5-DKG concentrations these results were verified with the spectrophotometric assay, both methods gave similar results. The derived Michaelis constants for NADPH (K_a) is 0.014 ± 0.003 mM, which is very low and allows to use a low concentration of the coenzyme in the process (50–70 μM). The Michaelis constant for the substrate 2,5-DKG (K_b) is 23.1 ± 3.8 mM and the turnover number

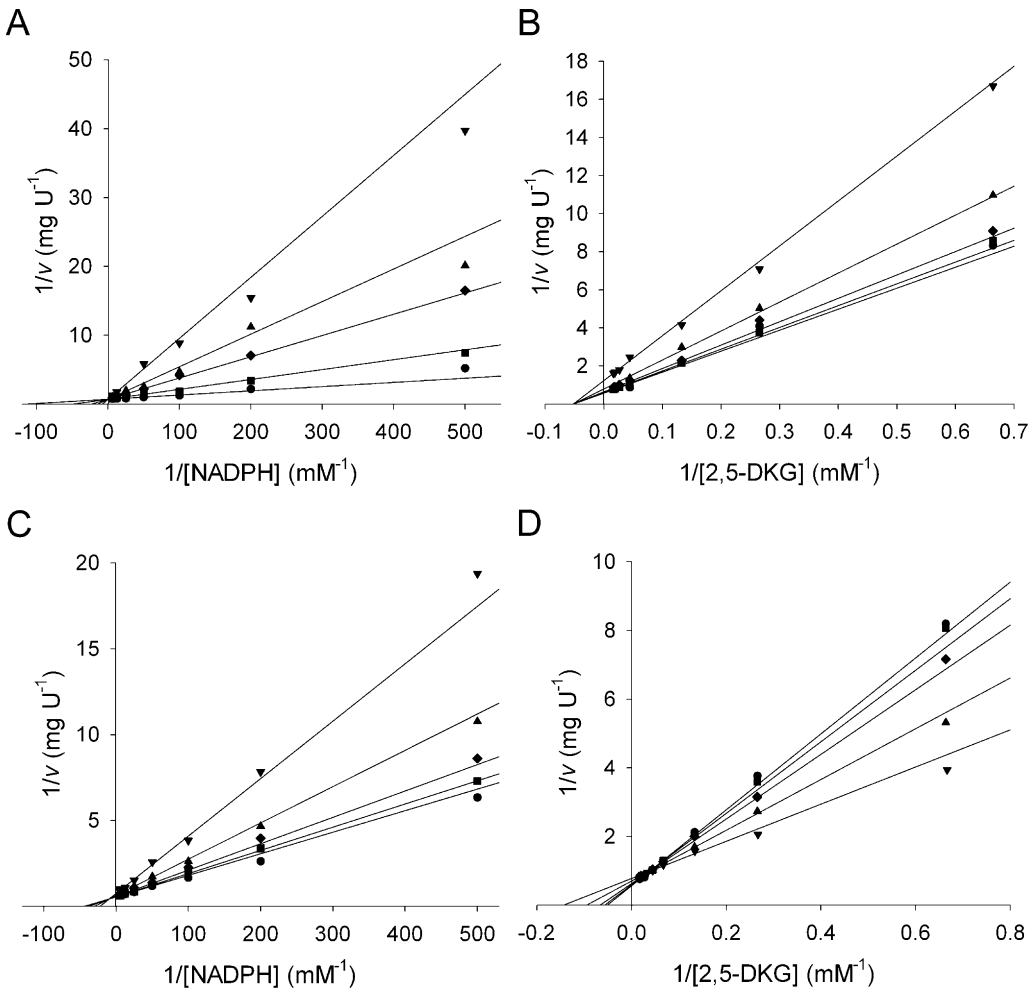

Fig. 5. (A) Lineweaver–Burk plot for the inhibition of recombinant 2,5-DKG reductase by NaCl with respect to NADPH. Without inhibitor (circles), 10 mM NaCl (squares), 30 mM NaCl (diamonds), 50 mM NaCl (triangles up), and 100 mM NaCl (triangles down) at a 23 mM 2,5-DKG concentration. (B) Lineweaver–Burk plot for the inhibition of recombinant 2,5-DKG reductase by NaCl with respect to 2,5-DKG. Without inhibitor (circles), 10 mM NaCl (squares), 30 mM NaCl (diamonds), 100 mM NaCl (triangles up), and 300 mM NaCl (triangles down) at a 0.2 mM NADPH concentration. (C) Lineweaver–Burk plot for the inhibition of recombinant 2,5-DKG reductase by 2-KLG with respect to NADPH. Without inhibitor (circles), 10 mM 2-KLG (squares), 30 mM 2-KLG (diamonds), 100 mM 2-KLG (triangles up), and 300 mM 2-KLG (triangles down) at a 23 mM 2,5-DKG concentration. (D) Lineweaver–Burk plot for the inhibition of recombinant 2,5-DKG reductase by 2-KLG with respect to 2,5-DKG. Without inhibitor (circles), 10 mM 2-KLG (squares), 30 mM 2-KLG (diamonds), 100 mM 2-KLG (triangles up), 300 mM 2-KLG (triangles down) at a 0.08 mM NADPH concentration.

(k_{cat}) is $1.87 \pm 0.18\ s^{-1}$. When assuming a 2,5-DKG concentration of 300 mM, most of the substrate (80%) can be converted before reaching the rate limiting concentration below 60 mM ($3 \times K_b$). The product 2-KLG was found to be an inhibitor of the forward reaction. Analysis of the results by double-reciprocal plots (Fig. 5C and D) indicates that 2-KLG is a hyperbolic mixed inhibitor [31] with respect to NADPH ($K_{i2\text{-}KLG;NADPH} = 110 \pm 28$ mM; $\alpha = 3.02 \pm 0.82$; $\beta = 0.44 \pm 0.12$) and 2,5-DKG ($K_{i2\text{-}KLG;2,5\text{-}DKG} = 375 \pm 26$ mM; $\alpha = 0.20 \pm 0.085$; $\beta = 0.66 \pm 0.038$). The factor α modifies the Michaelis–Menten constant, whereas the factor β modifies the maximal velocity for a given substrate in the presence of an inhibitor (2-KLG). $K_{i2\text{-}KLG}$ is the equilibrium dissociation constant of 2-KLG. The determined constants show that inhibition under process conditions is low (concentrations of 2,5-DKG above 10 mM and NADPH above 20 μM) and affects the conversion rate only minimally. Inhibition by the oxidized coenzyme $NADP^+$ was not observed.

4. Conclusions

2,5-DKG reductase from *C. glutamicum* can be recombinantly produced by *E. coli* in high yields and easily purified by affinity chromatography. Its physical and catalytic properties make the enzyme ideally suited for the application in the synthesis of 2-KLG, a precursor of L-ascorbic acid production. The investigated inhibition mechanism of 2,5-DKG reductase by anions and cations is of high relevancy for further process engineering. Having the recombinant enzyme in hands, powerful protein engineering tools can be used to further optimize the enzyme for its application in the production of ascorbic acid.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2012.07.014.

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