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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

Vanja Kaswurm, Claudia Pacher, Klaus Dieter Kulbe, Roland Ludwig*

*Food Biotechnology Laboratory, Department of Food Science and Technology, BOKU – University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria*

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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]

* Corresponding author at: Department of Food Sciences and Technology, University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria. Tel.: +43 147654 6149; fax: +43 147654 6199.
*E-mail address:* roland.ludwig@boku.ac.at (R. Ludwig).

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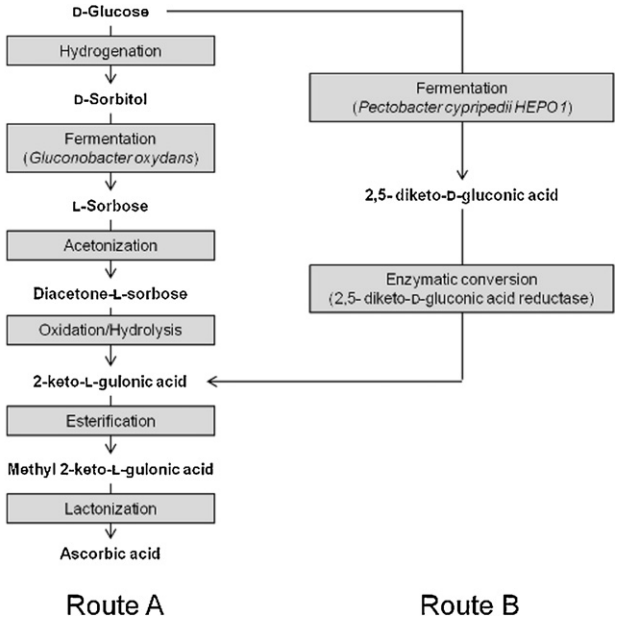


**Fig. 1.** Synthetic routes to ascorbic acid. Route A depicts the classical Reichstein process [26,27], Route B shows the process for utilizing the 2-KLG intermediate produced by 2,5-DKG-reductase [28].

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