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# Characterisation of a secondary alcohol dehydrogenase from *Xanthomonas campestris* DSM 3586

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**Abstract** The chromosomal locus NP_636946 of *Xanthomonas campestris* DSM 3586 (ATCC 33913) which was earlier presumed to encode a quinoprotein glucose dehydrogenase has been cloned, expressed in *Escherichia coli* and the recombinant enzyme has been characterised. It was found to have no glucose dehydrogenase activity but to be active on many different polyols and diols, aliphatic alcohols, certain aldonic acids and amino-sugars. The product of D-gluconic acid oxidation was 5-keto-D-gluconic acid. The enzyme differs from polyol/gluconate dehydrogenases found in *Gluconobacter* by its single-chain architecture, different substrate specificity and much higher (20- to 30-fold) expression level in *E.coli*.

We are interested in identifying enzymes that oxidise D-gluconic acid to 5-keto-D-gluconic acid, a compound that can be further converted into L-tartaric acid and some other useful fine chemicals (de Rooij 1984; Fleche 1998; Matzerath et al. 1995). So far, only certain species of the genus *Gluconobacter* are known to produce the enzymes catalysing this reaction (Shinagawa et al. 1983; Weenk 1984). Recently, it was discovered that the formation of 5-keto-D-gluconic acid in *G. suboxydans* IFO 3255 is catalysed by an enzyme that is also a polyol dehydrogenase (Matsushita et al., 2003). The polyol dehydrogenase of *G. suboxydans* IFO 3255 is a heterodimer encoded by two tandemly arranged genes—*sldA* and *sldB* (Miyazaki et al. 2002; Hoshino et al. 2003). We reached a similar conclusion by purifying polyol/gluconate 5-dehydrogenase (GA 5-DH) from *G. suboxydans* IFO 12528, cloning the two genes encoding it and characterising the recombinant enzyme (Salusjärvi et al. 2004). In our previous study, we identified two problems in using the cloned GA 5-DH gene from *G. suboxydans* as a basis for developing a fermentation process for 5-keto-D-gluconic acid. First, the enzyme had a relatively low activity with D-gluconic acid (about 10% of the activity for the same enzyme with D-sorbitol as substrate). Second, expression of GA 5-DH gene was quite toxic to *Escherichia coli* (as evidenced by e.g. extensive cell lysis during cultivation at 37°C in the presence of isopropyl-β-D-thiogalactopyranoside; IPTG) and only modest expression levels (about 0.03 mU/ml $*A_{600}$) were obtained using a *tac* promoter-based expression vector. One approach to solving these problems is to search for other enzymes capable of producing 5-keto-D-gluconic acid.

A BLAST search of the GenBank database, using combined sequences of small and large subunits of *G. suboxydans* IFO12528 GA 5-DH identified a number of homologues. Among these homologues, only a few have been biochemically characterised, including the closely related sorbitol dehydrogenase from *G. suboxydans* IFO 3255 (84% identity) and several glucose dehydrogenases, e.g. those from *E. coli* and *Acinetobacter calcoaceticus* (both having about 38% identity). The closest (48% sequence identity) uncharacterised homologue of GA 5-DH from *G. suboxydans* is found in the genomic DNA sequence of *Xanthomonas campestris* ATCC 33913 /DSM 3586 (NP_636946). In terms of sequence homology, NP_636946 is slightly closer to the polyol/gluconate dehydrogenases from *Gluconobacter* than the known glucose dehydrogenases, but its apparent single-chain architecture is typical for a glucose dehydrogenase. Also, *Xanthomonas* is not known to convert glucose to 5-keto-D-gluconic acid. Moreover, we could not measure any GA 5-DH activity with cell suspensions or cell extracts of *X. campestris* DSM 3586. Despite these uncertainties, we decided to follow the lead provided by homology comparisons and study the substrate specificity and other basic enzymatic properties of the *X. campestris* DSM 3586 (ATCC 33913) dehydrogenase encoded by the chromosomal locus NP_636946.

The coding region of NP_636946 was amplified by PCR, using the primers oXCGAD5 (5′-GAGAATTCATGTCGACATTGCTTCGTCC-3′) and oXCGAD3

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(5′-GACTGCAGCTCGAGAAGCTTTTACCGCTGCGG-CAACGCGT-3′) and cloned into expression vector pTAC under the control of a *tac* promoter, resulting in plasmid pTAC(GADX). The structure of the expression vector pTAC, the description of the induction procedure used with the *tac* promoter, the enzymatic assays and the rest of the experimental methodology used in this study were described in detail by Salusjärvi et al. (2004). When the culture of *E. coli* strain XL1-Blue MRF′ transformed with pTAC(GADX) was induced with IPTG in the presence of externally added pyrroloquinoline quinone (Matsushita et al. 1997), gluconate dehydrogenase activity was readily detected. The expression level (typically about 0.6–0.8 mU/ml *$A_{600}$) was 20-30 times higher than that of the *G. suboxydans* GA 5-DH gene tandem cloned in a similarly designed expression vector pTAC(GAD). So far, we have not made any attempt to purify the recombinant *X. campestris* dehydrogenase and measure its specific activity. However, qualitative estimates based on SDS-PAGE analysis of the two *E. coli* strains expressing the gluconate dehydrogenases from *G. suboxydans* and *X. campestris* suggest that most of the difference in expression levels is explained by a higher specific activity of the *Xanthomonas* enzyme. Both pTAC(GAD) and pTAC(GADX) were similarly toxic to the host cells of *E. coli*, causing quick cell lysis if induction was carried out with a high concentration of IPTG (over 25 mg/l) and/or at high temperature (above 30°C).

As the data presented in Table 1 show, the enzyme encoded by locus NP_636946 of *X. campestris* is definitely not a glucose dehydrogenase and its current GenBank annotation is erroneous. There are many similarities in terms of substrate specificity between this enzyme and the GA 5-DH from *G. suboxydans*. All good substrates of GA 5-DH are also efficiently oxidised by the *X. campestris* enzyme. In particular, this includes polyols matching the definitions of the so-called Bertrand–Hudson rule (Kulhánek 1989) such as D-arabitol, D-sorbitol, ribitol etc. Polyols not conforming to this rule (galactitol, L-arabitol) are poor substrates or not accepted at all. More interestingly, there are striking differences in the specificity of the two enzymes. Many poor substrates of *G. suboxydans* GA 5-DH are oxidised by the *X. campestris* dehydrogenase with high efficiency: 5- to 10-fold increases in the relative reaction rates are observed with the aliphatic alcohols 2-pentanol and isobutanol, the amino-sugar N-methylglucamine and a seven carbon polyol perseitol. Both D-gluconic and D-glucosaminic acids are quite good substrates. Estimates of kinetic constants (Table 2) show that the maximum reaction rate with D-gluconic acid is about one-half of the maximum rates with D-sorbitol or D-arabitol while $K_m$ is 20- to 50-fold higher for D-gluconic acid than for polyol substrates. Most aldoses and ketoses are not accepted as substrates, presumably because the reactive hydroxyl is protected by cyclic hemiacetal formation in solution. The only exception is D-ribose. It is oxidised efficiently, presumably because of its unusually high equilibrium concentration of a linear (aldehyde) form in water solution (Pigman and Horton 1972). For several substrates, the reaction products have been identified by HPLC. It was found that ribitol is oxidised to ribulose, D-arabitol to xylulose, D-sorbitol to sorbose, D-mannitol to fructose and D-gluconic acid to 5-

**Table 1** Substrate specificity of the secondary alcohol dehydrogenase from *X. campestris* DSM 3586 in comparison with the specificity of the GA 5-DH from *G. suboxydans*. The values in the table are expressed relative to the activity of each enzyme with 30 mM arabitol as substrate (100%). The values are averages of at least two experiments with two different cell extracts. As a control, *E. coli* cells transformed with pTAC were used. These cells had no activity with any of the tested substrates except glucose. – Activity not determined

| Substrate | Relative activity at different substrate concentrations | | |
|---|---|---|---|
| | *X. campestris* secondary alcohol dehydrogenase | | *G.suboxydans* GA 5-DH |
| | 30 mM | 200 mM | 30 mM |
| D-Arabitol | 100 | - | 100 |
| D-Sorbitol | 91 | - | 77 |
| Ribitol | 91 | - | 51 |
| Galactitol | 0 | - | 0 |
| D-Mannitol | 89 | - | 57 |
| *meso*-Erytritol | 100 | - | 109 |
| 2,4-Butandiol | 116 | - | 129 |
| Glycerol | 66 | - | 106 |
| N-Methylglucamine | 49 | 91 | 2.7 |
| 2-Pentanol | 34 | 102 | 6.5 |
| Isobutanol | 8.1 | 11 | 1.5 |
| 2-Propanol | 4.9 | 24 | 5.0 |
| L-Arabitol | 0 | - | 0 |
| Perseitol | 13 | 49 | 2 |
| L-Fucitol | 14 | - | 3 |
| Acetoin | 0 | - | 0 |
| D-Glucuronic acid | 0 | 0 | 0 |
| L-Gulonic acid | 0 | 0 | 0 |
| D-Galactonic acid | 0 | 0 | 0 |
| D-Gluconic acid | 36 | 51 | 6.1 |
| 2-Ketogluconic acid | 0 | - | |
| D-Glucosaminic acid | 40 | 0 | 5.6 |
| D-Fructose | 0 | 0 | 0 |
| D-Galactose | 0 | 0 | 0 |
| D-Ribose | 28 | 46 | 1.7 |
| D-Glucose | 0 | 0 | 0 |

**Table 2** Kinetic constants of the secondary alcohol dehydrogenase from *X. campestris* with selected substrates. The values listed are averages of at least four independent measurements. Confidence intervals at 95% probability are also shown. $V_{max}$ values could only be measured in relative terms because no pure preparation of secondary alcohol dehydrogenase was available

| Substrate | $K_m$, mM | Relative $V_{max}$, ($V_{max\ arabitol}$=1) |
|---|---|---|
| D-Sorbitol | 5.6±0.09 | 0.93±0.07 |
| D-Arabitol | 1.4±0.04 | 1 |
| D-Gluconic acid | 109±17 | 0.51±0.06 |

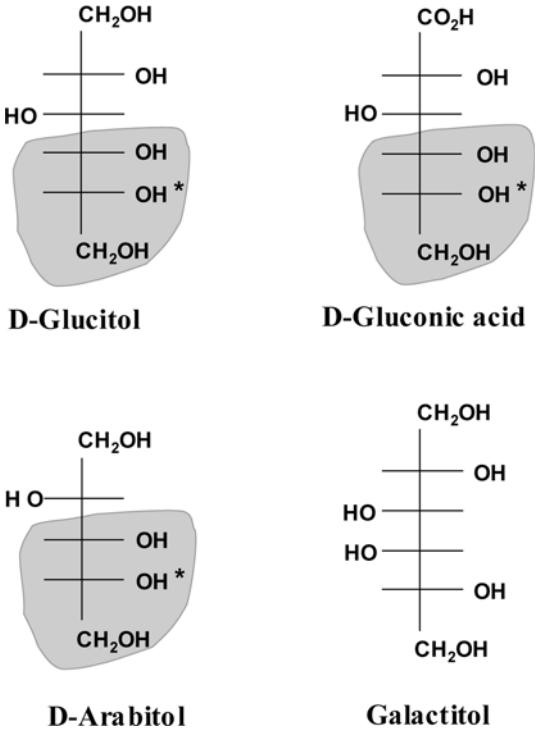


**Fig. 1** Substrate specificity of the secondary alcohol dehydrogenase from *X. campestris.* For hexitols, the configuration of both C-5 and C-4 appears essential (C-4, C-3 for pentitols). The fragment of the molecule determining the specificity of polyol oxidation is indicated by *shading*. The hydroxyl group oxidised in the reaction is marked with an *asterisk*. D-Sorbitol, D-arabitol and D-gluconic acid are all good substrates for the secondary alcohol dehydrogenase from *X. campestris*, but galactitol is not a substrate. This pattern practically coincides with the so-called Bertrand–Hudson rule (Kulhánek 1989)

keto-D-gluconic acid. All of these products match the predictions of the Bertrand–Hudson rule (Kulhánek 1989; Fig. 1). In conclusion, the substrate specificity of the enzyme encoded by the *X. campestris* chromosomal locus NP_636946 overlaps that of several dehydrogenases from *Gluconobacter*, described under a variety of different names (glycerol, sorbitol, arabitol, gluconate dehydrogenases; Adachi et al. 2001; Sugisawa et al. 2002; Salusjärvi et al. 2004); but it is distinctly broader. It is not an easy task to give this enzyme a short but sufficiently descriptive name to cover all the substrates it accepts. In our opinion, "secondary alcohol dehydrogenase" may be the most suitable name.

The pH–activity profile of the secondary alcohol dehydrogenase from *X. campestris* was very similar to that of the GA 5-DH from *G. suboxydans* with a maximum at pH 6.0, but the thermostability of the two enzymes was quite different. The enzyme from *Xanthomonas* retained full activity after 20 min at 45°C while the enzyme from *Gluconobacter* was almost completely inactivated under these conditions. The different thermostabilities were also reflected in the different temperature optima (in a 20-min assay) of the two enzymes (42.5°C, 35.0°C, respectively).

Like all quinoproteins, the secondary alcohol dehydrogenase from *X. campestris* is dependent on the presence of divalent cations for its activity and it can be completely inactivated by treatment with EDTA. Full re-activation of the EDTA-treated enzyme requires the presence of both $Ca^{2+}$ and $Mg^{2+}$ ions, while about 40% of activity can be re-gained by incubation with $Ca^{2+}$ alone. No reactivation was observed when apo-enzyme was incubated with $Mg^{2+}$ only. In its requirement for the simultaneous presence of both $Ca^{2+}$ and $Mg^{2+}$, the enzyme is similar to the gluconate 5-dehydrogenase from *Gluconobacter*. However, the metal-dependence patterns of the two enzymes are not identical, since apo-gluconate 5-dehydrogenase could be partly re-activated with $Mg^{2+}$ in the absence of $Ca^{2+}$.

The biotechnological potential of the secondary alcohol dehydrogenase form *X. campestris* can be illustrated by model experiments on the preparative conversion of D-gluconic acid into 5-keto-D-gluconic acid, using the cells of recombinant *E. coli* transformed with either pTAC (GAD) or pTAC(GADX). To achieve 75% conversion with the cells expressing the gluconate dehydrogenase gene from *G. suboxydans*, a long incubation (100 h) with a concentrated suspension of catalytic cell mass [optical density at 600 nm ($OD_{600}$) = 20] was required (Salusjärvi et al. 2004). Using *E. coli* cells expressing *X. campestris* enzyme, a comparable conversion yield was achieved in a much shorter time (16 h), with a much lower cell density ($OD_{600}$=2). Obviously, the secondary alcohol dehydrogenase from *Xanthomonas* could also be a useful biotechnological tool for production of L-sorbose, rare pentuloses such as L-ribulose and amino-sugar derivatives (such as those used as intermediates in Miglitol production; Deppenmeier et al. 2002). It is quite probable that closest homologues of this enzyme (e.g encoded by other *Xanthomonas* chromosomal loci: NP_641965, NP_638430, NP_643520) would be similarly applicable.

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