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Journal of Molecular Catalysis B: Enzymatic 47 (2007) 43–50
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Purification and characterization of 2-keto-D-galactonate reductase from *Pseudomonas fluorescens*

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Received 24 September 2006; received in revised form 16 February 2007; accepted 19 March 2007
Available online 30 March 2007

Abstract

NADPH-dependent 2-keto-D-galactonate reductase from *Pseudomonas fluorescens* IFO 14808, which was present in the soluble protein fraction, was purified to apparent homogeneity using a five-step procedure. The enzyme catalyzed NADPH-dependent reduction of 2-keto-aldonate to aldonate as well as $NADP^+$-dependent oxidation of aldonate and aldonate-6-phosphate to 2-keto derivatives. The enzyme consisted of two subunits with molecular masses of about 37 kDa. The gene encoding the enzyme was identified in the complete sequence of the *P. fluorescens* pf-5 genome based on the N-terminal amino acid sequence derived from the purified enzyme. The enzyme sequence exhibited 75 and 72% amino acid identities to the probable 2-keto-D-gluconate reductase from *Pseudomonas aeruginosa* and 2-keto-D-gluconate-6-phosphate reductase from *Pseudomonas putida*, respectively. The enzyme was found to belong to the 2-keto-aldonic acid reductase family in bacteria, although its substrate specificities differed from those of previously characterized members. Its relative reduction activities were 1.0 and 3.9 for 2-keto-D-gluconate and 2-keto-D-galactonate, respectively, while its relative oxidation activities were 1.0, 4.3, 1.4 and 0.4 for D-gluconate, 6-phospho-D-gluconate, D-galactonate and D-galactono-1,4-lactone, respectively. The 6-phospho-D-gluconate reductase activity was inhibited either competitively by 6-phospho-D-glucose and 2-keto-D-gluconate or uncompetitively by dilute 2-keto-D-gluconate. The possibility of D-erythorbic acid formation from D-galactose by *P. fluorescens* is discussed.

Keywords: 2-Keto-D-aldonate reductase; NADPH; 2-Keto-D-galactonate; Gluconate-6-P; *Pseudomonas fluorescens*; Erythorbic acid

1. Introduction

In a previous study, we found that 2-keto-D-gluconate (2KGlcA) and 2-keto-D-galactonate (2KGalA) were produced in high yields from D-glucose (D-Glc) and D-galactose (D-Gal) by cell cultures of *Pseudomonas fluorescens*, and that 2KGlcA was formed more rapidly than 2KGalA [1]. These observations indicated that D-Glc dehydrogenase and D-GlcA 2-dehydrogenase in the membrane catalyzed the above reactions for both D-Glc and D-Gal in *P. fluorescens* cells, and that the products were transported to the outside of the membrane in a similar manner to some oxidative bacteria [2,3]. D-GlcA 2-dehydrogenase and 2KGlcA reductase have been purified from the membrane and cytoplasm of some oxidative bacteria, respectively, and their physicochemical properties have been described [4–8]. The membrane-bound enzyme (EC 1.1.99.3) is a heme-FAD protein with a molecular mass of about 140 kDa comprising three subunits with different molecular masses. D-GlcA oxidation producing 2KGlcA is the main reaction. Furthermore, the enzyme is involved in L-ascorbic acid production from D-glucose [3]. The soluble enzyme (EC 1.1.1.215) is an $NADP^+$-dependent enzyme with a molecular mass of 120 kDa composed of three or eight subunits [9–12]. These enzymes use various compounds as substrates. The enzyme from *Acetobacter rancens* catalyzes the reduction of 2KGlcA (relative activity: 1), 2-keto-L-gulonate (1.8) and 2KGalA (0.8), although hydroxypyruvate (7.3) and glyoxylate (3.5) are its predominant substrates. In the present study, 2-keto-aldonate reductase was purified from the soluble fraction of *P. fluorescens* and characterized. Subsequently, the complete amino acid sequence of 2-keto-aldonate reductase from *P. fluorescens* pf-5 was identified. 2KGalA reductase predominantly catalyzed the reduction of 2KGalA

Abbreviations: D-Glc, D-glucose; D-Gal, D-galactose; D-GalA, D-galactonate; D-GlcA, D-gluconate; 2KGalA, 2-keto-D-galactonate; 2KGlcA, 2-keto-D-gluconate; Glc-6P, 6-phospho-D-glucose; GlcA-6P, 6-phospho-D-gluconate

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doi:10.1016/j.molcatb.2007.03.008

in buffers at neutral pH, and also catalyzed the oxidation of non-phosphorylated D-gluconate (D-GlcA), D-galactonate (D-GalA) and phosphorylated 6-phospho-D-gluconate (GlcA-6P). The active sites in the enzyme are discussed according to the kinetics of its inhibition. Finally, the formation of D-erythorbic acid (the C-5 epimer of L-ascorbic acid) from D-Gal in *P. fluorescens* is discussed.

2. Materials and methods

2.1. Materials

The following reagents were obtained commercially: 2KGlcA (Nacalai Tesque, Kyoto, Japan); 5KGlcA, 6-phospho-D-glucose (Glc-6P) and GlcA-6P (Sigma, St. Louis, MO); NADPH, $NADP^+$, NADH and NAD^+ (Oriental Yeast Co. Ltd., Tokyo, Japan). 2KGalA was prepared as described previously [1]. All other compounds were of the highest purity commercially available.

2.2. Protein analysis methods

The molecular masses of the enzyme subunits were estimated by 12.5% SDS-PAGE using the Laemmli method [13] and a molecular mass calibration kit (LMW; Bio-Rad, Richmond, CA). The apparent molecular mass of the protein under non-denaturing conditions was determined using a Cellulofine column (1 cm × 100 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.0; buffer A). Molecular weight marker proteins (BSA: 66.2 kDa; LADH: 44 kDa; chymotrypsin: 25.2 kDa) were used. Protein concentrations were measured using a CBB kit (Nacalai Tesque) with BSA as the protein standard.

2.3. N-terminal amino acid analysis

For N-terminal amino acid sequencing, the purified enzyme was separated by SDS-PAGE and transferred to a PVDF membrane (Millipore Co., Bedford, MA). Following excision of the appropriate band from the PVDF membrane, the N-terminal amino acid sequence of the protein was determined using an automatic amino acid sequencer; Procise 491 cLC (Applied Biosystems at Protein Research, Osaka University). The obtained N-terminal amino acid sequence was used to search the *P. fluorescens* pf-5 genome available at the Institute for Genomic Research (http://www.tiger.org).

2.4. Enzyme assay and kinetic analysis

Enzymatic activity was measured at 25 °C using a Model UV-1600 spectrophotometer (Shimadzu, Tokyo, Japan) according to the included kinetics program. The increases in absorbance of NADPH (A340 nm, $\varepsilon = 62.5\,mM^{-1}\,cm^{-1}$) formed from $NADP^+$, accompanied by conversion of D-GlcA (D-GalA or GlcA-6P) to 2-keto-aldonate for 1 min after the initiation of the reaction, were monitored. Reaction mixtures (0.7 ml) containing 40 mM sugar, 1.1 mM $NADP^+$ and enzyme in 80 mM Na_2CO_3–HCl buffer (pH 9.0 for D-GlcA and D-GalA; pH 8.5 for GlcA-6P) were used. The initial velocity values, which were the averages of two or three determinations, were used to represent the enzymatic activity. One unit of enzymatic activity was defined as the amount of enzyme catalyzing the formation of 1 μmol NADPH/min. In the reduced reaction, 2KGlcA or 2KGalA and NADPH (0.11 mM) were used instead of D-GlcA, D-GalA and $NADP^+$ and the mixtures were incubated in potassium phosphate buffer (80 mM, pH 7.0 or 8.5).

2.5. Microorganism growth and purification of 2KGalA reductase

P. fluorescens IFO 14808 was grown for 40 h as described previously [1]. Harvested cells were washed with buffer A (0.01 M potassium phosphate buffer, pH 7.0) and subjected to a five-step purification of 2KGalA reductase as described below.

In step 1, cell-free extracts were prepared. Briefly, washed cells (106 g wet weight) were suspended in 250 ml of buffer A, and 50 ml aliquots were individually sonicated with a Model UR-200 p Ultrasonic Disrupter (TOMY SEIKO, Tokyo, Japan) for 15 min. The intact cells, cell debris and membrane fractions were removed following centrifugation at 140,000 × *g* for 45 min, and the supernatants containing 2KGalA reductase were combined (total volume: 330 ml).

In step 2, the supernatant was subjected to ammonium sulfate fractionation. Solid ammonium sulfate was added to the supernatant to give 30% saturation. After removal of the precipitate by centrifugation at 14,000 × *g* for 20 min, further solid ammonium sulfate was added to the supernatant to give a final saturation of 60%. The precipitate was collected by centrifugation as described above, dissolved in buffer A (100 ml), dialyzed against buffer A (5 l) overnight and centrifuged.

In step 3, DEAE-Sephacel column chromatography was performed. The enzyme solution (100 ml) was loaded onto a DEAE-Sephacel column (3 cm × 22 cm), washed with buffer A and eluted with buffer A containing 0.1 M KCl. The active fractions were collected and pooled (136 ml).

In step 4, Octyl-Sepharose 4 fast-flow column chromatography was carried out. Following addition of solid ammonium sulfate to 20% saturation, the enzyme solution (136 ml) was loaded onto an Octyl-Sepharose column (2.5 cm × 15 cm) and equilibrated with buffer A supplemented with ammonium sulfate (20% saturation). After washing with the same buffer, the column was eluted with buffer A containing a linear gradient (20–0%) of ammonium sulfate. Active fractions were collected (54 ml), and concentrated to 5 ml.

In step 5, Cellulofine column chromatography was performed. The enzyme solution (5 ml) was loaded onto a Cellulofine GCL-2000-m (1.2 cm × 85 cm) equilibrated with buffer A and eluted with buffer A. The active fractions were collected and pooled as the purified enzyme.

3. Results and discussion

3.1. Purification of 2KGalA reductase

Using the five-step procedure, 2KGalA reductase was purified from cell-free extracts of *P. fluorescens* as described in

Table 1
Purification of 2KGalA reductase from *P. fluorescens*

Step	TP (mg)	TA (U)	SA (U/mg)	Yield (%)
Cell-free extract	5092.0	36.8	0.007	100
DEAE-Sephacel	285.0	28.1	0.099	76
Octyl-Sepharose 4F	25.5	17.8	0.700	48
Cellulofine	2.0	2.9	1.440	8

TP: total protein; TA: total activity; SA: specific activity. The activities were measured with D-GlcA as the substrate.

Section 2. The enzyme was purified by about 200-fold to apparent homogeneity with a yield of about 8% (Table 1). The purified enzyme produced a single band upon SDS-PAGE (Fig. 1). The specific activity of the final preparation was 1.44 U/mg protein. The native molecular mass of the enzyme was determined to be 75 kDa by gel filtration. On SDS-PAGE, one band with a relative molecular mass of 37 kDa was detected (Fig. 1). These results suggest that the native enzyme has a dimeric configuration.

3.2. Enzymatic properties

3.2.1. Substrate specificities of the reaction catalyzed by 2KGalA reductase

The 2KGalA reductase purified from *P. fluorescens* catalyzed NADPH-dependent reduction of 2KGalA to D-GalA, and this activity was 3.9-fold higher than its reducing activity toward 2KGlcA. Furthermore, its $NADP^+$-dependent oxidation activities toward D-GalA and GlcA-6P were 1.4- and 4.3-fold higher than its oxidation activity toward D-GlcA (Table 2). A compari-

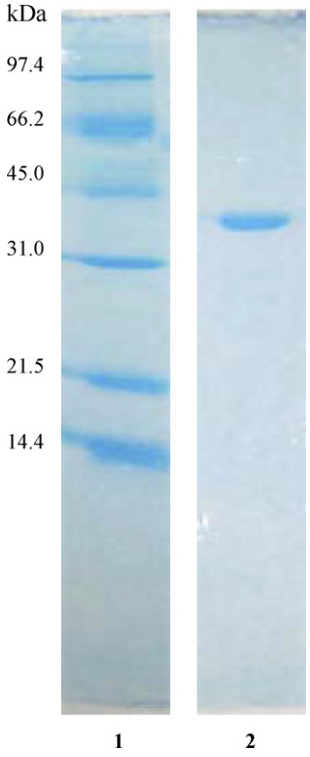


Fig. 1. SDS-PAGE of purified 2KGalA reductase. Lane 1: molecular mass marker proteins; lane 2: 1 μg of 2KGalA reductase. The proteins were resolved in a 12.5% acrylamide gel and stained with Coomassie Brilliant Blue.

Table 2
Comparison of the properties of soluble 2-keto-aldonate reductases from bacteria

	Pseudomonas fluorescens	*Acetobacter rancens* [9]	*Gluconobacter liquefaciens* [8,9]	*Brevibacterium ketosoreductum* [11]
Molecular mass	74 kDa (dimer)	120 kDa (octamer)	110 kDa (trimer)	72 kDa (dimer)
K_m (μM) for cofactor (substrate)				
NADPH	14.9 (2KGlcA)	10 (2KGlcA)	15.2 (2KGlcA)	10 (2,5-di-KGlcA)
$NADP^+$	360 (GlcA)	83 (GlcA)	12.5 (GlcA)	ND
Relative activity (K_m) for substrate				
Reduction				
2-KGlcA	100 (12.6 mM)	100 (0.86 mM)	100 (6.6 mM)	100 (ND)
2-KGalA	385 (12.7 mM)	79 (16 mM)	79 (ND)	ND
5-KGlcA	0	11 (ND)	0	0
2.5-di-KGlcA	ND	ND	ND	848 (5 mM)
2-K-L-gulonate	ND	177 (91 mM)	25 (ND)	92 (ND)
Hydroxypyruvate	ND	733 (65 μM)	ND	ND
Glyoxylate	ND	350 (0.38 mM)	ND	ND
Oxidation				
GlcA	70 (18.9 mM)	9 (2.4 mM)	67 (13 mM)	0
GalA	100 (15.8 mM)	6 (ND)	47 (ND)	ND
GlcA-6P	304 (5.1 mM)	0	0	ND
L-Idonate	ND	11 (ND)	94 (ND)	ND
Optimal pH				
Oxidation	8.5–10.0	12.0	10.5	ND
Reduction	7.0–8.5	7.0	6.5	6.0
Optimal temperature (°C)	35–45	50	50	50

ND: not determined.

son of the substrate specificities of the 2KGlcA reductases from *Acetobacter* and *Gluconobacter* has previously been reported [9]. These enzymes catalyzed NADPH-dependent reduction of 2KGlcA and also 2KGalA, but GlcA-6P was not a substrate. The reduction activity ratios (2KGalA/2KGlcA) were 0.8–0.9-fold for both enzymes, while the oxidation activity ratios (GalA/GlcA) were 0.1–0.7-fold for *Acetobacter* enzymes and 0.7–0.8-fold for *Gluconobacter* enzymes. Recently, 2KGlcA reductases have been purified from *Brevibacterium ketosoreductum* [11] and *Escherichia coli* [12]. These dimeric enzymes had molecular masses of 72–74 kDa and catalyzed NADPH-dependent reduction of 2,5-di-KGlcA to 5KGlcA and, to a lesser extent, 2KGlcA to D-GlcA and 2-keto-L-gulonate to L-idonate. These data demonstrate that the 2KGalA reductase purified from *P. fluorescens* shows a higher specificity for 2KGalA reduction and also has GlcA-6P oxidation activity.

3.2.2. K_m values for substrates and coenzymes

The following K_m values were determined for the reduction and oxidation reactions catalyzed by the 2KGalA reductase from *P. fluorescens*—reduction: 2KGlcA (12.6 mM) and 2KGalA (12.7 mM); oxidation: D-GlcA (18.9 mM) and D-GalA (15.8 mM), GlcA-6P (5.1 mM). The K_m for $NADP^+$ (360 μM) with D-GlcA as a substrate was 24-fold higher than that for NADPH (14.9 μM) with 2KGlcA as a substrate (Table 2). V_{max}/K_m values ($\times 10^3$) were 0.076 (D-GlcA), 0.13 (D-GalA), 1.21 (D-GlcA-6P), 0.17 (2KGlcA), and 0.66 (2KGalA) suggested that the enzyme preferred to D-galcto-configulation than D-gluco-configulation, and that D-2KGlaA-6P may be the best substrate.

3.2.3. Optimal pH

The optimal pH values for the reduction of 2KGalA and 2KGlcA were 7.0–8.5 and 8.5, respectively (Fig. 2A), while those for the oxidation of GlcA-6P, D-GlcA and D-galactono-1,4-lactone (Fig. 2B) were 8.5, 9 and 10, respectively.

3.2.4. Effects of temperature on the reaction activity and stability of the enzyme

The optimal temperatures for the reduction of 2KGalA and 2KGlcA were determined to be 45 and 35–40 °C, respectively (Fig. 2C). The heat stability of the enzyme maintained at each temperature for 5 min before the assay was measured using 2KGalA and 2KGlcA as the substrates. The enzyme was stable until 40 °C and then rapidly became unstable at 50 °C. However, when NADPH was added to the reaction solution, the enzyme was stable up to 50 °C (Fig. 2D).

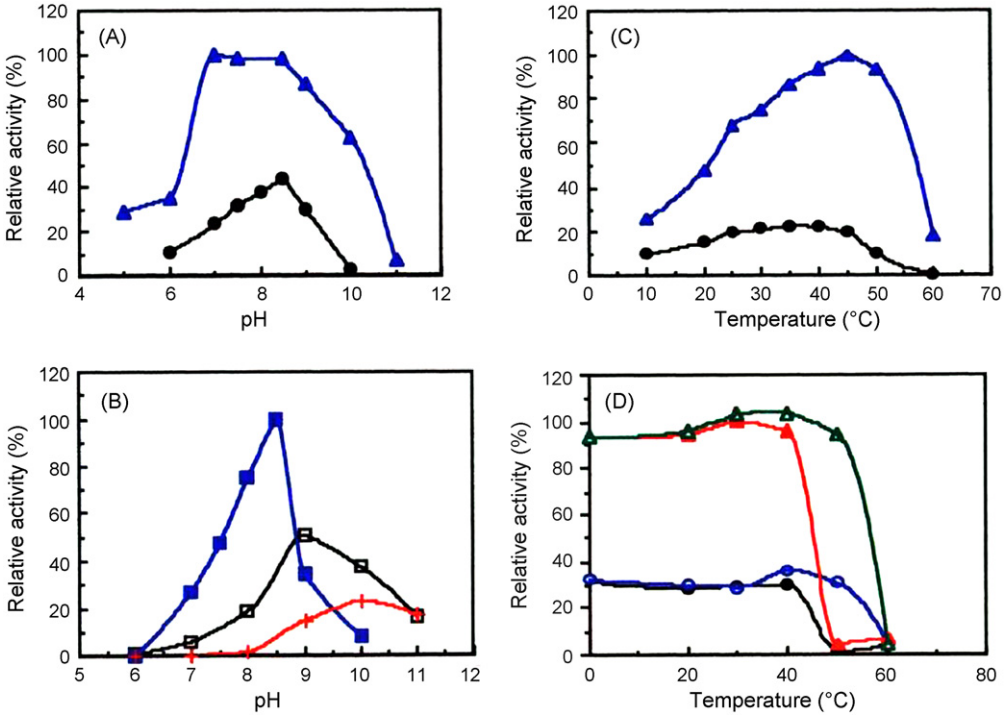


Fig. 2. Effects of pH and temperature on the 2KGalA reductase activity. (A) Effects of pH on the reduction activity. The activity was measured as described in Section 2. The buffers used were 0.1 M concentrations of KMES (pH 5.0 or 6.0), KPB (pH 7.0 or 7.5) and Na_2CO_3–HCl (pH 8.0, 8.5, 9.0, 10 or 11). The enzyme (4.7 μg) was incubated with 2KGalA (▲) or 2KGlcA (●). The relative activities for 2KGalA (100: activity at pH 7.0) are shown. (B) Effects of pH on the oxidation activity. The enzyme (5.2 μg) was incubated with GlcA-6P (■), GlcA (□) or galactono-1,4-lactone (+). The relative activities of GlcA-6P (100: activity at pH 8.5) are shown. (C) Effects of temperature on the activity. The enzyme (4.7 μg) was incubated with 2KGalA (▲) or 2KGlcA (●) at a range of temperatures from 10 to 60 °C. The relative activities for 2KGalA (100: activity at 45 °C) are shown. (D) Effects of temperature on the stability of the enzyme in the presence of 2KGalA (▲) or 2KGlcA (●) as substrates. The enzyme (4.7 μg) was pre-incubated in buffer A in the presence or absence of NADPH (19.5 μM) for 5 min at each temperature, before the enzymatic activity was measured as described above. The results for 2KGalA + NADPH (△) and 2KGlcA + NADPH (◯) are also depicted. The relative residual activities for 2KGalA (100: activity at 30 °C) are shown.

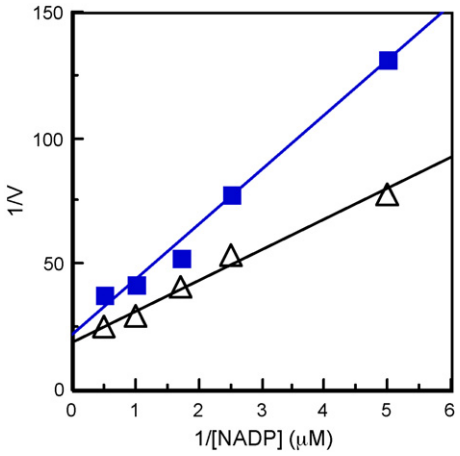


Fig. 3. $NADP^+$-binding sites for GlcA and GlcA-6P in the enzyme. The oxidation activity of GlcA-6P was measured under various concentrations of $NADP^+$ in the presence or absence of GlcA. Double-reciprocal plots of the initial velocity against the concentration of $NADP^+$ (0.2–2 mM) in the presence (■) or absence (△) of 2.6 mM GlcA are shown. *V*: U/min.

3.3. Effect of inhibitor on the activity of 2KGalA reductase

The 2KGalA reductase from *P. fluorescens* catalyzed the dehydrogenation of both phosphorylated and non-phosphorylated substrates. In order to determine whether or not the active sites for both types of substrates were similar, the following kinetic approaches were utilized.

3.3.1. Active sites for cofactors

The enzymatic activity with GlcA-6P as a substrate (40 mM) was measured at $NADP^+$ concentrations ranging from 0.2 to 2 mM in the absence or presence of D-GlcA (2.6 mM) (Fig. 3). The activity for GlcA-6P was inhibited almost competitively in the presence of D-GlcA, indicating that GlcA-6P and D-GlcA competed for the same active site of $NADP^+$ during the reaction process.

3.3.2. Inhibition of GlcA-6P oxidation

The $NADP^+$-dependent oxidation of GlcA-6P was measured in the absence or presence of D-GlcA, 2KGlcA or 5KGlcA. In the presence of 2.6 mM D-GlcA, a Lineweaver–Burk plot of the activity against the substrate concentration was almost parallel to that in the absence of D-GlcA. In the presence of 6.6 mM D-GlcA, the Lineweaver–Burk plot revealed competitive inhibition (Fig. 4A). 2KGlcA (6.6 mM), Glc-6P (2.6 mM) and 5KGlcA (2.6 mM) each inhibited the GlcA-6P oxidation activity competitively. In the presence of 2.6 mM 2KGlcA, a Lineweaver–Burk plot of the activity against the substrate concentration gave a parallel line similar to that for 2.6 mM D-GlcA in Fig. 4A (Fig. 4B). From the results shown in Fig. 4A and B, in the presence of higher concentration (6.6 mM) of D-GlcA, 2KGlcA, and 2.6 mM of Glc-6P and 5KGlcA, each bound to the GlcA-6P-binding site in the active site of the enzyme, indicating that these compounds inhibited GlcA-6P oxidation competitively in the ground state. In the presence of dilute D-GlcA or 2KGlcA (2.6 mM each), the inhibition patterns suggested

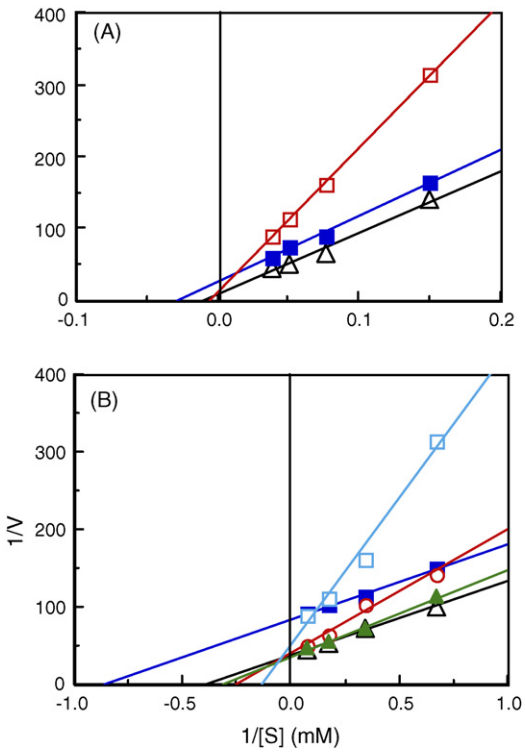


Fig. 4. Effects of sugars on the oxidation of GlcA-6P. (A) The enzymatic activities of 2KGalA reductase (20 μg) were measured in the presence or absence of GlcA as described in Section 2 with GlcA-6P (0.66–26 mM) as the substrate. Double-reciprocal plots of the initial velocity against the concentration of GlcA-6P in the presence of GlcA (2.6 mM; ■), GlcA (6.6 mM; □) or GlcA-6P alone (△) are shown. (B) Effects of sugars on the oxidation of GlcA-6P. The procedure was similar to that in (A), except that other sugars were used. Double-reciprocal plots of the initial velocity against the concentration of GlcA-6P in the presence of 5KGlcA (2.6 mM; ▲), Glc-6P (2.6 mM; ◯), 2KGlcA (2.6 mM; ■), 2KGlcA (6.6 mM; □) and GlcA-6P alone (△) are shown.

uncompetitive inhibition. The compounds that showed uncompetitive inhibition have analogous structures to the transition state of the enzyme and the affinities of these compounds for the transition state are higher than those for the ground state [14,15]. At 2.6 mM or less 2KGlcA (formed from 2.6 mM D-GlcA) could bind to its transition state and inhibit the enzyme uncompetitively.

These results suggest that D-GlcA and GlcA-6P are catalyzed in similar active sites of the enzyme and that the phosphorylated compound is a better substrate, although the phosphoryl group is not necessary for the activity. D-GlcA and 2KGlcA inhibit the dehydrogenation of GlcA-6P during the two reaction steps, namely the ground and transition states. Furthermore, the results suggest that the oxidation product of GlcA-6P is 2KGlcA-6P, rather than a 5-keto derivative, since 5KGlcA inhibited the reaction slightly and competitively in the ground state only.

3.4. Complete DNA and amino acid sequence analyses of the 2KGalA reductase from P. fluorescens pf-5

The N-terminal part of the 2KGalA reductase sequence determined by automatic Edman degradation was MKKHLVLYKKLSPTLMARLQEQAQVTLIE. This sequence was used

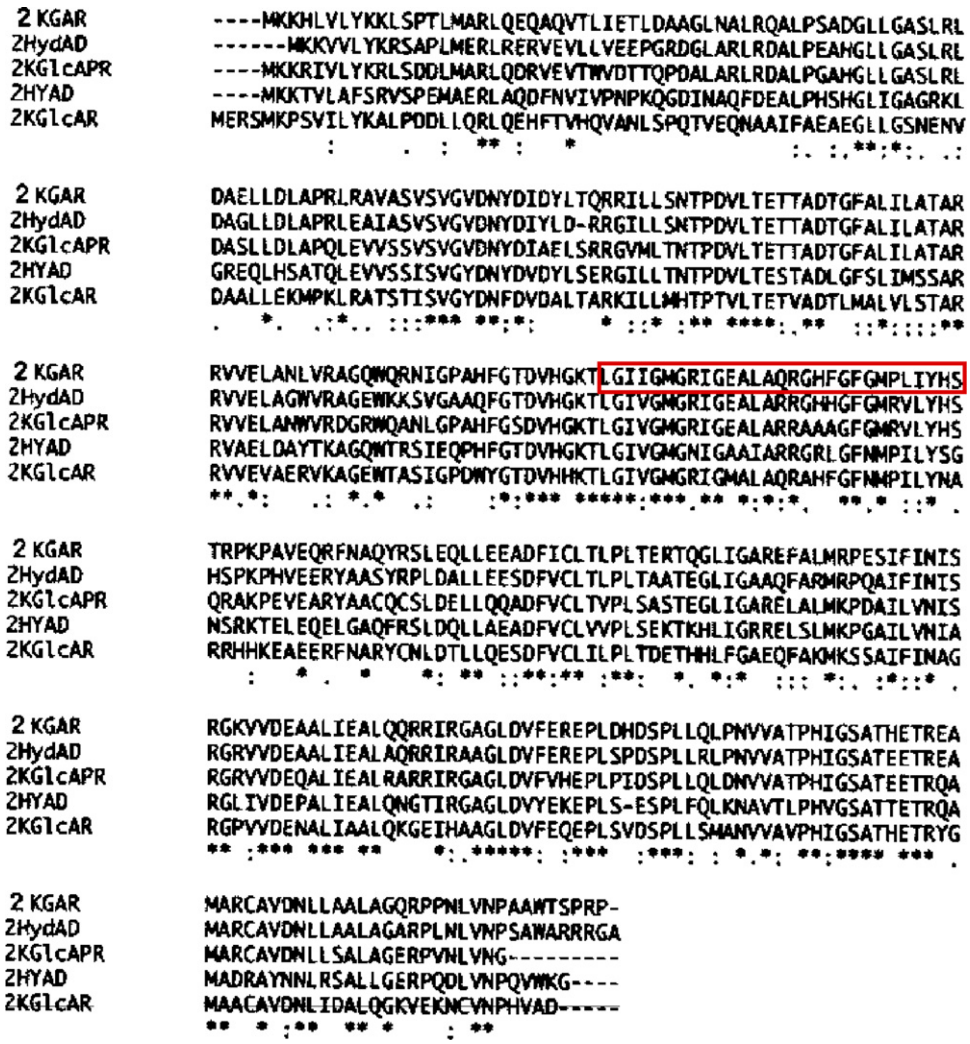
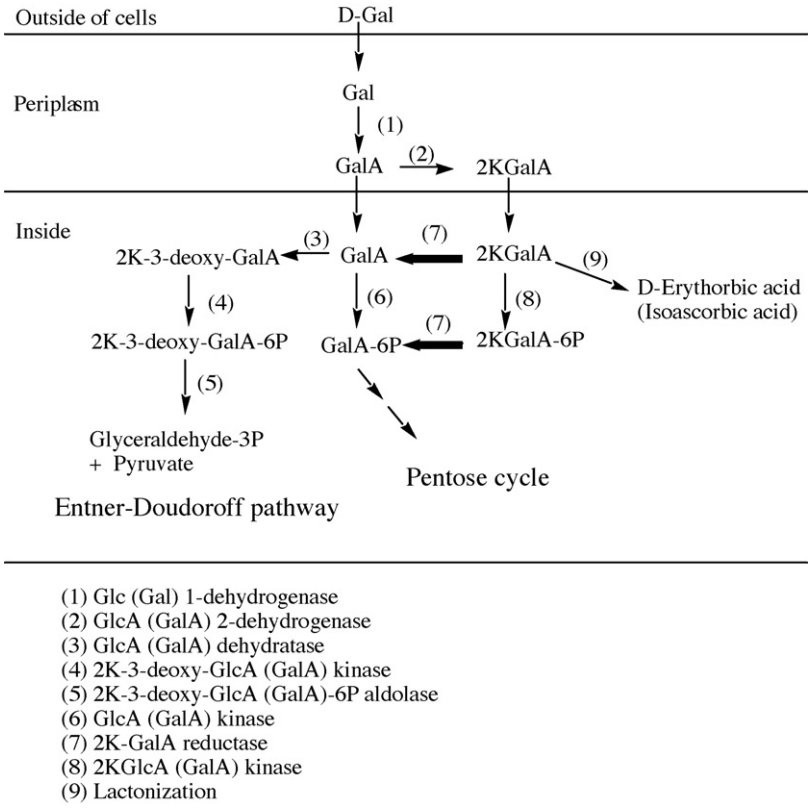


Fig. 5. Multiple sequence alignment of bacterial 2-keto-aldonic acid reductases. The amino acid sequence of 2KGalAR (2KGalA reductase) from *P. fluorescens* (BAD 69623) was compared with those of 2HydAD (putative 2-hydroxyacid dehydrogenase from *P. aeruginosa*, PA 2263), 2KGlcAPR (2KGlcA-6P reductase from *P. putida*, PLF 2717), 2HYAD (2-hydroxyacid dehydrogenase from *S. typhiminium*, STY 4156) and 2KGlcAR (2KGlcA reductase from *E. coli*, C4372). The NADPH-binding site is boxed.

to search the *P. fluorescens* pf-5 gene sequence database using genomic Blast with microbial genomes, and 100% matches were found. The open reading frame containing the N-terminal sequence of the enzyme encoded a polypeptide of 328 amino acids with a predicted molecular mass of 35,890 Da and a p*I* of 8.1 (calculated from the amino acid composition) (DDBJ Accession No. BAD 69623). This molecular mass was consistent with the SDS-PAGE result. Multiple alignment of the amino acid sequences of 2KGalA reductases by the CLUSTALW program revealed that it was a member of the 2-keto-D-aldonic acid reductase family in bacteria. Specifically, the probable 2KGlcA reductase from *P. aeruginosa* (identity: 76%; positivity: 85%) and the 2KGlcA-6P reductase from *Pseudomonas putida* (identity: 72%; positivity: 84%) were the enzymes with the highest scores for similarity, while the 2KGlcA reductase from *E. coli* showed 57% identity and 71% positivity. The $NADP^+$-binding motif conserved in all the other enzymes in Fig. 5 is also shown.

3.5. Putative metabolic pathway of aldonate in P. fluorescens

Similar to D-Glc, D-Gal is used as a source of carbon and energy in living cells via conversion to D-Glc by the Leloir pathway [16] and enters into the Embden–Meyerhof pathway. In some oxidation bacteria, D-Gal is oxidized to D-GalA and then dehydrated to 2-keto-3-deoxy-D-GalA and metabolized by the Entner-Doudoroff pathway similar to D-Glc [17]. According to previous results for the D-glucosamine metabolic pathway [18] and ketogluconate operon searches of *P. fluorescens* pf-5 by the Blast program (data not shown), the presence of the Entner-Doudoroff pathway was confirmed in *P. fluorescens*. L-Ascorbic acid is made from D-Glc or D-sorbitol. Specifically, D-Glc is dehydrogenated to D-GlcA, followed by conversion to 2KGlcA by 2-hydroxyacid dehydrogenase and 2,5-di-KGlcA by 2KGlcA dehydrogenase, then 2,5-di-KGlcA is converted to 2-keto-L-gulonate by 2,5-di-KGlcA reductase and finally to



(1) Glc (Gal) 1-dehydrogenase
(2) GlcA (GalA) 2-dehydrogenase
(3) GlcA (GalA) dehydratase
(4) 2K-3-deoxy-GlcA (GalA) kinase
(5) 2K-3-deoxy-GlcA (GalA)-6P aldolase
(6) GlcA (GalA) kinase
(7) 2K-GalA reductase
(8) 2KGlcA (GalA) kinase
(9) Lactonization

Scheme 1. Putative catabolic pathway of D-GalA in *P. fluorescens*.

L-ascorbic acid [3]. All these enzymes, except for 2KGlcA reductase, are membrane-bound and prefer D-Glc [19,20]. Since all the enzymes involved in the above pathway are known to be present in *P. fluorescens*, L-ascorbic acid may be made from D-Glc in this bacterium. In the case of Gal, L-Gal is known to be converted to L-ascorbic acid via oxidation by L-galactono-1,4-lactone oxidase [21]. In the present study, 2KGalA was formed via D-GalA oxidation by 2-hydroxy-acid dehydrogenase or the 2KGalA reductase, and subsequent lactonization of 2KGalA to its 1,4-lactone either spontaneously or enzymatically [22] may result in the formation of D-erythorbic acid (isoascorbic acid, the C-5 epimer of L-ascorbic acid) (Scheme 1). D-Erythorbic acid has very similar chemical properties to L-ascorbic acid, but very low vitamin C activity. D-Erythorbic acid has been used as a food antioxidant for many years, and several efforts toward the production of D-erythorbic acid from D-Glc have been reported [22], but not from D-Gal. Therefore, a fermentation method of D-Gal to 2KGalA by *P. fluorescens* cells [1] along with a spontaneous or chemical lactonization may be a useful method for D-erythorbic acid production from D-Gal. Further studies toward this aim are currently underway.

Acknowledgements

We thank Professor A. Nakagawa (Osaka University) and Ms. Y. Yoshimura (Protein Research, Osaka University) for the protein sequence analysis.

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