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Journal of Molecular Catalysis B: Enzymatic 23 (2003) 291–298
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Enzymatic synthesis of 2-keto-D-gluconate and 2-keto-D-galactonate from D-glucose and D-galactose with cell culture of *Pseudomonas fluorescens* and 2-keto-galactonate from D-galactono 1,4-lactone with partially purified 2-ketogalactonate reductase

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Received 5 February 2003; accepted 30 April 2003

Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

2-Keto-D-gluconate and 2-keto-D-galactonate were prepared from D-glucose (with a yield of 40%) and D-galactose (with a yield of 25%), respectively, with cell culture of *Pseudomonas fluorescens*. However, 2-keto-D-mannoate was not prepared in this method. The time courses of the reactions showed that 2-keto-D-gluconic acid and 2-keto-D-galactonic acid were produced from D-glucose and D-galactose through D-gluconate and D-galactonate, respectively. When using D-galactono 1,4-lactone as a starting material, 2-keto-D-galactonate was produced with partially purified NADP-dependent 2-ketogalactonate reductase from *P. fluorescens*. Some fundamental properties of the 2-ketogalactonate reductase were compared with those of 2-ketogluconate reductase from *Acetobacter* and *Gluconobacter*.

Keywords: 2-Ketogluconate; 2-Ketogalactonate reductase; NADP; NADPH; *Pseudomonas fluorescens*

1. Introduction

Many biological transformation of organic compounds by cell culture of fungi and bacteria have been reported [1,2]. The reactions catalyzed by these bioorganisms are regio- and enantio-selectivity of glucosilation of phenols, steroids, terpenes, and organic acids. Chemical methods for the synthesis of anomerically pure glycosides involved protection and deprotection step and are inherently circuitous. Urose (keto-sugar) has been reported as a useful intermediate for the synthesis of amino-sugar [3,4], deoxy-sugar [5], and branched-sugar [6,7]. 2-Keto-aldonic acid might be a useful intermediate for the synthesis of similar derivatives of aldonic acid. In the chemical synthesis of 2-ketogluconic acid from D-glucose, before oxidation of aldehyde group of C_1 to carboxyl group and hydroxyl group of C_2 to keto group, protection of hydroxyl groups at $C_3 \sim C_6$ of glucose then after oxidation, removal of these

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doi:10.1016/S1381-1177(03)00092-4

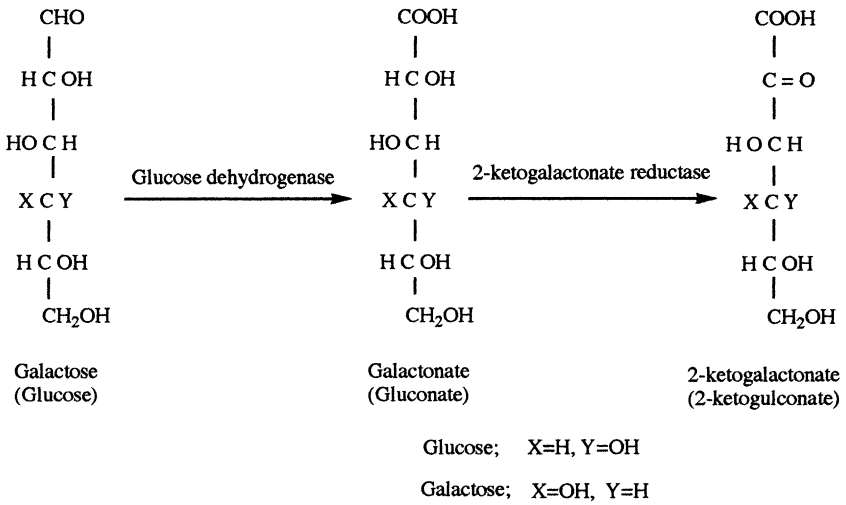

Fig. 1. Reaction of D-galactose (glucose) to 2-keto-D-galactonate (2-keto-D-gluconate) through D-galactonate (D-gluconate).

protected groups are needed. Contrarily, biosynthetic technique is specific for the position of the reaction and further it proceeds under mild experimental conditions and organic reagents that are dangerous for the circumstances were not used. In this work, 2-keto-D-gluconate and 2-keto-D-galactonate were synthesized with cell culture of *P. fluorescens* from D-glucose and D-galactose, respectively. These sugars are rich in nature and cheap. 2-Ketogalactonate reductase was purified partially from soluble fraction of *P. fluorescens* and its properties are reported Fig. 1.

2. Materials and methods

2.1. Materials

D-Glucose, D-galactose, D-mannose, D-gluconate K, galactono 1,4-lactone, 2-keto-D-gluconate Ca and other sugars were obtained from NACALAI TESQUE (Kyoto, Japan). DEAE-sepharose Cl-6B, octyl sepharose 4 F, and sephadex G-200 were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Cellulofine GCL-2000-m was purchased from Seikagaku Kogyo (Tokyo, Japan). NADP, NADPH, NAD, and NADH were purchased from ORIENTAL YEAST (Tokyo, Japan). Other chemicals were of analytical grade.

2.2. Analysis

Thin layer chromatography (TLC) employed a Merck silica gel (Kieselgel 60 F_{254}) with *n*-butanol: pyridine:water (1:1:1 by volume). Reaction products were detected by ethanol–H_2SO_4 or Orcinol reagents. IR spectra were measured by a Shimadzu FTIR-8400 spectrometer (Kyoto, Japan). Protein was measured with a NACALAI TESQUE protein assay kit. Crystalline bovine serum albumin was used as a standard.

2.3. Culture conditions

P. fluorescens (IFO 14808) was grown at 28 °C for 48 h with aeration in a medium of 1.5% peptone, 0.2% KH_2PO_4, 0.2% K_2HPO_4, 0.01% $MgSO_4$, and 0.01% yeast extract. Harvested cells were washed with a 0.01 M potassium phosphate buffer (pH 7.0) and used for purification of 2-ketogalactonate reductase.

2.4. Assay of the 2-ketogalactonate reductase activity

The enzymatic activity was determined at 25 °C using a Shimadzu UV-1600 spectrophotometer according to the included kinetic program. The increase in absorbance of NADPH formed from NADP, which are accompanied by the conversion of D-galactono lactone to 2-ketogalactonate (or D-gluconate to 2-keto-

gluconate), was monitored at 340 nm. Reaction mixtures (0.7 ml) containing 40 mM sugars, 1.1 mM NADP, and enzyme in a 80 mM Na_2CO_3–HCl buffer (pH 10 for galactono lactone or pH 9 for gluconate), were incubated. Initial velocity values, which were averages of two or three determinations, were used as the enzymatic activity. One unit of enzymatic activity was defined as the amount of enzyme catalyzing the formation of 1 μM NADPH/min.

For reverse reactions, 2-ketogluconate and 2-ketogalactonate, and NADPH (0.11 mM) were used instead of D-gluconate, D-galactonate, and NADP.

2.5. Biosynthesis of 2-ketogalactonate and 2-ketogluconate from D-galactose and D-glucose

Biosynthesis of 2-ketogalactonate and 2-ketogluconate was performed similarly to the methods reported by Asai et al [8] with some modifications. Preculture of *P. fluorescens* (1.5% peptone, 0.2% KH_2PO_4, 0.2% K_2HPO_4, 0.01% $MgSO_4$, and 0.01% yeast extract; 5 ml), which was grown overnight at 28 °C, was added to 100 ml of main culture (0.5% yeast extract, 2% glucose or galactose, 0.4% $CaCO_3$) and incubated at 28 °C for 75 h (glucose) or 145 h (galactose). Aliquots (0.2 ml) were withdrawn from culture each day and decreases of galactose or glucose, and increases of 2-ketogalactonate or 2-ketogluconate were measured using Orcinol or Semicarbazide methods [9]. After most of the starting materials were changed to the products, the cultures were centrifuged and supernatants containing product were desalted with Dowex 50 (H^+). The desalted solutions were neutralized (pH 8) with 1 N KOH and concentrated to about 10 ml. Crystals were obtained by adding methanol or ethanol to the solution and then kept at 4 °C.

Almost pure 2-ketogluconate (0.8 g, yield: 40%) was obtained. Recrystallization of 70% pure of 2-ketogalactonate (0.9 g) utilizing a similar method gave pure 2-ketogalactonate (0.4 g, yield: 20%) and its lactone (0.1 g).

2.6. Purification of 2-ketogalactonate reductase from P. fluorescens

All operations were done at 0–5 °C, and 0.01 M potassium phosphate buffer (pH 7.0), containing 0.01% 2-mercaptoethanol (the buffer) was used. Column chromatography was done at room temperature (about 10 °C).

2.6.1. Step 1: preparation of cell-free extracts

Washed cells (wet weight, 10 g) were suspended in 70 ml of buffer, and 40 ml samples were sonicated with a TOMMY SEIKO ultrasonic disruptor model UR-200p for 10 min. The intact cells and cell debris were removed by centrifugation at 10,000 × *g* for 30 min. The supernatant solution obtained containing 2-ketogalactonate reductase was then treated as follows.

2.6.2. Step 2: ammonium sulfate fractionation

Solid ammonium sulfate was added to the supernatant solution up to 30% saturation. After standing for 20 min at 4 °C, the precipitate was removed by centrifugation at 10,000 × *g* for 30 min and discarded. The ammonium sulfate concentration of the supernatant solution was 60% saturation by the addition of solid ammonium sulfate. The precipitate was collected by centrifugation at 10,000 × *g* for 30 min after standing for 20 min at 4 °C, and then dissolved in the buffer (50 ml).

2.6.3. Step 3: octyl sepharose 4 fast flow column chromatography

The enzyme solution (50 ml), suspended with ammonium sulfate (20% saturation), was put on a column (0.8 cm × 16 cm), and equilibrated with buffer supplemented with ammonium sulfate (20% saturation). After the column was washed with the same buffer, the enzyme was eluted with the buffer supplemented with ammonium sulfate (10% saturation). Active fractions were collected (22 ml) and dialyzed against the buffer (500 ml) overnight.

2.6.4. Step 4: DEAE-sepharose Cl-6B column chromatography

The enzyme solution (32 ml) was charged to a DEAE-sepharose Cl-6B column (1.5 cm × 10 cm) and washed with the buffer. The enzyme was eluted through fractions. The pooled fractions were then concentrated (5 ml).

2.6.5. Step 5: cellulofine column chromatography

The enzyme solution (4.8 ml) was put on a cellulofine GCL-2000-m (2.8 cm × 46 cm) that had been equilibrated with the buffer, and the enzyme was then

eluted with the buffer. The active fractions were collected.

2.6.6. Step 6: second DEAE-sepharose Cl-6B column chromatography

The enzyme solution after gel-filtration, was rechromatographed in the same manner as the first DEAE-sepharose Cl-6B column chromatography. In this case, the enzyme was eluted with the buffer containing 0.1 M KCl.

3. Results

3.1. Formation of 2-keto-D-galactonate and 2-keto-D-gluconate from D-galactose and D-glucose by cell culture of P. fluorescens

Cell cultures of *P. fluorescens* containing galactose were incubated at 28 °C and the time courses of the decreases of galactose and increases of 2-ketogalactonate were measured (Fig. 2). Almost complete conversion was obtained under the conditions established. Further, TLC of the reaction mixture showed the complete conversion of galactose to 2-ketogalactonate (Fig. 3). With glucose, similar results were obtained though the reaction time was shorter than that for galactose (data not shown). IR spectra of crystallized 2-ketogalactonate and 2-ketogluconat are shown in Fig. 4. The formation of 2-ketogalactonate and 2-ketogluconate were also confirmed by these spectra.

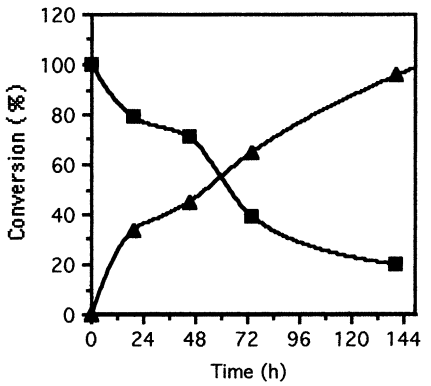

Fig. 2. Time course of formation of 2-ketogalactonate from D-galactose: galactose (■) and 2-ketogalactonate (▲). Experimental conditions as described in Section 2.

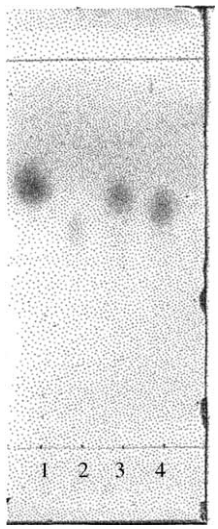

Fig. 3. TLC of crystallized 2-ketogalactonate: (1) galactose, (2) galactonate, (3) 2-ketogluconate, and (4) sample (2-ketogalactonate). Conditions as described in Section 2.

3.2. Purification of 2-ketogalactonate reductase from P. fluorescens

2-Ketogalactonate reductase was purified as described in Section 2. Native-PAGE of the purified enzyme showed several protein bands. The enzyme was determined by activity staining (Fig. 5). The purification steps are showed in Table 1. The enzyme was purified about 44 times at 6.8% yield.

3.2.1. Enzymatic properties

The molecular mass of the enzyme was determined to be 80 kDa by cellulofine column chromatography. Standard proteins (BSA, 66 kDa; LADH 40 kDa; chymotrypsinogen, 25.2 kDa) were used as markers for the preparation of the calibration curve; from which the molecular weight was estimated (Fig. 6). The pH optimum for 2-ketogalctonate reductase was determined. The dehydrogenase reaction (gluconate to 2-ketogluconate, or galactonate to 2-ketogalactonate) exhibits pH 9.0, while the reduced reaction (2-ketogluconate to gluconate and 2-ketogalactonate to galactonate) exhibits pH 6.5. But galactono lactone to 2-ketogalactono lactone exhibits pH 10.0 (Fig. 7). D-Glucose, D-galactose, D-mannose, and D-sorbitol were not substrates for the enzyme. NADP and NADPH are the main cofactors for the

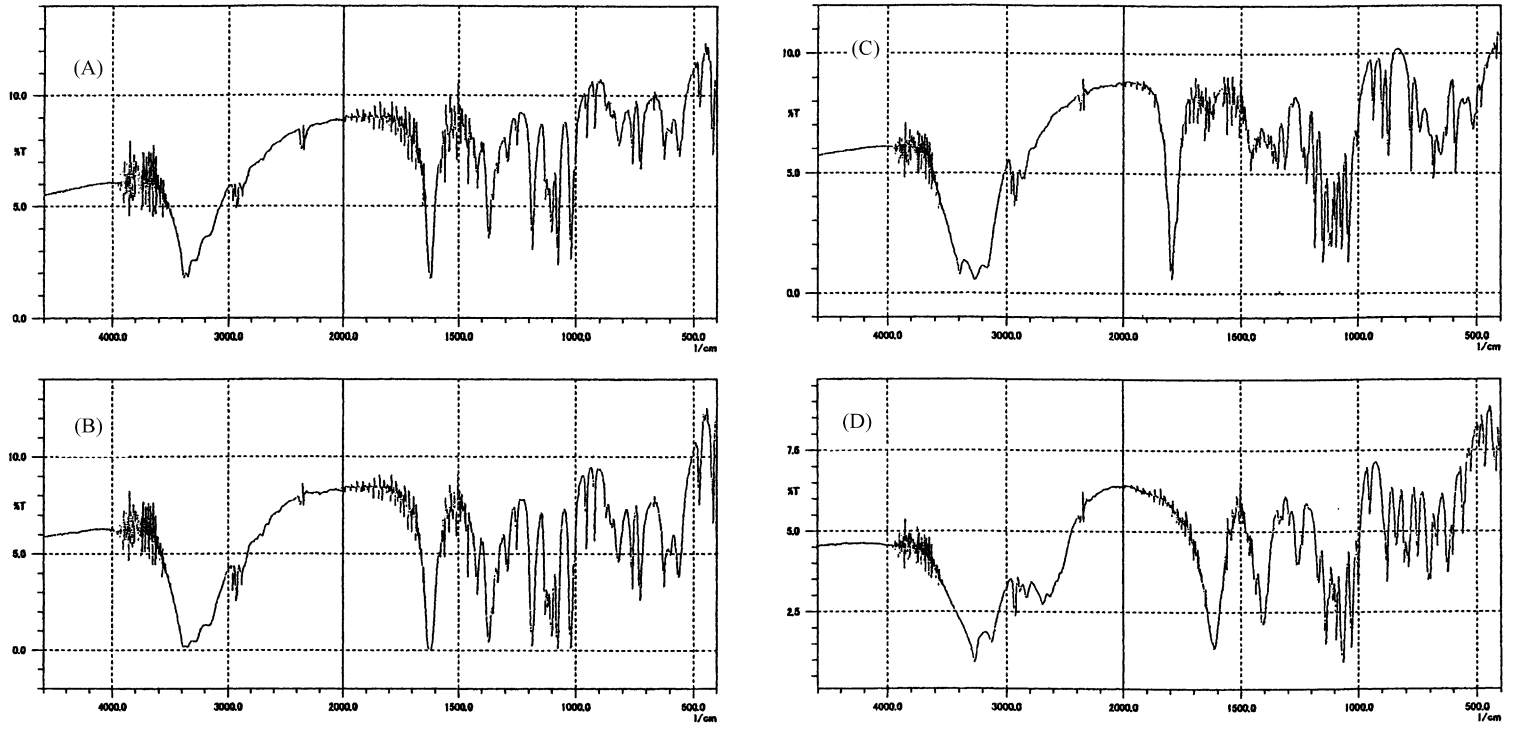

Fig. 4. IR spectra of 2-ketogluconate standard (A), crystallized 2-ketogluconate (B), galactono lactone (C), and crystallized 2-ketogalactonate (D).

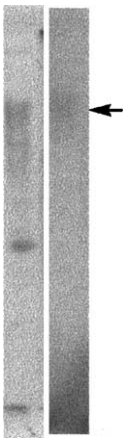

Fig. 5. Native-PAGE of purified enzyme: a sample (after 2nd DEAE-sepharose Cl-6B enzyme; 7 μg) was electrophoresed using 7.5% polyacrylamide gel. After the run, protein was stained with 1% CBB in 7% acetic acid (left). Activity staining of phenazin methosulfate and NBT [9] was undertaken (right column).

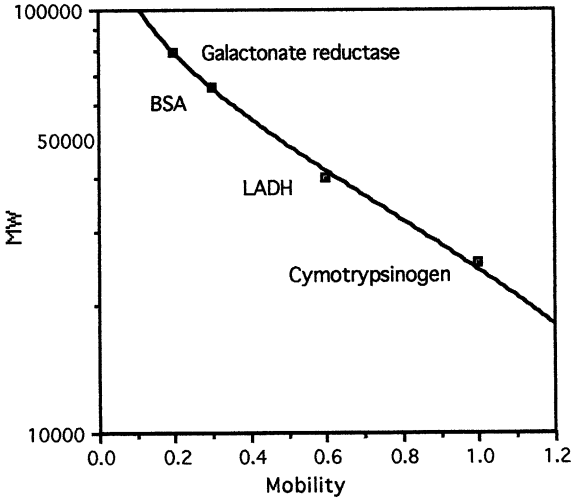

Fig. 6. Estimation of molecular mass by gel filtration. The enzyme and marker proteins were applied on a cellulofine column (0.8 cm × 25 cm) and eluted with the buffer.

enzyme. NAD reacted 10.9% of NADP for oxidation of gluconate, and NADH reacted 8.5% of NADPH for reduction reaction of 2-ketogluconate. The inhibition against oxidation reaction of gluconate was studied (Table 2). The enzyme was inhibited a little by EDTA, Mg^{2+}, Ca^{2+}, and 77.5% by Zn^{2+}. Mn^{2+} and monoiodate acetic acid activated enzyme a little.

3.2.2. Kinetic constants for substrate and cofactor

K_m and V_{max} values were determined by the Linewaver-Burk method of the Michaelis–Menten equation (Table 3). Relatively large K_m values for gluconate and galactonate (31.6 mM and 31.4 mM, respectively), and similar values for NADP and NADPH (0.27 μM and 0.14 μM, respectively) were determined. The V_{max} of reduction step was larger than the oxidation step (about 22 times for 2-ketogluconate and 30 times for 2-ketogalactonate).

3.3. Small-scale reaction of oxidation of galactonate lactone by the partially purified 2-ketogalactonate reductase

Preparation of 2-ketogalctonate from D-galactono lactone was performed by small-scale reaction (similar to activity assay conditions, but 2.5 times the amount of enzyme was used). Incubation of the reaction mixture at 37 °C for 16 h, then formation of product was determined by TLC as described in Section 2. Most galactono lactone was converted to 2-ketogalactonate (data not shown). To scale up the production of 2-ketogalactonate from galactono lactone that was obtained commercially, NADPH

Table 1
Purification of 2-ketogalactonate reductase

Step	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Yield (%)
Cell-free extract[a]	790	1400	1.8	100
Octyl sepharose 4 F	64	650	10	46
1st DEAE-sepharose Cl-6B	34	260	7.6	19
Cellulofine	11	140	13	10
2nd DEAE-sepharose Cl-6B	1.2	95	79	6.8

[a] In membrane fractions, no 2-ketogalactonate reductase activity was shown.

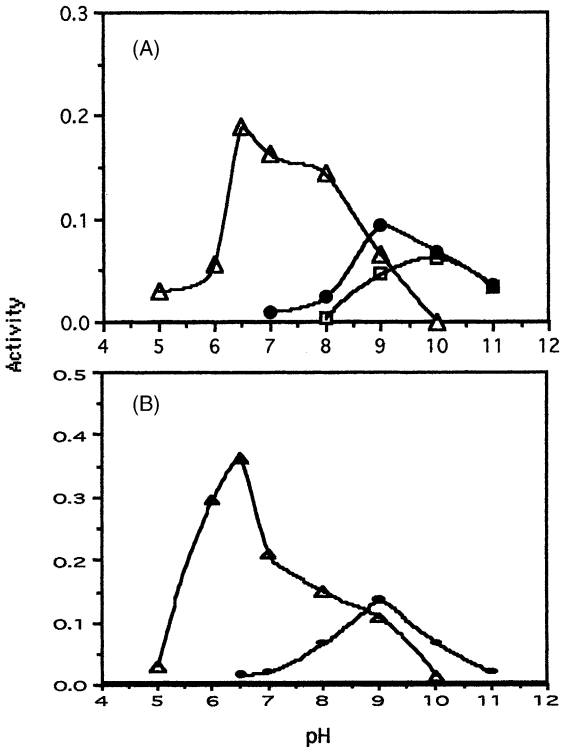

Fig. 7. Effect of pH on enzyme activity: (A) galactonate (●), galactono lactone (□), and 2-ketogalactonate (△) were used as substrates; (B) gluconate (●) and 2-ketogluconate (△) were used as substrates. 6.8 and 1.7 μg of purified enzymes were used for oxidation and reduction reactions, respectively. Activity was measured under the standard assay conditions using the following buffers: KMES (pH 5–6.5), potassium phosphate (pH 7–8), Na_2CO_3–HCl (pH 9–11).

Table 2
Effect of inhibitors on enzyme activity

Inhibitor (mM)	Relative activity (%)
EDTA (0.65)	77.5
$MgSO_4$ (0.65)	76.1
$CaCl_2$ (0.26)	84.5
$ZnCl_2$ (0.26)	22.5
$MnCl_2$ (0.26)	117
ICH_2COOH (1.3)	106

4.9 μg of the enzyme was used. Enzyme activity was measured under the standard assay conditions with addition of each inhibitor. The reaction was initiated by additions of substrate and NADP after the enzyme was preincubated with each inhibitor.

Table 3
Kinetic constant of 2-ketogalactonate reductase

	K_m			V_{max} (U/mg)
	Substrate	NADP	NADPH	
GlcA	32 mM	0.27 μM		0.36
GalA	31 mM	ND		0.55
GalA lactone	210 mM	0.13 μM		1.6
2-KGlcA	31 mM		0.14 μM	7.9
2-KgalA	15 mM		0.11 μM	16

ND: not determined; GlcA: gluconate; GalA: galactonate; 2-KGlcA: 2-ketogluconate; 2-KGalA: 2-ketogalactonate.

recycling methods and purification of the product are presently under investigation.

4. Discussion

Oxidation bacteria such as acetic acid bacteria, *Klebseilla*, *Pseudomonas*, and *Serratia* were reported, in that D-glucose oxidized to D-gluconate and 2-ketogluconate, in nonphosphorylation pathway [10]. 2-Ketogluconate reductase was reported by the presence of two types in oxidative bacteria [11–13]. One is membrane bound, and depends on cytochrom c-flavin, and the other is soluble enzyme that is dependent on NAD (P) of *Acetobacter* and *Gluconobacter* cells. The biosynthesis of 2-ketogluconate from D-gluconate was catalyzed by the membrane bound enzyme not by the soluble enzyme was reported. Because specificity of the membrane bound enzyme is high, the enzyme did not catalyze the conversion of D-galactonate to 2-ketogalactonate. We propose that soluble 2-ketogalactonate reductase (D-galactonate 2-dehydrogenase) may be a main enzyme in the biosynthesis of 2-ketogalactonate from D-galactose in these cells. In this study, the cell culture of *P. fluorescens* was used for the preparation of 2-ketogluconate and 2-ketogalactonate from D-glucose and D-galactose. These products were obtained in about 40% (glucose) or 20% (galactose) yield, but the reaction was over 90% of completion in the reaction mixture by the measurements of the product by Semicarbazide and Orcinol methods, so the conditions of crystallization (purification) must be improved. In some *Acetobacter*, the gluconate pathway is present both in the membrane and the soluble

Table 4
Comparison of relative activities between oxidation (GlcA to 2-KGlcA) and reduction (2-KGlcA to GlcA) reactions

	Oxidation		Reduction	
Gluconobacter liquefaciens	GlcA	67.6	2-KGlcA	100
	GalA	47.3	2-KGalA	79.7
Acetobacter rancens	GlcA	9.0	2-KGlcA	100
	GacA	6.0	2-KGalA	177
Pseudomonas fluorescens	GlcA	4.6	2-KGlcA	100
	GalA	7.0	2-KGalA	208
	GalA lactone	20.7		

GlcA: gluconate; GalA: galactonate; 2-KGlcA: 2-ketogluconate; 2-KGalA: 2-ketogalactonate.

fraction, though at a lower level than in *Gluconobacter*. Comparing the oxidation and reduction activities of gluconate reductase (Table 4), the *P. fluorescens* used in this work belongs to the *Acetobacter* type of gluconate pathway: a low level of gluconate reductase is present in the soluble fractions, and the reduction of 2-ketogluconate to gluconate is much higher than the reverse reaction. But interestingly, in the *P. fluorescens* used, galactonate and 2-ketogactonate are better substrates than gluconate or 2-ketogluconate. We called this enzyme D-galactonate reductase. The molecular mass of the purified 2-ketogalactonate reductase is 80 kDa (information regarding the subunit is not yet known). The effect of EDTA, Zn^{2+}, and Mn^{2+} on the enzyme activity suggested that some metals are concerted to the active sites. Further purification and sequence analyses of the enzyme are needed for the activity–structure relationship to be ascertained.

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