2-Keto-L-gulonic Acid Fermentation

Part IV. L-Sorbose Metabolism in *Pseudomonas aeruginosa*

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L-Sorbose metabolism in *Pseudomonas aeruginosa* IFO 3898 was studied. When the strain was cultivated in L-sorbose medium, L-idonic and 2-keto-L-gulonic acids were detected in the culture broth.

From the results on the metabolism of various sugars and sugar acids with the cell suspension and the metabolites accumulated, the following pathway was proposed for the L-sorbose metabolism in *Ps. aeruginosa* IFO 3898.

L-Sorbose $\longrightarrow$ L-idose $\longrightarrow$ L-idonic acid $\longrightarrow$ 2-keto-L-gulonic acid.

It has been reported by Tengerdy¹⁾ and Huang²⁾ that L-sorbose is metabolized by many pseudomonads to 2-keto-L-gulonic acid.

Huang²⁾ described that *Pseudomonas aeruginosa* was not able to produce the acid from L-sorbose. No detailed study on the mechanism of 2-keto-L-gulonic acid formation from L-sorbose has been carried out except our previous studies^{3,4)} in which the metabolic sequence of sorbitol and L-sorbose in acetic acid bacteria was presented.

Subsequent studies in this series³⁻⁵⁾ demonstrated that organisms belonging to *Ps. aeruginosa* which had been described by Huang as a non-producer of 2-keto-L-gulonic acid from L-sorbose were the most prominent bacteria among pseudomonads for the production of

2-keto-L-gulonic acid. The present paper deals with the identification of L-sorbose metabolites and the possible metabolic pathway of L-sorbose in *Ps. aeruginosa* IFO 3898 which was the most active strain in the L-sorbose metabolism.

MATERIALS AND METHODS

Organism and cultivation. *Ps. aeruginosa* IFO 3898 was used throughout this study. The seed medium consisted of 1.0% of L-sorbose and 2.5% of corn steep liquor, and the main medium was composed of 5.0% of L-sorbose, 2.5% of corn steep liquor, 0.01% of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01% of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03% of KH_2PO_4 , 0.07% of K_2HPO_4 , 0.05% of NaCl and 0.05% of glycerol.

The seed culture was grown in a 200 ml conical flask containing 20 ml of the seed medium at 30°C for 24 hr on a rotary shaker.

One ml of the seed was transplanted into a 200 ml conical flask containing 20 ml of the main medium and the flask was incubated at 30°C for an appropriate period on the rotary shaker.

The 120 hr culture was used for the isolation and identification of the metabolites and the 40 hr culture was employed for the preparation of cell suspensions, respectively.

1) R. P. Tengerdy, *J. Biochem. Microbiol. Technol. Eng.*, **3**, 241 (1961).

2) H. T. Huang, U. S. Patent, 3043749 (1962).

3) H. Okazaki, T. Kanzaki, M. Doi, K. Nara and K. Motizuki, *Agr. Biol. Chem.*, **32**, 1250 (1968).

4) H. Okazaki, T. Kanzaki, K. Sasajima and Y. Terada, *ibid.*, **33**, 207 (1969).

5) M. Isono, I. Nakanishi, K. Sasajima, K. Motizuki, T. Kanzaki, H. Okazaki and H. Yoshino, *Agr. Biol. Chem.*, **32**, 424 (1968).

Preparation of cell suspensions. After a mild centrifugation (10 min, $1000\times g$) to separate large solid materials, the 40 hr culture was recentrifuged for 10 min at $15,000\times g$.

The sedimented cells were washed three times with 0.9% saline solution and suspended in 0.2 M phosphate buffer (pH 6.5) amounting to one tenth in volume to the initial culture broth.

Incubation with intact cells. A test tube containing 1 ml of 3% substrate solution, 1 ml of 0.2 M phosphate buffer (pH 6.5) and 1 ml of the cell suspension was incubated at 30°C for 12 or 24 hr with shaking under aerobic conditions.

Cells were removed from the mixture by centrifugation after the reaction had terminated. The resulted clear supernatant was treated with Amberlite IR-120, H⁺-form to eliminate cations and submitted to paper chromatography or paper electrophoresis.

For the detection of metabolites, silver nitrate reagent⁶⁾ was employed.

Separation of metabolites. Separation of metabolites was performed by ion-exchange column chromatography with a linear gradient elution of formic acid as described in the previous paper.³⁾

Paper chromatography and paper electrophoresis. Toyo Roshi No. 51A was used for both paper chromatography and paper electrophoresis.

Paper chromatography was carried out using the following solvent systems by ascending method: water saturated phenol; pyridine-ethylacetate-acetic acid-water (5:5:1:3) and *n*-butanolacetic acid-water (4:1:1).

Paper electrophoresis was conducted in 0.1 M formate buffer (pH 3.0) for 45 min at 50 v/cm and 1 mA/cm.

RESULTS

Fractionation of metabolites

After one liter of the culture broth was centrifuged, the supernatant was passed through a column of Amberlite IR-120 H⁺-form to eliminate cations and treated with small amounts of activated carbon to remove colored materials.

To separate neutral and acidic components

each other, the solution was added to a column of Amberlite IR-45 OH⁻-form (4×40 cm).

The acidic components were adsorbed on the column while neutral components passed through the column (Fraction I).

The acidic components were eluted from the column with 0.5% ammonium hydroxide and the eluate was concentrated *in vacuo* to a syrup. The syrup was chromatographed on Amberlite CG-400, formate-form (3×40 cm) with a linear gradient elution of formic acid up to 1.0 N, and the eluate was fractionated into every 20 ml portions. Fractions II and III were tube numbers 30 to 35 and 50 to 61, respectively.

The paper chromatogram and the paper electrophoregram of the culture filtrate, Fractions II and III are presented in Fig. 1.

Isolation and identification of neutral sugar

Fraction I was concentrated to a syrup *in vacuo*, and chilled in a refrigerator to give crystals. Recrystallization from water; mp 156°C, $[\alpha]_D^{22} -42^\circ$ ($c=1.0$ in water). *Anal.* Found: C, 40.02; H, 6.70. Calcd. for C₆H₁₂O₆: C, 40.00; H, 6.71%.

These characteristics were the same as those of authentic L-sorbose. The infrared spectrum of the crystals in KBr disc was also identical with that of L-sorbose.

Isolation and identification of hexonic acid

As shown in Fig. 2, Fraction II contained only one component behaving as L-idonic acid in paper chromatography using the solvent systems of pyridine-ethylacetate-acetic acid-water (5:5:1:3) and *n*-butanol-acetic acid-water (4:1:1).

To isolate the component, Fraction II was concentrated *in vacuo* to a syrup, neutralized with sodium hydroxide and supplemented with ethanol until the solution became faintly cloudy.

Crystals were obtained by standing the solution in a refrigerator. Recrystallization was performed in the ethanol solution in the same

6) W. W. Trevelyan, D. P. Procter and J. S. Harrison, *Nature*, **166**, 444 (1950).

FIG. 1. Paper Chromatogram and Paper Electrophoregram of Culture Filtrate, Fractions I, II and III.

A: Paper chromatogram. Solvent: water-saturated phenol

B: Paper electrophoregram. Solvent: 0.1 M formate buffer (pH 3.0)

1: culture filtrate, 2: Fraction I, 3: Fraction II, 4: Fraction III.

Other abbreviations are the same as those in Fig. 4.

FIG. 2. Paper Chromatograms of Fraction II.

A: Pyridine, ethylacetate, acetic acid, water (5:5:1:3)

B: *n*-Butanol, acetic acid, water (4:1:1)

1: Fraction II, 2: L-idonolactone,

3: D-mannonolactone

4: L-gulonolactone, 5: D-gluconolactone.

Fraction II was treated with acid to give the lactone of the component.

method mentioned above; mp 179°C, $[\alpha]_D^{24} + 8.0^\circ$ ($c=1.0$ in H_2O). *Anal.* Found: C, 30.58; H, 5.49. Calcd. for $C_6H_{11}O_7 \cdot Na \cdot H_2O$: C, 30.52; H, 5.55%. These characteristics indicate that the crystals are sodium L-idonate.

The infrared spectrum of the crystals (Fig. 3) also shows that they are identical with the authentic sodium L-idonate.

Isolation and identification of ketohexonic acid

The component of Fraction III behaved as

2-keto-L-gulonic acid by paper electrophoresis as shown in Fig. 1 and 2-keto-D-gluconic acid which was detected in case of acetic acid bacteria³⁾ was not demonstrated.

Fraction III was concentrated *in vacuo* to give a syrup followed by neutralization with sodium hydroxide. After the solution was supplemented with ethanol until 70% (v/v) concentration, the crystals were obtained by standing the ethanol solution in a refrigerator. These were recrystallized by the same method mentioned above and submitted to physical analyses; mp 145°C, $[\alpha]_D^{22} - 25.5^\circ$ ($c=2.0$ in water). *Anal.* Found: C, 30.95; H, 4.73. Calcd. for $C_6H_9O_7 \cdot Na \cdot H_2O$: C, 30.78; H, 4.77%.

The infrared spectrum of the crystals in KBr disc shows that they are identical with that of authentic sodium 2-keto-L-gulonate.

Oxidation of various substrates by intact cells

To examine the metabolic pattern of sugars and sugar acids by *Ps. aeruginosa* IFO 3898, various substrates were incubated with the intact cells under aerobic conditions.

The data in Figs. 4 and 5 lead us to conclude that L-sorbose was metabolized to 2-keto-L-gulonic acid, but D-fructose was probably degraded completely during 12 or 24 hr incubation since any metabolite was not detected in the reaction mixture. D-Mannose

FIG. 3. Infrared Spectra of Crystals from Fraction II and Crystalline Sodium L-Idonate. (KBr disk)

A: Crystals from Fraction II, B: Crystalline sodium L-idonate.

FIG. 4. Paper Electrophoregrams of Incubation Products.

S : neutral sugar, SL: sorbitol, SR: L-sorbose, F: D-fructose, M: D-mannose, G : D-glucose, HA: hexonic acid, IA: L-idonic acid, MA: D-mannonic acid, GA: D-gluconic acid, GUA: L-gulonic acid, KG: 2-keto-D-gluconic acid, KU: 2-keto-L-gulonic acid, 5KG: 5-keto-D-gluconic acid, 5KM: 5-keto-D-mannonic acid.

gave a substance corresponding to D-mannonic acid. In case of D-glucose, a substance behaving as 2-keto-D-gluconic acid was formed after 12 hr incubation but the greater part of it disappeared after 24 hr incubation. This might be due to a further degradation of the acid to substances other than sugars and sugar acids. Sorbitol was not metabolized.

In sugar acids, substances corresponding to 2-keto-L-gulonic acid and 2-keto-D-gluconic acid were produced from L-idonic acid and D-gluconic acid, respectively.

In case of D-mannonic, L-gulonic and 2-keto-L-gulonic acids, no changes occurred. 2-Keto-D-gluconic acid disappeared rapidly from the reaction mixture.

DISCUSSION

The results presented in this paper show that *Ps. aeruginosa* IFO 3898 accumulates L-idonic and 2-keto-L-gulonic acids in the culture broth when grown on L-sorbose.

On the other hand, as described in the pre-

FIG. 5. Paper Chromatograms of Incubation Products.

Abbreviations are the same as those in Fig. 4.

Solvent: water-saturated phenol.

1: D-mannonolactone, 2: L-gulonolactone,

3: L-idonolactone and D-gluconolactone.

vious papers, acetic acid bacteria belonging to *Pseudomonadales* produced L-sorbose, D-fructose, D-mannonic, L-idonic, 2-keto-D-gluconic, 2-keto-L-gulonic and 5-keto-D-mannonic acids from sorbitol,³⁾ and D-fructose and all of the sugar acids described above were produced from L-sorbose,⁴⁾ respectively. The metabolic activity of the present strain was different from that of *Gluconobacter melanogenus* in respect that the former could not metabolize sorbitol and did not accumulate D-fructose, D-mannonic, 2-keto-D-gluconic, and 5-keto-D-mannonic acids from L-sorbose in the culture broth.

Both organisms however accumulate L-idonic and 2-keto-L-gulonic acids from L-sorbose.

Although D-fructose formation from L-sorbose was not clear in the present paper, the facts that *Ps. aeruginosa* IFO 3898 could grow on L-sorbose and D-fructose disappeared rapidly from the reaction mixture and that L-sorbose was metabolized *via* D-fructose in acetic acid bacteria⁴⁾ might suggest the possibility of D-fructose formation from L-sorbose by *Ps. aeruginosa* IFO 3898.

Under the experimental conditions, D-mannose- or D-mannonic acid-forming system was presumed to be absent in the present strain

since D-mannose, D-mannonic and 2-keto-D-gluconic acids were not detected in the broth and also D-mannonic acid was formed and accumulated from D-mannose with intact cells.

As for the 2-keto-L-gulonic acid formation, it seems reasonable to assume that it is formed *via* L-idonic acid because it is formed from L-idonic acid but not from L-gulonic acid.

The results and possibilities described above suggest that the L-sorbose metabolism in *Ps. aeruginosa* IFO 3898 involves the following sequence; L-sorbose $\rightarrow$ L-idose $\rightarrow$ L-idonic acid $\rightarrow$ 2-keto-L-gulonic acid.

L-Idose is a possible intermediate and exists between L-sorbose and L-idonic acid based on the studies^{3,4)} about the metabolic sequence of sorbitol and L-sorbose in acetic acid bacteria.

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