NITRILASE-CATALYZED PRODUCTION OF PYRAZINOIC ACID, AN ANTIMYCOBACTERIAL AGENT, FROM CYANOPYRAZINE BY RESTING CELLS OF *RHODOCOCCUS RHODOCHROUS* J1

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Using resting cells of *Rhodococcus rhodochrous* J1, in which a large amount of nitrilase is induced, a simple and efficient bioconversion process for the production of pyrazinoic acid, an antimycobacterial agent, through catalysis by a nitrilase was developed. The reaction conditions for production of pyrazinoic acid were optimized. Under optimum conditions, 3.5 M cyanopyrazine was converted to pyrazinoic acid, with a molar conversion yield of 100%. The highest yield achieved corresponded to 434 g of pyrazinoic acid per liter of reaction mixture. The synthesized pyrazinoic acid was isolated and identified physico-chemically.

Various nitrile compounds are manufactured as starting materials for industrially important compounds. Nitriles are normally hydrolyzed to the corresponding acids through a chemical process. However, the chemical conversion of nitriles has several disadvantages: Reactions require either strongly acidic or basic media, energy consumption is high, and unwanted by-products (toxic substances such as hydrogen cyanide or large amounts of salt) are formed. The application of enzymes to organic chemical processing is attracting increasing attention. The advantages of bioconversions are that the pH and temperature conditions are less severe than those for chemical processes, and very pure products are obtained without by-products. In addition, microbial conversions are characteristics as to stereo- and regio-specificities.

Nitrilase and nitrile hydratase have great potential as catalysts for the processing of organic chemicals because they can convert nitriles to the corresponding higher-value acids or amides. Recently, we optimized culture conditions for the preparation of *Rhodococcus rhodochrous* J1 cells with high nitrilase activity[1] and established the optimal reaction conditions for the production of nicotinic acid from 3-cyanopyridine using resting cells[2]. We have already purified and crystallized the *R. rhodochrous* J1 nitrilase[3].

Pyrazinoic acid has been reported to be an antimicrobial agent[4,5] against *Mycobacterium tuberculosis* and is used as a precursor of pyrazinamide, an active pharmaceutical compound. In the present paper, we describe the microbial transformation of cyanopyrazine to pyrazinoic acid (Eqn. 1) using resting cells of *R. rhodochrous* J1.

(1)

Materials and Methods

Microorganism, Culture Conditions and Preparation of Resting Cells

R. rhodochrous J1, previously isolated and identified in our laboratory[6], was used. Subculture was carried out at 28°C for 24 hours with reciprocal shaking in test tubes containing 4 ml of basal medium

(pH 7.2) consisting of 10 g of glycerol, 5 g of Polypepton (Daigo, Japan), 3 g of malt extract and 3 g of yeast extract (Oriental Yeast, Japan) per liter of tap water. Culture broth in each test tube was added to each 2-liter flask containing 500 ml of the basal medium supplemented with 0.5 ml of isovaleronitrile and incubation was carried out at 28°C with reciprocal shaking. After 57 hours and 78 hours, 0.5 ml and 1.0 ml of isovaleronitrile was added, respectively, and the cultivation was performed for a further 17 hours.

The above cultures (1.5 liters) were pooled and centrifuged at 12,000 × *g* for 30 minutes at 4°C. The harvested cells were washed with 0.1 M potassium phosphate buffer (pH 7.5). The cells were suspended in 150 ml of 0.1 M potassium phosphate buffer (pH 7.5) (32.4 mg (dry weight) of cells per ml). This cell suspension was used for the resting cell reaction.

Assay for Nitrilase Activity

Nitrilase activity in the culture medium was measured as described previously[1,3]. The standard reaction mixture (2 ml) for pyrazinoic acid production contained 1 mmol of cyanopyrazine, 20 μmol of potassium phosphate buffer (pH 8.0), 1 μmol of dithiothreitol and 0.2 ml of the cell suspension (6.5 mg (dry weight) of cells). The reaction was carried out with shaking at 30°C and stopped by adding 0.2 ml of 1 M HCl to the reaction mixture. The amounts of pyrazinoic acid, pyrazinamide and cyanopyrazine were determined by analytical HPLC. HPLC was performed with a Shimadzu LC-5A equipped with an M&S pack C_{18} column (reversed-phase, 4.6 × 150 mm; M&S Instruments Inc., Japan) at a flow rate of 1.0 ml/minute, using the following solvent system: 5 mM KH_2PO_4-H_3PO_4 buffer (pH 2.9)-acetonitrile (4:1). The amount of NH_3 produced in the enzyme reaction was colorimetrically determined by the phenol/hypochlorite method[7] using a Conway micro-diffusion apparatus[8]. One U of nitrilase activity is defined as the amount of cells that catalyzes the formation of 1 μmol of benzoic acid per minute from benzonitrile as a substrate. The specific activity of the cell suspension corresponded to 52 U/mg dry cell weight at 25°C.

Analytical Methods

^1H and ^{13}C NMR spectra were recorded in DMSO with Nihondenshi JNM GX-270 (270 MHz, Japan) and Perkin-Elmer 1710 FT-IR (68 MHz, U.S.A.) spectrophotometers, respectively. Chemical shifts are given in relation to internal standards (TMS and DMSO). IR and electron ionization mass spectra were recorded, respectively, with a Shimadzu IR 27G and a Hitachi M-80 (70 eV, 300 μA). Elemental analysis was carried out with a Perkin-Elmer 240-B (U.S.A.).

Results and Discussion

Accumulation of Pyrazinoic Acid

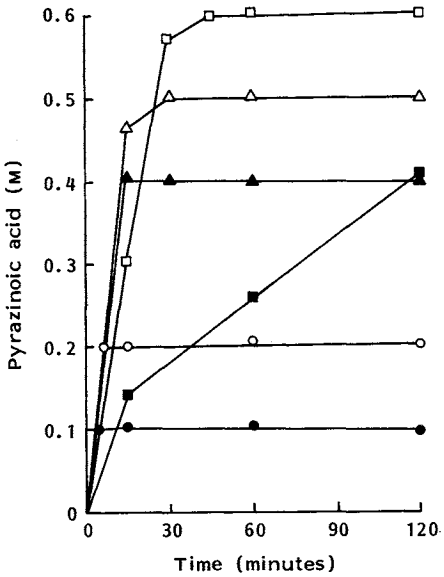
Various reaction parameters for the efficient production of nicotinic acid using *R. rhodochrous* J1 cells, *i.e.*, pH, temperature and nature of the buffer, were previously optimized in our laboratory[2]. The optimum pH for the hydrolysis of 3-cyanopyridine to nicotinic acid had been found to be between 8 and 9, and no change in activity had been observed on replacement of the potassium phosphate buffer, pH 8, by Tris-hydrochloride, H_3BO_3-NaOH or HEPES-KOH buffer. We investigated the effect of pH on the hydrolysis of cyanopyrazine by *R. rhodochrous* J1 nitrilase and obtained similar results. As for the reaction temperature, the optimum temperature for the hydrolysis of 3-cyanopyridine was 45°C[2]. However, since cyanopyrazine is volatile at such a high temprature, the accumulation of pyrazinoic acid was carried out at 30°C.

When cells were added to the reaction mixture at concentrations of 0.65, 1.3 and 3.25 mg/ml, 0.5 M cyanopyrazine was completely converted to pyrazinoic acid within 260, 60 and 30 minutes, respectively. The cell concentration of 3.25 mg/ml was selected for the following experiments.

We examined the inhibitory effect of cyanopyrazine on nitrilase activity (Fig. 1). The rate of synthesis of pyrazinoic acid decreased with an increase in the concentration of cyanopyrazine only at concentrations

Fig. 1. Effect of the concentration of cyanopyrazine on the synthesis of pyrazinoic acid.

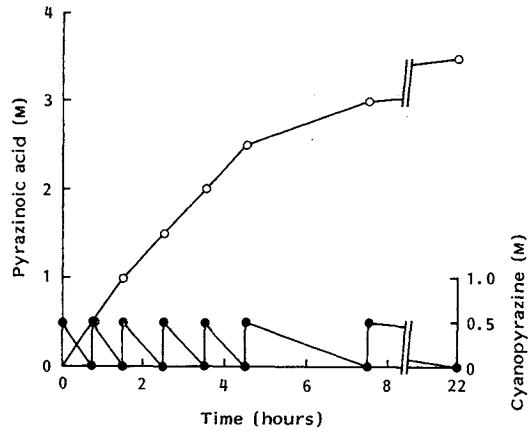
● 0.1 M, ○ 0.2 M, ▲ 0.4 M △ 0.5 M, □ 0.6 M, ■ 0.8 M cyanopyrazine.



The assays were performed as described under Materials and Methods but with different concentrations of cyanopyrazine.

Fig. 2. Accumulation of pyrazinoic acid by resting cells of *Rhodococcus rhodochrous* J1.

● cyanopyrazine, ○ pyrazinoic acid.



The incubation was carried out as described in the text.

higher than 0.5 M. However, even 0.6 M and 0.8 M cyanopyrazine was converted completely to pyrazinoic acid, after 45-minute and 6.5-hour (data not shown) incubation, respectively. Thus, 0.5 M cyanopyrazine was added at one time to the reaction mixture during the course of the reaction in the following experiments to avoid the inhibitory effect due to a high concentration of the substrate.

Next, we investigated the inhibition of the nitrilase activity by the product, pyrazinoic acid. The rate of conversion of 0.5 M cyanopyrazine to pyrazinoic acid was compared in the presence of 1 M to 3.5 M pyrazinoic acid. The conversion rate decreased in parallel with an increase in the concentration of pyrazinoic acid added. However, 0.5 M cyanopyrazine added was converted completely to pyrazinoic acid in a 7-hour incubation, even in the presence of 3.5 M pyrazinoic acid.

High level accumulation of pyrazinoic acid was attempted using resting cells of *R. rhodochrous* J1. The reaction mixture (100 ml) consisted of 50 mmol of cyanopyrazine, 1 mmol of potassium phosphate buffer (pH 8.0), 50 μmol of dithiothreitol and resting cells (390 mg (dry weight) of cells harvested from 120 ml of culture broth). The incubation was performed at 30°C with shaking. During the course of the reaction, 0.5 M cyanopyrazine was fed at intervals. In parallel with an increase in the amount of pyrazinoic acid accumulated, the rate of synthesis of pyrazinoic acid decreased due to product inhibition. With feeding of 0.5 M cyanopyrazine 7 times during a 22-hour incubation, 3.5 M pyrazinoic acid was produced, with a 100% conversion yield (Fig. 2). Ammonia was formed stoichiometrically with the consumption of cyanopyrazine, but no formation of pyrazinamide was detected. The accumulation of pyrazinoic acid corresponded to 434 g/liter of reaction mixture.

Isolation and Identification of Pyrazinoic Acid

In order to isolate the pyrazinoic acid produced, the above reaction mixture was centrifuged briefly and then filtered through a membrane (0.45 μm; Millipore, U.S.A.). The filtrate was applied to a Dowex

1X2 column (2.5×32 cm, OH^- form). After washing of the column with distilled water and then with 0.1 M acetic acid, the synthesized pyrazinoic acid was eluted with 0.5 M acetic acid. The fractions containing pyrazinoic acid were pooled and then concentrated *in vacuo* at 40°C. The concentrated residue was dissolved in a small amount of distilled water and then kept on ice overnight. White crystals of pyrazinoic acid appeared. Recrystallization was carried out in the same manner. The crystals formed were collected by filtration and then dried in a vacuum desiccator over P_2O_5. 39.4 g of pure crystalline pyrazinoic acid was obtained.

The identification of this product was performed by comparison with the ^1H, ^{13}C NMR, IR and EI mass spectra of authentic pyrazinoic acid. The ^1H, ^{13}C NMR and IR spectra of the product agreed very closely with those of authentic pyrazinoic acid. The analytical data were as follows: ^1H NMR (DMSO) δ 8.87 (2H, m), 9.23 (1H, s), 13.75 (1H, br s); ^{13}C NMR (DMSO) δ 144 (s, C-2), 145 (d, C-3), 146 (d, C-5), 148 (d, C-6), 166 (s, *C*OOH); IR (KBr) cm^{-1} 1740 (C=O), 1400 (O–H), 1320 (C=O); EI mass spectrum *m/z* 124 (M^+, 33), 106 (7), 80 (100); elemental analysis of the synthesized pyrazinoic acid was carried out (*Anal* Calcd for $C_5H_4N_2O_2$: C 48.39, H 3.25, N 22.57, O 25.78. Found: C 48.54, H 3.26, N 22.58). The disagreement between the calculated and found values was within the usual limits of variation for elemental analysis.

Pyrazinoic acid is chemically produced through the monodecarboxylation of pyrazine-2,3-dicarboxylic acid[9] or through the oxidation of alkylpyrazines[10] in the presence of a catalyst. However, these conventional procedures are not completely satisfactory in several respects: Several steps are required for the synthesis of pyrazinoic acid, the yield is low (30 to 60%), and the pyrazinoic acid produced is contaminated by the catalyst. The biological conversion of cyanopyrazine to pyrazinoic acid proceeds smoothly under mild conditions, with a 100% molar conversion yield, without the formation of any undesirable secondary products. The pyrazinoic acid synthesized can be easily purified. It is also noteworthy that the production of pyrazinoic acid (434 g/liter) catalyzed by *Rhodococcus* nitrilase surpassed that of nicotinic acid described previously (172 g/liter)[2]. Thus, an economical synthetic method for pyrazinoic acid has been established with the combination of the present microbiological synthsis and the chemical production of cyanopyrazine. *R. rhodochrous* J1 produces an enormous amount of nitrilase, corresponding to about 30% of the soluble protein in cells cultivated under optimum culture conditions[1]. The *R. rhodochrous* J1 cells can be stored for more than 1 year at −20°C without loss of activity. In addition, the nitrilase acts on various kinds of aromatic and heterocyclic nitriles[3]. In addition to its activity towards benzonitrile (100%), the *Rhodococcus* nitrilase also acts on cyanopyrazine as a suitable substrate (relative rate, 37.3%), and therefore the enzyme appears to be a promising catalyst for the production of various acids from nitriles.

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