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MONOHYDROLYSIS OF AN ALIPHATIC DINITRILE COMPOUND BY NITRILASE FROM RHODOCOCCUS RHODOCHROUS K22

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Summary: Nitrilase from Rhodococcus rhodochrous K22 catalyzes the conversion of various aliphatic nitrile derivatives to the corresponding acids. Using this resting cells, 4-cyanobutyric acid was synthesized from glutaronitrile with 100% molar conversion.

Various nitrile compounds are manufactured as starting materials for industrially important compounds. Nitriles are hydrolyzed to the corresponding acids or amides 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 such as large amounts of salts are formed. Our attention is currently focused on the application of enzymes to organic chemical processing. 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 secondary products. In addition, microbial conversions are characteristic as to stereo- and regio-specificities.

Nitrilase and nitrile hydratase are expected to 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, the use of Pseudomonas chlororaphis B23 nitrile hydratase, which was found in our laboratory, for the industrial production (6,000 tonnes per year) of the important chemical commodity, acrylamide, was pioneered in Japan[1]. We optimized the culture conditions for the preparation of

Rhodococcus (R.) rhodochrous J1 cells with high nitrilase activity[2] a established the optimal reaction conditions for the production of nicoti acid[3] and p-aminobenzoic acid[4] using resting cells. We have alre purified and crystallized the R. rhodochrous J1 nitrilase[5]. Furthermo we reported the regiospecific hydrolysis of aromatic dinitriles, using t R. rhodochrous J1 nitrilase[6].

Nitrilases that utilize benzonitrile and related aromatic nitriles substrates have been purified from Pseudomonas[7], Nocardia[8], Fusarium Arthrobacter[10], R. rhodochrous J1[5], and Escherichia coli transformed w a Klebsiella ozaenae plasmid DNA[11]. However, aliphatic nitriles a inert as substrates for all these nitrilases. To best of our knowled no nitrilases which act on aliphatic nitriles are previously reporte Very recently, we found the occurrence of high aliphatic nitril hydrolyzing activity in cells of Rhodococcus (R.) rhodochrous K22. In t present paper, we describe the monohydrolysis of an aliphatic dinitri i.e., glutaronitrile, using the R. rhodochrous K22 nitrilase.

$$NC\text{-}(CH_2)_3\text{-}CN + 2H_2O \longrightarrow NC\text{-}(CH_2)_3\text{-}COOH + NH_3$$

A general procedure employed here is as follows. The reaction mixt (20 ml) consisted of 2 mmol potassium phosphate buffer (pH 8.0), 1 m glutaronitrile, 10 μmol dithiothreitol and 15 nmol (7.2 units) of t enzyme. The reaction was carried out at 30°C in an air-tight Erlenme flask. An aliquot (0.1 ml) of the reaction mixture was taken at appro ate times and the reaction was terminated by adding 50 μl of 1 M HCl.

Throughout the experiment (Fig. 1), glutaronitrile w stoichiometrically hydrolyzed, with the concomitant formation of cyanobutyric acid and ammonia. The substrate, glutaronitrile, w completely consumed in 30 min and quantitatively converted to cyanobutyric acid. The formation of glutaric acid was not detected at a

Compared to that towards crotononitrile (100%), the activity towa glutaronitrile was 345%. Other aliphatic dinitriles, e.g., malononitri

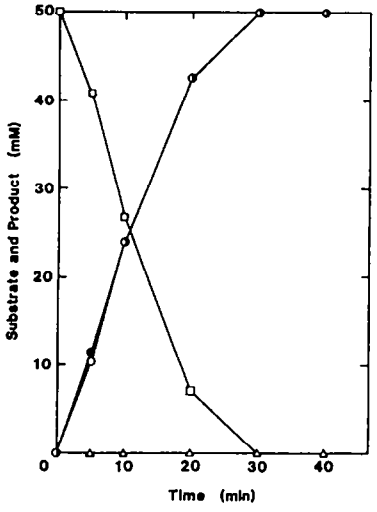

Fig. 1. Time course of 4-cyanobutyric acid synthesis.
Glutaronitrile (□), 4-cyanobutyric acid (●), ammonia (○) and glutaric acid (△).

(45.1%), succinonitrile (271%), adiponitirle (110%), pimelonitrile (27.3%), suberonitrile (21.4%) and sebaconitrile (15.7%) were also attacked by the R. rhodochrous K22 nitrilase (submitted for publication). R. rhodochrous K22 produces enormous amounts of nitrilase and the enzyme is very stable for more than 1 year at -20°C without a loss of activity. In the present study, the Rhodococcus K22 nitrilase was capable of hydrolyzing only one cyano group of aliphatic dinitriles to a carboxyl group. This transformation could also be attained using resting cells of R. rhodochrous K22 (unpublished data). From this point of view, this nitrilase seems to be promising as a useful biocatalyst for the production of mono-cyano-monocarboxylic acids; for example, 5-cyanovaleric acid, as a precursor of nylon-6. An economical synthetic method for cyanocarboxylic acids will be established with the combination of the present enzymatic synthesis and the chemical production of aliphatic dinitriles.

Experimental:

Analysis.
The amounts of glutaronitrile, 4-cyanobutyric acid and glutaric acid in the reaction mixture were determined with a Shimadzu gas-liquid chromatograph, model GC-7A, equipped with a flame ionization detector. A glass column, of 2 mm internal diameter, packed with Porapak type PS (80-100 mesh) was used. The operational conditions were: column temperature, 210°C; and injection and detector temperature, 230°C. N_2 was used as the carrier gas at the flow rate 50 cm^3/min. The integration and calibration of peak areas were carried out with a Shimadzu Chromatopac C-R1B. The amount of NH_3 produced in the enzyme reaction was colorimetrically determined by the phenol/hypochlorite method[12]

using a Conway micro-diffusion apparatus[13].

The ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ with a Nihondenshi JNM GX-2 (270 MHz) and a Perkin Elmer 1710 FT-IR (68 MHz) spectrophotometer, respectivel Chemical shifts are given in relation to an internal standard (tetramethylsilane). and electron ionization mass spectra were recorded, respectively, with a Shimadzu IR 2 and a Hitachi M-80 (70 ev, 300 μA).

Culture conditions and preparation of nitrilase.

R. rhodochrous K22, which was isolated from soil as a crotononitrile-catabolizi microorganism and was identified in our laboratory (submitted for publication), was use The subculture was carried out at 28°C for 36 h with reciprocal shaking in test tub containing 4 ml of basal medium (pH 7.2) consisting of 50 g of sorbitol, 3 g of yea extract, 7 g of NZ amine and 7 g of urea/liter of tap water (pH 7.2). Then the cultu broth in three test tubes (12 ml) was added to each of a 2-liter flask containing 500 of the basal medium supplemented with 0.5 ml of isovaleronitrile and then incubation w carried out at 28°C with reciprocal shaking. After 76 h and 100 h, 0.5 ml and 1.0 ml isovaleronitrile was added, respectively, and the cultivation was performed for a furth 20 h. The R. rhodochrous K22 nitrilase was purified by ammonium sulfate fractionation DEAE-Sephacel, Phenyl-Sepharose and gel filtration column chromatographies. One unit the enzyme is defined as the amount needed to catalyze the formation of 1 μmol croton acid/min from crotononitrile at 25°C.

Isolation and identification of 4-cyanobutyric acid.

The reaction mixture was centrifuged briefly and then filtered through a membra (0.45 μm; Millipore, USA). The pH of the filtrate was adjusted to 11 with 1 M $NaHCO_3$ a then the solution was washed fully with ether. After adjusting the pH to 4 with 1 HCl, the water layer was extracted with ether. The ether layer was evaporated in vac at 45°C and the product was purified. On purification, 100 mg of 4-cyanobutyric ac was obtained. Identification of the product as 4-cyanobutyric acid was established the IR, ^{1}H NMR, ^{13}C NMR and electron ionization mass spectra. The analytical data we as follows: ^{1}H NMR ($CDCl_3$), 2.01(2H, m), 2.50(2H, t), 2.58(2H, t), 7.29(1H, br.s) pp ^{13}C NMR ($CDCl_3$), 16.8, 20.9, 32.3, 119, 178 ppm; electron ionization mass spectrum, m/ 41, 54, 60, 96; IR spectrum (KBr), 1720, 2250, 2900-3300 cm^{-1}.

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References

1. Asano, Y., Yasuda, Y., Tani, Y. and Yamada, H. (1982) Agric. Biol. Chem., **46**, 118 1189.; Ryuno, K., Nagasawa, T. and Yamada, H. (1988) Agric. Biol. Chem., **52**, 1813- 1816.
2. Nagasawa, T., Kobayashi, M. and Yamada, H. (1988) Arch. Microbiol., **150**, 89-94.
3. Mathew, C.D., Nagasawa, T., Kobayashi, M. and Yamada, H. (1988) Appl. Environ. Microbiol., **54**, 1030-1032.
4. Kobayashi, M., Nagasawa, T., Yanaka, N. and Yamada, H. (1989) Biotechnol. Lett., 1 27-30.
5. Kobayashi, M., Nagasawa, T. and Yamada, H. (1989) Eur. J. Biochem., **182**, 349-356.
6. Kobayashi, M., Nagasawa, T. and Yamada, H. (1988) Appl. Microbiol. Biotechnol., 2 231-233.
7. Robinson, W.G. and Hook, R.H. (1964) J. Biol. Chem., **239**, 4257-4262.; Hook, R.H. a Robinson, W.G. (1964) J. Biol. Chem., **239**, 4263-4267.
8. Harper, D.B. (1977) Bichem. J., **165**, 309-319.; Harper, D.B. (1985) Int. J. Biocher **17**, 677-683.
9. Harper, D.B. (1977) Biochem. J., **167**, 685-692.
10. Bandyopadhyay, A.K., Nagasawa, T., Asano, Y., Fujishiro, K., Tani, Y. and Yamada, (1986) Appl. Environ. Microbiol., **51**, 302-306.
11. Stalker, D.M., Malyj, L.D. and McBride, K.E. (1988) J. Biol. Chem., **263**, 6310-631
12. Fawcett, J.K. and Scott, J.E. (1960) J. Clin. Pathol., **13**, 156-159.
13. Conway, E.J. and Byrne, A. (1933) Biochem. J., **27**, 419-429.