NITRILASE-CATALYZED PRODUCTION OF p-AMINOBENZOIC ACID FROM p-AMINOBENZONITRILE WITH RHODOCOCCUS RHODOCHROUS J1

Michihiko Kobayashi*, Toru Nagasawa, Noriyuki Yanaka and Hideaki Yamada

Department of Agricultural Chemistry, Kyoto University, Kitashirakawa, Oiwake-cho, Sakyo-ku, Kyoto 606, Japan

SUMMARY

The reaction conditions for the enzymatic production of p-aminobenzoic acid were optimized, using a nitrilase in cells of Rhodococcus rhodochrous J1. The highest accumulation, 110 g p-aminobenzoic acid /liter of reaction mixture, was attained from p-aminobenzonitrile, with a conversion yield of 100%.

INTRODUCTION

Nitrilase catalyzes the conversion of nitriles directly into the corresponding carboxylic acids and ammonia. In contrast to the conventional organic chemical methods for the hydrolysis of nitriles, with the accompanying formation of a large amount of salt which makes purification and isolation of the products difficult, the enzymatic conversion of nitriles can proceed smoothly under mild conditions and without the formation of unwanted salt. Thus, nitrilase is expected to have great potential as a catalyst in organic chemical processing.

Recently, we found high nitrilase activity in cells of Rhodococcus rhodochrous J1 (Nagasawa et al., 1988a) and established the optimal conditions for the production of nicotinic acid from 3-cyanopyridine with Rhodococcus resting cells (Mathew et al., 1988). We also reported the regiospecificity of the Rhodococcus nitrilase for dicyanobenzenes (Kobayashi et al., 1988). In addition, we recently purified and crystallized the Rhodococcus nitrilase (Kobayashi et al., in preparation).

p-Aminobenzoic acid (PABA) is a vitamin Bx and is an important intermediate for sunburn preventives, dyes and developers. PABA esters are also used as local anesthetics.

In the present study, we attempted the microbial conversion of p-aminobenzonitrile to PABA (Eq. 1) using resting cells of R. rhodochrous J1 containing high nitrilase activity.

\[
\text{H}_2\text{N}-\begin{array}{c} \text{CN} \\ + \text{2H}_2\text{O} \end{array} \xrightarrow{} \text{H}_2\text{N}-\begin{array}{c} \text{COOH} \\ + \text{NH}_3 \end{array}
\]

MATERIALS AND METHODS

Rhodococcus rhodochrous J1, which was isolated from soil samples and
identified in our laboratory (Nagasawa et al., 1988b), was used. The subculture was carried out at 28°C for 24 h with reciprocal shaking in test tubes containing 4 ml of the basal medium (pH 7.2), which consisted of 10 g of glycerol, 5 g of Polypeptone (Daigo, Japan), 3 g of malt extract and 3 g of yeast extract (Oriental Yeast, Japan) (pH 7.2) per liter of tap water. Then the contents of three test tubes were added to each of 2-liter flasks containing 500 ml of the basal medium supplemented with 0.5 ml of isovaleronitrile, and then the incubation was carried out at 28°C with reciprocal shaking. After 55 h and 77 h, 0.5 ml and 1.0 ml of isovaleronitrile were added, respectively, and the cultivation was continued until 96 h.

Nitrilase activity toward benzonitrile was measured as described previously (Nagasawa et al., 1988a). The standard reaction mixture (2 ml) for PABA production contained 0.2 M p-aminobenzonitrile, 0.1 M potassium phosphate buffer (pH 8.0), 1 mM dithiothreitol and cell suspension (32.6 mg [dry weight] of cells) obtained from 12 ml of culture broth. The reaction was carried out at 30°C with shaking and stopped by adding 0.25 ml of 1 N HCl. The amounts of PABA, p-aminobenzamide and p-aminobenzonitrile were determined by HPLC under the same conditions as used for the determination of benzonitrile previously (Nagasawa et al., 1988a), except for the solvent system: 5 mM KH$_2$PO$_4$-H$_3$PO$_4$ buffer (pH 2.9)/acetonitrile, 4/1 (v/v).

The $^1$H- and $^{13}$C-nuclear magnetic resonance (NMR), and infrared spectra were recorded with a Nihondenshi JNN-GX270 (Japan) and a Perkin Elmer 1710-FTIR (USA), respectively. Elemental analysis was carried out with a Perkin Elmer 240-B (USA).

RESULTS

At first, we examined the inhibitory effect of the substrate, p-aminobenzonitrile, on the nitrilase activity. The concentration of p-aminobenzonitrile in the reaction mixture was varied. The rates of PABA synthesis at 300, 400 and 500 mM were low compared with that at 200 mM. However, after 6 h, 500 mM p-aminobenzonitrile had been converted completely to PABA.

Next, we examined the product inhibition of the nitrilase activity. The rates of enzymatic conversion of 200 mM p-aminobenzonitrile into PABA were compared in the presence of 0.4 M to 1.2 M PABA. As shown in Fig. 1, the rate decreased with increasing concentration of PABA added. However, in the presence of 1.0 M PABA, 200 mM p-aminobenzonitrile could be converted completely into PABA by 9 h of incubation.

Thus, the accumulation of PABA was attempted through an enzymatic process using resting cells of R. rhodochrous J1. During the course of the reaction, 200 mM p-aminobenzonitrile was fed at intervals, because a high concentration of p-aminobenzonitrile is inhibitory toward the nitrilase activity. With the feeding of 200 mM p-aminobenzonitrile four times over a 10-h period at 30°C, 800 mM PABA was accumulated, with a 100% conversion yield (Fig. 2). The accumulation of PABA corresponded to 110 g/liter of reaction mixture. No formation of p-aminobenzamide was detected in the reaction mixture. After the 5th feeding of 200 mM p-aminobenzonitrile (total, 1.0 M p-aminobenzonitrile) to the reaction mixture described above, the conversion rate decreased due to product inhibition.