Superiority of *Pseudomonas chlororaphis* B23 nitrile hydratase as a catalyst for the enzymatic production of acrylamide

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Summary. In this paper an explanation is given of how *Pseudomonas (P.) chlororaphis* B23 can accumulate so much acrylamide of such high purity. One reason is that *P. chlororaphis* B23 exhibits much greater nitrile hydratase activity than amidase activity; the rate of formation of acrylamide through the nitrile hydratase reaction is at least 4000 times higher than its breakdown catalyzed by amidase. Furthermore, acrylonitrile, a powerful nucleophilic reagent, inactivates the active thiol residue of the amidase, whereas nitrile hydratase is not so susceptible to acrylonitrile. Thus acrylamide is produced but not transformed further. In addition, the nitrile hydratase purified from *P. chlororaphis* B23 exhibits high resistance to a high concentration of acrylamide. Some other explanations, and the results of evaluation of the *P. chlororaphis* B23 enzyme as a catalyst for the production of acrylamide are discussed.
Key words. Acrylamide; nitrile hydratase; *P. chlororaphis* B23.

Introduction

Acrylamide is one of the most important chemical commodities, being in great demand as a starting material for the production of various polymers used as flocculants, stock additives and polymers for petroleum recovery. Conventional synthesis involves the hydration of nitriles with the use of a cooper salt as a catalyst. Galzy and colleagues[4,5,7,11] in France, our Kyoto University group[2,21,27] and Nitto Chemical Industry Company, Ltd.[23–25] have each proposed a process for the enzymatic production of acrylamide from acrylonitrile involving the nitrile hydratases of *Brevibacterium* R312, *Pseudomonas (P.) chlororaphis* B23, and *Rhodococcus* species N-774, respectively. Very recently, using *P. chlororaphis* B23, the industrial production of acrylamide (6,000 tonnes per year) was started in Japan. This is the first successful example of a bioconversion process for the production of a commodity chemical. In the present paper, an explanation for the mechanism of accumulation of acrylamide and the results of evaluation of *P. chlororaphis* B23 cells as a catalyst for the production of acrylamide are presented.

Materials and methods

Bacterial strains and cultivation. P. chlororaphis B23 isolated in our laboratory as an isobutyronitrile-utilizing bacterium from soil, and *Brevibacterium* B312, deposited as a patent microorganism, No. FERM-P2722[6,8], and obtained from the Fermentation Research Institute, Ministry of International Trade & Technology, Japan, were used in this study. *P. chlororaphis* B23 was cultivated at 25 °C for 28 h in the optimized medium containing methacrylamide as an inducer, as described previously[21,27]. The cultivation of *Brevibacterium* R312 was carried out at 28 °C for 45 h in a nutrient medium containing peptone and glucose, as described previously[16]. Growth was determined turbidimetrically as the absorbance at 610 nm and plotted as a dry weight calibration curve.

Enzyme assays. The nitrile hydratase activity of resting cells was assayed using 424 mM acrylonitrile as a substrate at 10 °C, as described previously[27]. The nitrile hydratase activity of a cell-free extract was assayed using 100 mM acrylonitrile as a substrate at 20 °C, as described previously[15]. The cell-free extract was prepared as follows; cells obtained from the culture medium by centrifugation at 10,000 × g at 5 °C for 20 min were suspended in 0.1 M potassium phosphate buffer (pH 7.0) containing 44 mM *n*-butyric acid and then disrupted with an ultrasonic oscillator (19 kHz at 0 °C for 60 min). The sonificate was centrifuged at 12,000 × g at 5 °C for 20 min and the resultant supernatant was used as the cell-free extract. The amount of acrylamide formed in the reaction mixture was determined with a Shimadzu gas-liquid chromatograph (model GC-4CM)[20]. One unit of acrylamide-forming activity was defined as the amount of cells or enzyme that catalyzed the formation of 1 μmol of acrylamide per min. Specific acitivity was expressed as units per mg of dry cells or protein.

For the amidase activity assay, cells were suspended in 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol and a cell-free extract was prepared in a similar manner. The reaction mixture (1 ml) consisted of 40 μmol of acrylamide, 20 nmol of dithiothreitol and an appropriate amount of the enzyme solution. The reaction was carried out at 30 °C for 30 min and stopped by adding 0.2 ml of 1 N HCl. Ammonia formed was determined by the indophenol method[10]. Acrylic acid formed was determined by analytical high-performance liquid chromatography (HPLC). HPLC was performed with a Toyosoda CCPM equipped with a Lichrosorb NH_2 column (Cica Merck) at the flow rate of 1.0 ml min^{-1}, using the solvent system of methanol/10 mM

KH_2PO_4 (pH 6.0), 7:3 (v/v), at 40 °C. The absorbance was measured at 250 nm. One unit of acrylamide-hydrolyzing activity was defined as the amount of cells or enzyme that catalyzed the formation of 1 µmol of ammonia per min. Specific activity was expressed as units per mg of dry cells or protein.

Other methods. Protein was determined by the Coomassie brilliant blue G-250 dye-binding method of Bradford[3] using dye reagent supplied by Bio-Rad. For some experiments, the nitrile hydratase was purified from *Brevibacterium* R312 and *P. clororaphis* B23 cells according to our previous reports[15,16]. Amidase was purified from methacrylamide-induced *P. chlororaphis* B23 cells by successive ammonium sulfate fractionation, and column chromatography on DEAE-Sephacel and Sephadex G-100[14]. SDS polyacrylamide gel electrophoresis was performed in 12.5% polyacrylamide slab gels using a Tris/glycine buffer system[12].

Results and discussion

P. chlororaphis B23, isolated through screening procedures, was found to exhibit high acrylamide-producing activity[2]. In order to use this strain as a catalyst for industrial purposes, we first optimized the culture conditions[27]. Methacrylamide was found to be the most effective inducer. The addition of ferrous or ferric ions to the medium greatly increased the formation of nitrile hydratase. Next, we isolated an advantageous mutant, strain Am-324, which can be easily precipitated by brief centrifugation and which exhibits high nitrile hydratase activity[21]. When resting cells of *P. chlororaphis* B23, in which nitrile hydratase was highly induced, were added to the reaction mixture, more than 400 g of acrylamide per liter of reaction mixture was accumulated after incubation for several hours, the molar conversion yield being more than 99%.

Why does the process lead to the accumulation of so much acrylamide of such high purity? The first reason is that *P. chlororaphis* B23 exhibits extremely high nitrile hydratase activity. The amount of nitrile hydratase formed in the cells was determined by SDS-polyacrylamide gel electrophoresis (fig. 1). An enormous amount of nitrile hydratase was formed in the cells, which corresponded to more than 5% of the total soluble protein.

For the accumulation of acrylamide, the acrylamide should not be transformed. We examined the amidase activity, i.e., the hydrolysis of acrylamide to acrylic acid, in cells of *P. chlororaphis* B23 and compared it with the nitrile hydratase activity (table). Amidase activity was detected in the cell-free extract of methacrylamide-induced *P. chlororaphis* B23 cells; however, the rate of ammonia formation through the amidase reaction was at least 4000 times lower than the rate of acrylamide formation catalyzed by nitrile hydratase. The formation of acrylic acid could barely be detected in the resting cell.

Methacrylamide was added as a powerful inducer for the production of nitrile hydratase by *P. chlororaphis* B23 cells. Methacrylamide was metabolized slowly through the amidase reaction during the cultivation. The efficient induction by methacrylamide seems to be due to its slow degradation in *P. chlororaphis* B23 cells. The purified *P. chlororaphis* B23 amidase shows higher activity and affinity for methacrylamide (V_{max} = 3.96 µmol · min^{-1} (mg protein)$^{-1}$, K_m = 0.345 mM) than for acrylamide (V_{max} = 2.42 µmol · min^{-1} · (mg protein)$^{-1}$, K_m = 11.4 mM). Compared to the metabolism of methacrylamide, acrylamide metabolism is slower in the resting cell.

In addition, we found that acrylonitrile inactivates the amidase of *P. chlororaphis* B23 cells (fig. 2). When acrylonitrile is incubated with amidase purified from *P. chlororaphis* B23 cells, the amidase activity is inhibited. Acrylonitrile is known to be a powerful nucleophilic reagent. The *P. chlororaphis* B23 amidase is a typical thiol-enzyme and contains an active thiol residue[17]. The acrylonitrile, which is a nucleophilic reagent, attacks the active thiol residue of the amidase and inactivates it. On the other hand, nitrile hydratase is not as susceptible to acrylonitrile. Thus, acrylamide is accumulated by *P. chlororaphis* B23 cells but not transformed further.

The optimum temperatures for the nitrile hydratase and amidase from *P. chlororaphis* B23 cells are shown in figure 3. The amidase activity increases in parallel with an increase in temperature up to 40 °C, whereas the optimum temperature of nitrile hydratase was found to be around 20 °C. Therefore, when acrylamide production is carried out between 5 and 10 °C, the amidase activity can be depressed fully. Acrylamide production should be carried out at a low temperature, because acrylamides readily polymerize under extreme conditions.

The nitrile hydratase in *P. chlororaphis* B23 cells is inducibly formed in response to aliphatic nitriles and amides[27], whereas the nitrile hydratase is constitutively formed in *Brevibacterium* R312[1] and *Rhodococcus* N-774[26] cells. We purified and crystallized

Relative activity of nitrile hydratase and amidase in *P. chlororaphis* B23 cells grown under conditions optimal for the production of acrylamide. *P. chlororaphis* B23 cells were cultivated for 28 h at 28 °C in a 2-l flask containing 100 ml of medium IV described previously[21]. The nitrile hydratase and amidase assays were carried out as described under 'Materials and methods'.

	Nitrile hydratase activity	Amidase activity
Resting cells		
Total activity (units/ml of culture broth)	1240	n.d.[a]
Specific activity (units/mg of dry cell weight)	135	n.d.[a]
Cell free extract		
Specific activity (units/mg of protein)	98.2	0.0232[b]

[a] The formation of acrylic acid was not detected. [b] The amount of ammonia formed was determined.

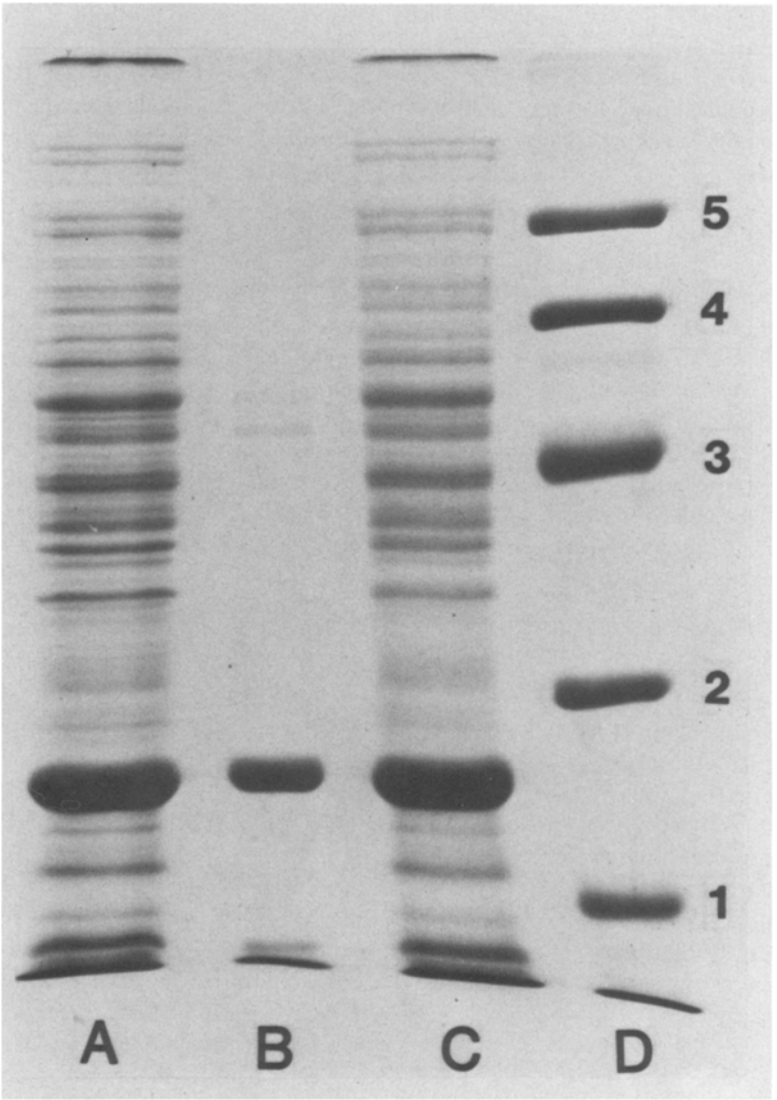

Figure 1. SDS polyacrylamide gel protein profiles of cells cultivated under optimal conditions for the production of nitrile hydratase. Lanes A and C were loaded with 56 µg of protein of a cell-free extract of *P. chlororaphis* B23 cells prepared under optimal conditions, as described previously[20,21]. Lane B was loaded with purified *P. chlororaphis* B23 nitrile hydratase (19 µg.). Lane D was loaded with the following mol. wt standards: 1, soybean trypsin inhibitor (20,100); 2, carbonic anhydrase (30,000); 3, ovalbumin (43,000); bovine serum albumin (67,000); and 5, phosphorylase b (94,000).

the nitrile hydratases from *P. chlororaphis* B23[15] and *Brevibacterium* R 312[16]. The *P. chlororaphis* B23 enzyme has a molecular mass of about 200 kDa and consists of four subunits identical in molecular mass (approximately 25 kDa)[15]. The *Brevibacterium* R312 and *Rhodococcus* N-774 enzymes are composed of the kinds of subunits, 26 kDa and 27.5 kDa[8], and 28.5 kDa and 29 kDa[9], respectively. The specific activity of the highly purified nitrile hydratase preparation was 1840 µmol · min^{-1} · (mg protein)$^{-1}$ at 20 °C under the standard assay conditions. The molecular activity of the *Pseudomonas* enzyme was calculated to be 184 kmol · min^{-1} · (mol nitrile hydratase)$^{-1}$ at 20 °C under the standard assay conditions.

On the other hand, the specific activity of the highly purified amidase preparation was 2.42 µmol · min^{-1} · (mg protein)$^{-1}$ at 30 °C under the standard assay conditions. As the molecular mass of this enzyme can be assumed to be 106 kDa (unpublished data), the molecular activity of the *Pseudomonas* enzyme was calculated to be 0.257 kmol · min^{-1} · (mol amidase)$^{-1}$ at 30 °C. Thus, the nitrile hydratase is more efficient than the amidase as a catalyst. It can be assumed that the high efficiency of the nitrile hydratase results from the correlative interaction of ferric ions and PQQ, which are cofactors for the nitrile hydratase[18,19,22]. Thus, the nitrile hydratase of *P. chlororaphis* B23 is suitable both in quality and in quantity as a catalyst for acrylamide production.

The *P. chlororaphis* B23 enzyme did not share any antigenic determinants with the hydratases from *Brevibacterium* R312 and *Rhodococcus* N-774, as judged by Ouchterlony double diffusion. It was reported that the

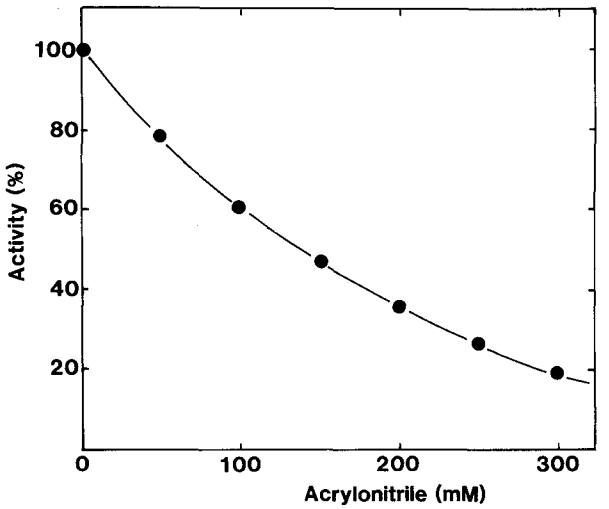

Figure 2. Effect of acrylonitrile on the *P. chlororaphis* B 23 amidase activity. After incubation of the purified amidase (0.0286 units) in 70 mM potassium phosphate buffer (pH 7.0) containing various concentrations of acrylonitrile at 30 °C for 20 min, the hydrolysis of acrylamide was started by adding 40 mM acrylamide, followed by incubation for 20 min at 30 °C under the standard conditions. The ammonia formation was determined. Relative activity is the percentage of the maximum activity attained under the experimental conditions.

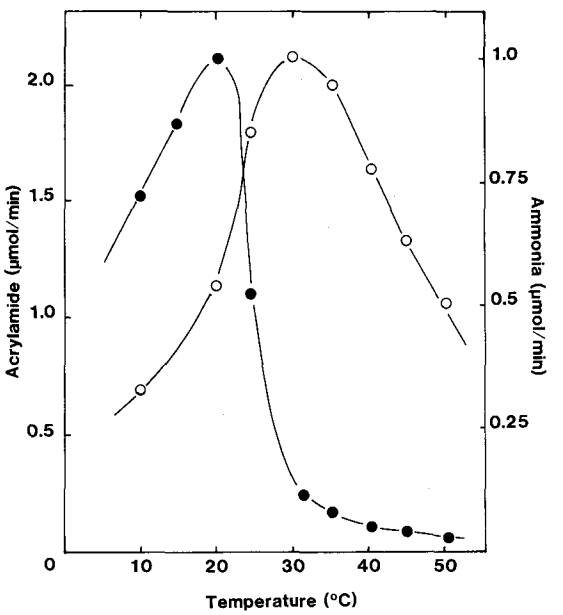

Figure 3. Optimum temperature for nitrile hydratase and amidase purified from *P. chlororaphis* B 23. The reactions were carried out at various temperatures for 30 min in the standard reaction mixture containing 2.2 units of the purified nitrile hydratase or 1.05 units of purified amidase.

nitrile hydratase activity in *Rhodococcus* sp. N-774 cells was enhanced by light-irradiation [20]. However, such light-enhancement was not observed for the *P. chlororaphis* B 23 nitrile hydratase. Therefore, light-irradiation equipment is not required for acrylamide production with *P. chlororaphis* B 23 cells.

One of the most important characteristics of the *P. chlororaphis* B 23 nitrile hydratase from the point of view

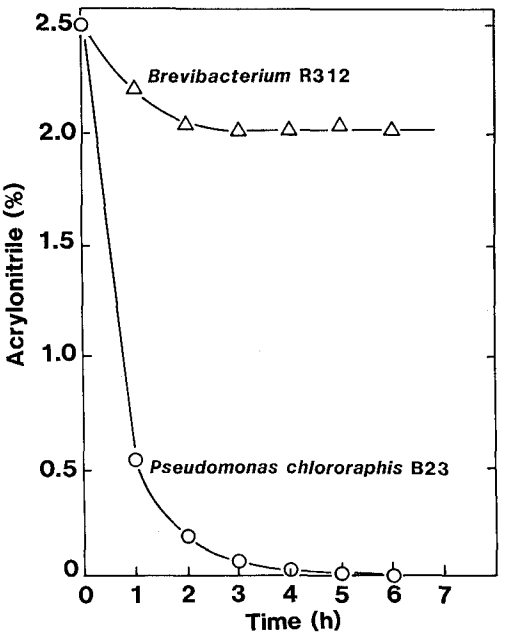

Figure 4. Effect of acrylamide on the activity of nitrile hydratases from *P. chlororaphis* B 23 and *Brevibacterium* R 312. The nitrile hydration reaction was carried out at 5 °C in the reaction mixture containing 15 units of nitrile hydratase of *P. chlororaphis* B 23 (○) or *Brevibacterium* R 312 (△), 17.5% (w/v) acrylamide, 2.5% (w/v) acrylonitrile, 0.2% (w/v) *n*-butyric acid and 10 mM potassium phosphate buffer (pH 7.5).

of acrylamide production is its tolerance of acrylamide. The tolerances of the nitrile hydratases purified from *P. chlororaphis* B 23 and *Brevibacterium* R 312 cells to a high concentration of acrylamide were compared (fig. 4). The acrylonitrile hydration reactions were carried out in the presence of 17.5% (w/v) acrylamide at 5 °C. The *P. chlororaphis* B 23 enzyme acts on acrylonitrile even in the presence of 17.5% (w/v) acrylamide, and the acrylonitrile added was found to be completely converted after a 6-h incubation. On the other hand, the *Brevibacterium* R 312 nitrile hydratase was inactivated by the high concentration of acrylamide and the production of acrylamide stopped after 3-h incubation. Thus the *P. chlororaphis* B 23 enzyme is a more suitable catalyst from the point of view of acrylamide accumulation.

At present, the *P. chlororaphis* B 23 enzyme seems to be one of the best catalysts for the production of acrylamide. Recently, we showed the presence of a cobalt-induced and cobalt-containing nitrile hydratase in *Rhodococcus rhodochrous* J1 [17]. This enzyme exhibits a broad substrate specificity and acts not only on aliphatic but also on aromatic nitriles. We have already established a process for the nitrile hydratase-catalyzed production of nicotinamide from 3-cyanopyridine with *Rh. rhodochrous* J1 cells [13]. Under optimum conditions, the highest yield achieved was 1465 g of nicotinamide per 1 of reaction mixture in 9 h, and in the course of the reaction, crystals of nicotinamide appeared. The enzyme is heat-stable and resistant to high concentrations of nitriles and amides. Therefore, the application of the cobalt-induced and

cobalt-containing enzyme seems to be promising for the production of acrylamide, too. However, there still seems to be considerable room for the improvement of microbial catalysts to increase the productivity of the process.

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Mixed substrates in environmental biotechnology

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Summary. The scope of environmental biotechnology is defined and four examples of recent research in environmental biotechnology involving various types of mixed substrates are discussed in detail and their probable impacts assessed. The four examples are: multiple carbon energy substrate (pollutant) biodegradation by both mono and mixed cultures, the biodegradation of whole microbial cells, the biodegradation of single compounds satisfying dual physiological requirements, i.e., mixed carbon and mineral nutrient sources, and simultaneous nitrification and denitrification.
Key words. Microbes; environment; biotechnology; process; treatment; biodegradation; mixed substrates; pollutants.