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Asymmetric hydrolysis of α-aminonitriles to optically active amino acids by a nitrilase of *Rhodococcus rhodochrous* PA-34

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Summary. *Rhodococcus rhodochrous* PA-34 isolated from soil as a propionitrile-utilizing microorganism, hydrolysed several α-aminonitriles to optically active amino acids. The hydrolysis of α-aminonitriles was found to be catalysed by a nitrilase. The characteristics of the purified enzyme revealed that this is a new nitrilase as it has a molecular mass of 45 kDa and acts as a monomer. The optimum pH and temperature for the activity of the purified enzyme were 7.5 and 35° C, respectively. Thiol-specific reagents caused inhibition whereas chelators did not significantly alter the activity of this enzyme. The amino acids produced were of L-form, except for alanine. In the case of leucine production from α-aminoisocapronitrile, the enantiomeric ratio of L-leucine to D-leucine was about 60.

Introduction

The α-aminonitriles are intermediates in the chemical synthesis of racemic amino acids and appear to be potentially attractive substrates for their hydrolysis to amino acids by microbial nitrilases. The production of racemic amino acids from α-aminonitriles by *Corynebacterium* sp. HR3 (Fukuda et al. 1971) and *Brevibacterium* sp. R312 (Arnaud et al. 1980) has been reported. A mutant of *Brevibacterium* sp. 312, which lost the non-stereospecific amidase but retained the L-specific amidase, produced L-amino acids from α-aminonitriles (Arnaud et al. 1980). Although the production of L-alanine by *Acinetobacter* sp. (Macadam and Knowles 1985) and L-phenylglycine by *Aspergillus fumigatus* (Choi and Goo 1986) from the corresponding α-aminonitriles has been reported, little is yet known about the mechanism of enantioselective hydrolysis of α-aminonitriles by the microorganisms.

In the past, several nitrilases have been purified and characterized from various microorganisms (Harper 1977a, b, 1985; Bandyopadhyay et al. 1986; Stalker et al. 1988; Goldlust and Bohak 1989; Kobayashi et al. 1989, 1990; Yamamoto and Komatsu 1991). However, none of these have been explored in detail for their catalytic activity to hydrolyse α-aminonitriles. Thus, in the present paper, we report the production of optically active amino acids from α-aminonitriles by *Rhodococcus rhodochrous* PA-34, and purification and characterization of the *R. rhodochrous* nitrilase that catalysed this asymmetric hydrolysis.

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Materials and methods

Materials

Sephacryl S-300 HR was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). DEAE Toyopearl 650S was purchased from Tosoh Corporation (Tokyo, Japan). Membrane filters were from Amicon (Danvers, Mass., USA). Aminonitriles used in the present studies were synthesized by Strecker's method in our laboratory. All other chemicals were of reagent grade and obtained from commercial sources.

Isolation and screening of nitrile-utilizing microorganisms

Nitrile-utilizing microorganisms were isolated from soil in media containing nitrile as the source of carbon, nitrogen or both. The medium used for isolation contained 2.5 g $Na_2HPO_4 \cdot 12H_2O$, 2.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.03 g $FeSO_4 \cdot 7H_2O$, 0.06 g $CaCl_2 \cdot 2H_2O$, 0.1 g yeast extract (Difco, Detroit, Mich., USA) and 2 g nitrile compound per litre of distilled water. Ten grams of glucose or 4 g $(NH_4)_2HPO_4$ or nothing was added to the medium to isolate microorganisms that could utilize nitrile as a source of nitrogen or carbon or both, respectively. Propionitrile, acetonitrile, benzonitrile and phenylacetonitrile were used as nitrile compounds. The isolated strains were cultivated in the isolation medium at 30° C for 2 days. The cells were centrifuged, washed and suspended in 100 mM ammonium chloride buffer (pH 10.0). The reaction mixture for the screening of amino-acid-producing strains contained 5 ml cell suspension (about 40 mg cells on a dry weight basis) and 20 mg α-aminoisovaleronitrile. The reaction was carried out at 30° C with shaking for 48 h and stopped by the ad-

dition of 1 M HCl. Strain PA-34, isolated in medium containing propionitrile as the nitrogen source and identified as *R. rhodochrous*, was selected for further experiments.

Culture conditions for R. rhodochrous PA-34

R. rhodochrous PA-34 was subcultured at 30° C for 24 h with reciprocal shaking in a test tube containing 4 ml medium consisting of 25 g Nutrient Broth no. 2 (Oxoid, Basingstoke, UK), 10 g glucose and 1 g yeast extract per litre distilled water (pH 7.5). Then the subculture was added to 100 ml medium with the following composition per litre: 10 g glucose, 2.5 g $Na_2HPO_4 \cdot 12H_2O$, 2.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.03 g $FeSO_4 \cdot 7H_2O$, 0.06 g $CaCl_2 \cdot 2H_2O$, 0.1 g yeast extract and 5 ml isobutyronitrile (pH 7.2). It was incubated at 30° C for 28 h in an incubator shaker. The growth was measured turbidometrically by a dry cell calibration curve of the absorbance at 660 nm.

Purification of nitrilase

All steps were performed at 4° C, and 100 mM potassium phosphate containing 5 mM dithiotreitol (DTT), pH 7.0, was used as a buffer throughout the purification process. The centrifugation (if not otherwise stated) was carried out at 13000 *g* for 20 min at 4° C.

Preparation of cell-free extract. R. rhodochrous PA-34 cells were harvested from 4 l culture broth by centrifugation (at 4800 *g* for 20 min), washed twice and suspended in the buffer. The cells were disrupted using a French press and the homogenate was centrifuged. The supernatant (cell-free extract) was collected and used for further experiments.

Ammonium sulphate fractionation. The cell-free extract was fractionated with ammonium sulphate (20–80% saturation). The precipitates were collected by centrifugation and suspended in the buffer. This enzyme suspension was subjected to centrifugation at 10^5 *g* for 60 min in a Beckman (Waldwick, USA) L8M Ultracentrifuge employing a Type 60 Ti rotor and the clear supernatant was collected.

First Sephacryl S-300 HR column chromatography. The enzyme preparation was applied to Sephacryl S-300 HR column (4.4 × 84 cm) and the column was eluted with the buffer at a linear flow rate of 23 cm/h. The enzyme-rich fractions were pooled, ammonium sulphate added to 80% saturation and the precipitates collected by centrifugation and suspended in the buffer.

Second Sephacryl S-300 HR column chromatography. The enzyme suspension was then loaded again on a Sephacryl S-300 HR column keeping all conditions the same as before.

DEAE Toyopearl 650S column chromatography. The enzyme preparation was then dialysed overnight against the buffer and applied to DEAE Toyopearl 650S column (2.2 × 24 cm). The elution was performed with buffer at a linear flow rate of 30 cm/h and the nitrilase-rich fractions were pooled and concentrated by ultrafiltration.

Enzyme and protein assays

Resting-cell reaction conditions. The cells were harvested in the mid-exponential growth phase by centrifugation at 10^4 *g* for 10 min at 4° C, washed in 100 mM potassium phosphate buffer (pH 7.0) and suspended in the same buffer to give an appropriate cell concentration. The reaction was initiated by the addition of aminonitrile compounds to the cell suspension (1 ml containing 16 mg cells on a dry weight basis) and incubated at 30° C with shaking (100 rpm). After 10 min the reaction was stopped by the addition of 1 M HCl, centrifuged and, the amount and optical purity of the amino acids in the supernatant were assayed with HPLC (Shimadzu LC-6A; Kyoto, Japan). The amino acid concentrations were estimated using a reversed phase chromatography column (Inertsil ODS, Gaskuro Kogyo, Tokyo, Japan) at a flow rate of 1.0 ml/min with buffer containing 10 mM acetic acid, 0.4 mM copper(II) acetate and 1.2 mM sodium heptane sulphonate (pH 5.6 adjusted by sodium acetate). The optical purity of the amino acids was determined using an optical resolution column (Chiralpak WH or WE, Daicel Chemical Industries, Tokyo, Japan) at a flow rate of 1.0 ml/min with 0.5 mM copper(II) sulphate as solvent. In each analysis the absorbance was measured at 254 nm.

Reaction conditions with nitrilase preparations. The nitrilase activity was assayed in the reaciton mixture (0.5 ml) containing 50 μmol potassium phosphate buffer (pH 7.0 if not otherwise stated), 2.5 μmol DTT, 37.5 μmol α-aminoisocapronitrile (as substrate) and an appropriate amount of enzyme. The reaction was carried out for 10 min at 30° C (if not otherwise stated) and stopped by the addition of 0.5 ml of 0.1 M HCl to the reaction mixture. The amount and optical purity of the amino acid produced were determined by HPLC as described in the preceding section. For testing the substrate specificity of the enzyme, the ammonia released during the reaction was assayed according to Fawcett and Scott (1960). The proteins were estimated following the dye-binding method (Bradford 1976) using BioRad (Richmond, Va., USA) reagents. One unit of enzyme activity was defined as the amount of enzyme that catalysed the production of 1 μmol leucine or ammonia/min under the assay conditions.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

This was performed on polyacrylamide gel slabs in the presence of SDS using the BioRad MiniProtean apparatus and reagents as described by Laemmli (1970).

Determination of molecular mass of nitrilase

The molecular mass of the enzyme was estimated by gel filtration of the purified nitrilase fraction on a Sephacryl S-300 HR column (2.2 × 96 cm). About 15 mg purified fraction of nitrilase was loaded on the column equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) containing 5 mM DTT and the column was run at a linear flow rate of 6.6 cm/h. The molecular size of this enzyme was also determined by applying 25 μg purified enzyme to HPLC (Shimadzu LC-6A) equipped with TSK G 3000 SW_{xL} column (0.78 × 30 cm, Toyo Soda) at a flow rate of 0.5 ml/min using 0.1 M potassium phosphate buffer (pH 7.0) containing 0.3 M NaCl at room temperature. The molecular mass was calculated from the standard curve obtained by calibrating the column with BioRad gel filtration reference proteins (molecular mass in parentheses): thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 KDa) and cyanocobalamin (1.35 kDa).

Results and discussion

Isolation and selection of α-aminonitrile-hydrolysing microorganisms

The microorganisms (65 strains) isolated from soil as nitrile-utilising microorganisms were screened for the production of valine from α-aminoisovaleronitrile. Among the 65 isolates, 63 strains produced L-valine. The optical purity of the L-valine produced exceeded 90% enantiomeric excess (ee) in 38 strains that belonged to the genera *Rhodococcus, Arthrobacter* and *Mycobacterium*. Although the organisms belonging to *Rhodococcus* have been reported to produce nitrile hydratase and nitrilase (Nagasawa et al. 1988, Kobayashi et al. 1989), the hydrolysis of α-aminonitrile has not yet been reported. The probability of obtaining α-aminonitrile-hydrolysing microorganisms reveals that these organisms are widely distributed in nature. From these microorganisms, strain PA-34, isolated as a propionitrile utilizer and identified as *R. rhodochrous*, was selected for further investigations because of the higher yield and optical purity of the amino acids produced.

Screening of compounds for induction of α-aminonitrile hydrolysing activity

Several nitrile compounds were examined for their ability to induce α-aminonitrile-hydrolysing activity (Table 1). Isobutyronitrile was found to be the most favorable compound under the present conditions. Dinitriles such as succinonitrile and adiponitrile inhibited the growth of this organism. Acetonitrile was a good nitrogen source but did not induce α-aminonitrile-hydrolysing activity. Propionamide, an inducer of some nitrile hydratase (Yamada et al. 1986), did not act as inducer. The time course of cultivation (Fig. 1) shows that α-aminonitrile-hydrolysing activity was induced in the exponential growth phase of cultivation and disappeared rapidly in the stationary phase.

Production of amino acids by resting cell reaction

Hydrolysis of various α-aminonitriles to amino acids was investigated and the results are shown in Table 2. Resting cells of *R. rhodochrous* PA-34 converted all of the substrates tested into the corresponding amino acids, which were optically active. L-Amino acids were produced from most of the substrates that have long or branched alkyl substituents whereas D-alanine was produced from α-aminopropionitrile. In an additional experiment, L-α-aminobutyric acid was produced from DL-α-aminobutyronitrile. The stereoselectivity of *R. rhodochrous* PA-34 differs from *Acinetobacter* sp., which produced L-alanine from DL-α-aminopropionitrile (Macadam and Knowles 1985).

Table 1. Screening of some compounds for induction of α-aminonitrile-hydrolysing activity in *Rhodococcus rhodochrous* PA-34

Compounds (0.5 ml/100 ml)	Growth (*A* 660 nm)	Aminonitrile-hydrolysing activity[a] (μmol leucine/mg dry cells)
None	2.29	0.00
Propionitrile	5.36	0.88
Propionitrile (1 ml)	5.32	2.57
Acetonitrile	17.00	0.00
n-Butyronitrile	8.04	1.88
Isobutyronitrile	8.28	2.84
Crotononitrile	6.40	2.73
Methacrylonitrile	7.44	0.99
Succinonitrile	No growth	—
Adiponitrile	2.02	0.00
Propionamide (0.5 g)	10.68	0.00

Cultivation was carried out under the conditions described in Materials and methods except that various compounds were used as the sole source of nitrogen in place of isobutyronitrile: *A*, absorbance

[a] For assaying aminonitrile-hydrolysing activity, α-aminoisocapronitrile was used as substrate

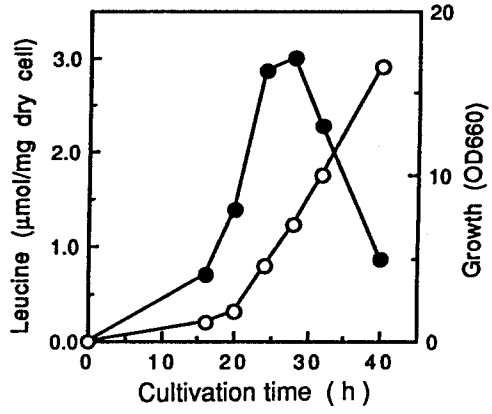

Fig. 1. Growth curve and activity of leucine production of *Rhodococcus rhodochrous* PA-34. The medium for cultivation is described in Materials and methods. The reaction mixture (1 ml), containing 100 mM potassium phosphate buffer (pH 7.0), 225 mM α-aminoisocapronitrile, and 16 mg (dry weight) of cells, was incubated at 30° C with shaking for 10 min: ●, leucine production; ○, cell growth, measured by optical density at 660 nm (OD_{660})

Time course of leucine production

The time course of L-leucine production from DL-α-aminoisocapronitrile by resting cells of *R. rhodochrous* PA-34 is shown in Fig. 2. The initial rate of L-leucine formation was fast but it slowed down as the reaction proceeded. The optical purity of the L-leucine produced also decreased as the conversion increased. These results imply that the mechanism of L-leucine production by this organism is a kinetic resolution. The enantiomeric ratio of L-leucine to D-leucine was about 60, which was calculated from conversion and optical purity according to the equation of Sih et al. (1982). Macadam and Knowles (1985) have suggested the ste-

Table 2. Substrate specificity of *R. rhodochrous* PA-34 for amino acid production

Substrates (75 mM)	Product	Activity (%)	Optical purity (% ee)
DL-α-Aminoisocapronitrile	L-Leucine	100.00[c]	89.7
DL-α-Aminopropionitrile[a]	D-Alanine	24.40	57.3
DL-α-Aminoisovaleronitrile[b]	L-Valine	0.16	100.0
DL-α-Amino-n-valeronitrile[b]	L-Norvaline	107.00	92.2
DL-α-Amino-n-capronitrile	L-Norleucine	77.50	100.0
DL-α-Amino-4-methylthio butyronitrile	L-Methionine	5.37	96.0

The reaction mixture (1 ml) contained 100 mM potassium phosphate buffer (pH 7.0), substrate and 16 mg (dry weight) of cells. The incubation was carried out at 30° C with shaking for 60 min: ee, enantiomeric excess

[a] 143 mmol/l

[b] 50 mmol/l

[c] 38.9 nmol/min per milligram dry cell weight

reochemical inversion of α-aminopropionitrile during the reaction employing *Acinetobacter* sp. because of the high yield of L-alanine (yield 94%; 87.5% ee), however, no such phenomenon was observed with *R. rhodochrous* PA-34 or *Brevibacterium* R312 (Arnaud et al. 1980) and *Corynebacterium* sp. HR3 (Fukuda et al. 1971).

Purification of nitrilase

Following the procedure described in Materials and methods, the enzyme was purified about 14-fold with a yield of 34.8% from the cell-free extract (Table 3). The enzyme preparations at various stages of purification were subjected to SDS-PAGE. The purified enzyme exhibited a single band on SDS-PAGE (Fig. 3). The enzyme produced propionic acid from propionitrile and exhibited neither amide-producing activity nor propionamide-hydrolysing activity, thus indicating that this enzyme is a nitrilase. In a reaction mixture (1 ml) containing 150 μg enzyme protein, 100 μmol potassium phosphate buffer, 5 μmol DTT and 75 μmol DL-α-aminoisocapronitrile, 25.5 μmol leucine (34%) with the L-form in 97% ee was obtained in 60 min. The Michaelis-Menten constant, K_m, and V_{max} of purified nitrilase of *R. rhodochrous* PA-34 with DL-α-aminoisocapronitrile were 45 mM and 5.8 μmol of leucine/min per milligram protein, respectively.

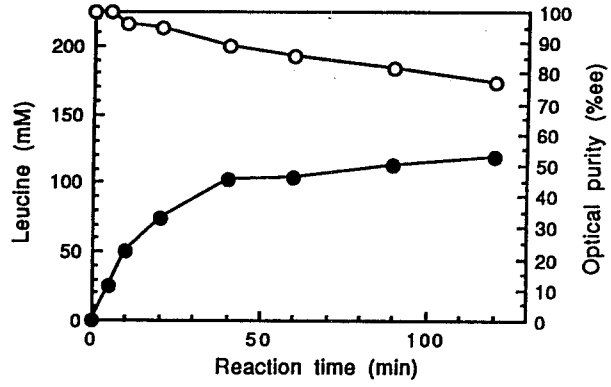

Fig. 2. Time course of leucine production by *R. rhodochrous* PA-34. The reaction mixture (1 ml) contained 100 mM potassium phosphate buffer (pH 7.0), 225 mM α-aminoisocapronitrile and 28 mg (dry weight) of cells. The incubation was carried out at 30° C and the reaction was stopped by 1 ml of 1 M HCl: ●, leucine production; ○, optical purity

Molecular mass and pH and temperature optima of nitrilase

The electrophoretic mobility of the purified enzyme and elution volume of the nitrilase on Sephacryl S-300 HR and TSK G 3000 SW_{xL} columns all corresponded to a moleclular mass of 45 kDa. The molecular mass of this enzyme is equal to that of a single subunit of *No-*

Table 3. Purification of nitrilase of *R. rhodochrous* PA-34

Stage of purification	Volume (ml)	Total protein (mg)	Total units	Specific activity	Purification (fold)	Yield (%)
CFE	96	1436	360	0.251	—	100
ASF	28	1241	356	0.287	1.14	98.8
SCC1	160	314	213	0.679	2.71	59.2
SCC2	83.6	135	155	1.15	4.57	43.1
DEAE	95	35.5	125	3.52	14.10	34.8

CFE, cell-free extract; ASF, ammonium sulphate fractionation; SCC1, first Sephacryl S-300HR column chromatography; SCC2, second Sephacryl S-300 HR column chromatography; DEAE, DEAE Toyopearl 650S ion exchange chromatography. One unit of enzyme activity is defined as the amount of enzyme that catalyses the production of 1 μmol leucine/min from α-aminoisocapronitrile under assay conditions

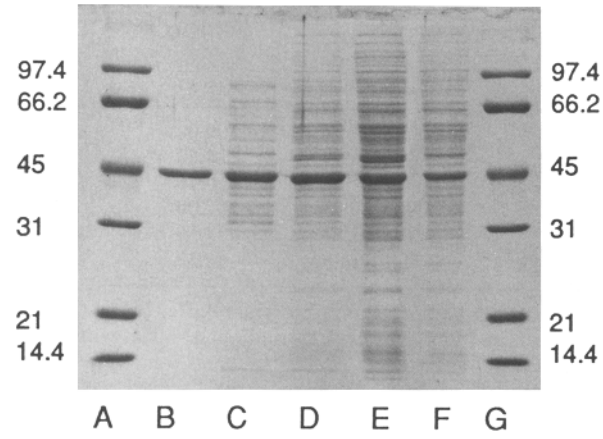

Fig. 3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of nitrilase preparations at various stages of purification. Electrophoresis was performed on 12% gel as described in Materials and methods section. *Lanes A* and *G* were loaded with following molecular mass standards: phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14.4 kDa). Approximately 15 μg protein of cell-free extract (*lane F*), 32 μg protein of the ammonium sulphate fraction (*lane E*), 10 μg protein of the first Sephacryl S-300 HR column chromatography fraction (*lane D*), 6 μg protein of the second Sephacryl S-300 HR fraction (*lane C*) and 1.5 μg protein of the DEAE Toyopearl 650S fraction (*lane B*) were applied to the gel

cardia (*rhodochrous* group) NCIB 11216 nitrilase (Harper 1977a). However, the latter enzyme is composed of 12 subunits while the nitrilase of *R. rhodochrous* PA-34 consists of a single polypeptide and acts as monomer like benzonitrilase A (molecular mass 30 kDa) of *Arthrobacter* sp. (Bandyopadhyay et al. 1986). The other reported nitrilases comprise two (Stalker et al. 1988; Kobayashi et al. 1989), eight (Harper 1977b), twelve (Harper 1977a, 1985) and fifteen to sixteen (Kobayashi et al. 1990) subunits.

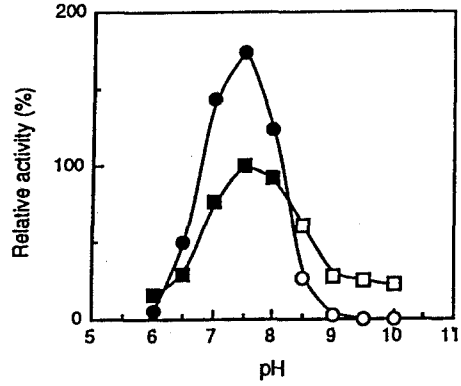

Fig. 4. Effect of pH on the activity of nitrilase. Assays were performed as described in Materials and methods using α-aminoisocapronitrile (■, □) and acrylonitrile (●, ○) as substrates (75 mM concentration). The buffers used were 100 mM potassium phosphate (■, ●) and 100 mM sodium borate (□, ○) containing 5 mM dithiothreitol. The relative activity is the percentage of highest activity observed at 7.5 pH with α-aminoisocapronitrile as substrate

The effect of pH on the activity of purified nitrilase was studied using two substrates (acrylonitrile and α-aminoisocapronitrile) and the results are shown in Fig. 4. The optimum pH for this enzyme is 7.5 with both the substrates. The pH optimum of the present nitrilase is similar to benzonitrilase B of *Arthrobacter* sp. (Bandyopadhyay et al. 1986) and differs from other nitrilases (Harper 1977b; Stalker et al. 1988; Kobayashi et al. 1989). The optimum temperature for the activity of nitrilase of *R. rhodochrous* PA-34 is 35° C and it shares this characteristic with the nitrilase of *Klebsiella ozaenae* (Stalker et al. 1988). The preincubation of the enzyme for 1 h above 35° C substantially influenced the activity of this nitrilase and subjection of the enzyme to 50° C for 1 h resulted in complete loss of activity.

Table 4. Substrate specificity of nitrilase of *R. rhodochrous* PA-34

Substrate	Relative activity (%)	Substrate	Relative activity (%)
Aliphatic nitriles		Hydroxy nitriles	
Acrylonitrile	156.2	Lactonitrile	20.1
Propionitrile	31.8	α-Hydroxyvaleronitrile	4.1
n-Butyronitrile	36.1	α-Hydroxycapronitrile	3.9
Crotononitrile	145.8	Mandelonitrile	9.9
Dinitriles		Amino nitriles	
Adiponitrile	0.8	α-Aminopropionitrile	9.5
Succinonitrile	9.6	α-Aminobutyronitrile	31.2
Glutaronitrile	1.6	α-Amino-*n*-valeronitrile	111.5
Methylated nitriles		α-Aminoisocapronitrile	100.0
Methacrylonitrile	4.3	α-Amino-*n*-capronitrile	51.2
α-Methylbutyronitrile	11.6	Phenylglycinonitrile	6.9
Aromatic nitriles		*N*-Methyl-α-amino-butyronitrile	0.8
Benzonitrile	695.2		
Phenylacetonitrile	5.5		

The concentration of various substrates used was 75 mM. The enzyme activity for this experiment was measured by estimating ammonia released during the reaction. The hydrolysis of α-amino-isocapronitrile corresponding to 3.52 μmol NH_3 released/min per milligram protein was taken as 100%

Table 5. Effect of some inhibitors and metal ions on nitrilase of *R. rhodochrous* PA-34

Compounds/metal ions	Concentration (mM)	Relative activity (%)
None (control)		100.0
Metal ions		
$AgNO_3$	1	78.3
	5	0.0
$CaCl_2$	1	100.5
$CoCl_2$	1	0.0
$CuCl_2$	1	0.0
$FeSO_4$	1	99.5
$HgCl_2$	1	57.8
	5	0.0
$MnCl_2$	1	101.3
$ZnCl_2$	1	0.0
Thiol reagents		
p-Chloromercuribenzoate	1	1.3
	5	0.0
DTNB	1	0.0
Iodoacetic acid	1	83.4
	5	1.4
Carbonyl reagents		
Hydroxylamine	1	121.7
	5	114.5
Phenylhydrazine	1	110.0
	5	95.0
Semicarbazide	1	102.0
Chelators	5	97.3
EDTA		
	1	99.2
Sodium azide	5	98.6
	1	98.5
o-Phenanthroline	5	97.7
	1	88.6
	5	0.0

The enzyme was incubated for 20 min at 30° C in the presence of various compounds under test using 0.1 M potassium phosphate buffer (pH 7.0) containing 1 mM dithiothreitol. The hydrolysis of α-aminoisocapronitrile (in the absence of any inhibitors/metal ions) corresponding to 3.52 μmol leucine production/min per milligram protein was taken as 100%: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid

Substrate specificity of purified nitrilase

The nitrilase of *R. rhodochrous* PA-34 exhibited wide substrate specificity and could hydrolyse a range of nitrile compounds (Table 4). This enzyme has a very high affinity for benzonitrile. However, it has shown fairly high activity with alkane nitriles and α-aminoalkane nitriles. Hitherto, there had been a conception that aromatic nitriles such as benzonitrile, are hydrolysed by nitrilases to ammonia and acids (Bandyopadhyay et al. 1986; Harper 1977a, b, 1985; Hook and Robinson 1964; Kobayashi et al. 1989) whereas aliphatic nitriles are catabolized in two steps: firstly these are converted to the corresponding amides by the enzyme 'nitrile hydratase' (Asano et al. 1980, 1982a) and then to carboxylic acids and ammonia by another enzyme 'amidase' (Asano et al. 1980, 1982b; DiGeronimo and Antonie 1976). However, this nitrilase could hydrolyse both aromatic and aliphatic nitriles. This observation, along with the recent report of Kobayashi et al. (1990), clearly demonstrates that these new nitrilases can hydrolyse aliphatic nitriles in a single step.

Effect of inhibitors and metal ions on nitrilase activity

The effect of various inhibitors, chelators, carbonyl reagents and metal ions on the activity of purified nitrilase is tabulated in Table 5. Among the thiol reagents, 5,5′-dithiobis(2-nitrobenzoic acid) and $CuCl_2$ were strong inhibitors of the enzyme activity (100% inhibition) followed by *p*-chloromercuribenzoate (98.7% inhibition), $HgCl_2$ (42.2% inhibition), $AgNO_3$ (21.7% inhibition) and iodoacetic acid (16.6% inhibiton) at 1 mM. However, in the presence of 5 mM thiol reagents, the enzyme activity was completely inhibited by all of them. In this respect the present nitrilase shows similarity to the nitrilase of *Fusarium oxysporum* f. sp. *melonis* (Goldlust and Bohak 1989). It is evident from this observation that the nitrilase of *R. rhodochrous* PA-34 contains sulphhydryl groups at the active site of the enzyme as in earlier reported nitrilases (Harper 1977b, 1985; Bandyopadhyay et al. 1986; Stalker et al. 1988; Kobayashi et al. 1989, 1990). The carbonyl reagents and metal ion chelators (except *o*-phenanthroline) did not significantly alter the activity of this nitrilase even at 5 mM. These findings suggest that this enzyme does not have a metal ion requirement for activity. However, the inhibition of nitrilase activity by *o*-phenanthroline seems to be unrelated to interaction with metal ions (Goldlust and Bohak 1989). Among metal ions Co^{2+} and Zn^{2+} caused complete inhibition of enzyme activity at 1 mM, to which the nitrilase of *K. ozaenae* is resistant (Stalker et al. 1988). These metal ions have been shown to cause inhibition of the nitrilase activity of *R. rhodochrous* J1 (Kobayashi et al. 1989); however, the extent of inhibition is greater for the present enzyme. The other metal ions tested (Fe^{2+}, Mn^{2+}, Ca^{2+}) had no appreciable effect on the activity of this nitrilase.

From the results discussed above it is evident that the nitrilase of *R. rhodochrous* PA-34, which produces optically active amino acids from the corresponding α-aminonitriles, is a new one as it has a molecular mass of 45 kDa and acts as a monomer. This enzyme can provide a new process for the production of optically active amino acids, not only of natural forms but also of unnatural forms.

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References

Arnaud A, Galzy P, Jallageas J (1980) Production d'acides α-amines stereospecifiques par hydrolyse biolofique d'α-aminonitriles racemiques. Bull Soc Chim Fr 2:87–90

Asano Y, Fujishiro K, Tani Y, Yamada H (1982a) Aliphatic nitrile hydratase from *Arthrobacter* sp. J-1: purification and characterization. Agric Biol Chem 46:1165–1174

Asano Y, Tachibana M, Tani Y, Yamada H (1982b) Purification and characterization of amidase which participates in nitrile degradation. Agric Biol Chem 46:1175–1181

Asano Y, Tani Y, Yamada H (1980) A new enzyme "nitrile hydratase" which degrades acetonitrile in combination with amidase. Agric Biol Chem 44:2251–2252

Bandyopadhyay AK, Nagasawa T, Asano Y, Fujishiro K, Tani Y, Yamada H (1986) Purification and characterization of benzonitrilases from *Arthrobacter* sp. strain J-1. Appl Environ Microbiol 51:302–306

Bradford M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Choi SY, Goo YM (1986) Hydrolysis of the nitrile group in α-aminophenyl acetonitrile by nitrilase; development of a new biotechnology for stereospecific production of S-α-phenylglycine. Arch Pharm Res 9:45–47

DiGeronimo MJ, Antonie AD (1976) Metabolism of acetonitrile and propionitrile by *Nocardia rhodochrous* LL100-21. Appl Environ Microbiol 31:900–906

Fawcett JK, Scott JE (1960) A rapid and precise method for the determination of urea. J Clin Pathol 13:156–159

Fukuda Y, Fukuni M, Harada T, Izumi Y (1971) Formation of α-amino-acid from α-aminonitrile by cell suspension of a strain of *Corynebacterium*. J Ferment Technol 49:1011–1016

Goldlust A, Bohak Z (1989) Induction, purification, and characterization of the nitrilase of *Fusarium oxysporum* f. sp. *melonis*. Biotechnol Appl Biochem 11:581–601

Harper DB (1977a) Microbiol metabolism of aromatic nitriles. Biochem J 165:309–319

Harper DB (1977b) Fungal degradation of aromatic nitriles. Biochem J 165:685–692

Harper DB (1985) Characterization of a nitrilase from *Nocardia* sp. (*rhodochrous* group) NCIB 11215, using *p*-hydroxybenzonitrile as sole carbon source. Int J Biochem 17:677–683

Hook RH, Robinson WG (1964) Ricinine nitrilase. II Purification and product. J Bio Chem 239:4263–4267

Kobayashi M, Nagasawa T, Yamada H (1989) Nitrilase of *Rhodococcus rhodochrous* J1: purification and characterization. Eur J Biochem 182:349–356

Kobayashi M, Yanaka N, Nagasawa T, Yamada H (1990) Purification and characterization of a novel nitrilase of *Rhodococcus rhodochrous* K22 that acts on aliphatic nitriles. J Bacteriol 172:4807–4815

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Macadam AM, Knowles CJ (1985) The stereospecific bioconversion of α-aminonitrile to L-alanine by an immobilized bacterium isolated from soil. Biotechnol Lett 7:865–870

Nagasawa T, Takeuchi K, Yamada H (1988) Occurrence of a cobalt-induced and cobalt containing nitrile hydratase in *Rhodococcus rhodochrous* J1. Biochem Biophys Res Commun 155:1008–1016

Sih CJ, Fujimoto Y, Chen C (1982) Quantitative analyses of biochemical kinetic resolutions of enantiomers. J Am Chem Soc 104:7294–7299

Stalker DM, Malyj LD, McBride KE (1988) Purification and properties of a nitrilase specific for herbicide bromoxynil and corresponding nucleotide sequence analysis of bxn gene. J Biol Chem 263:6310–6314

Yamada H, Ryuno K, Nagasawa T, Enomoto K, Watanabe H (1986) Optimum culture conditions for production by *Pseudomonas chlororaphis* B23 of nitrile hydratase. Agric Biol Chem 50:2859–2865

Yamamoto K, Komatsu K (1991) Purification and characterization of nitrilase responsible for the enantioselective hydroylsis from *Acinetobacter* sp. AK 226. Agric Biol Chem 55:1459–1466