APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1995, p. 998–1003 Vol. 61, No. 3
0099-2240/95/$04.00+0

Purification and Characterization of an Enantioselective Amidase from *Pseudomonas chlororaphis* B23

LAWRENCE M. CISKANIK,[1]* JOLANTA M. WILCZEK,[2] AND ROBERT D. FALLON[2]

Central Research and Development Department, E. I. duPont deNemours & Co., Experimental Station 328/341, Wilmington, Delaware 19880-0328,[1] and Glasgow Site 301, Newark, Delaware 19714-6101[2]

Received 28 September 1994/Accepted 21 December 1994

An amidase produced by *Pseudomonas chlororaphis* B23 was purified and characterized. The purification procedure used included ammonium sulfate precipitation and hydrophobic, anion-exchange, gel filtration, and ceramic hydroxyapatite chromatography steps. This amidase has a native molecular mass of about 105 kDa and is a homodimer whose subunits have a molecular mass of 54 kDa. The enzyme exhibited maximal activity at 50°C and at pH values ranging from 7.0 to 8.6. We found no evidence that metal ions were required, and the enzyme was inhibited by several thiol reagents. This amidase exhibited activity against a broad range of aliphatic and aromatic amides and exhibited enantioselectivity for several aromatic amides, including 2-phenylpropionamide (enantiomeric excess [ee] = 100%), phenylalaninamide (ee = 55%), and 2-(4-chlorophenyl)-3-methylbutyramide (ee = 96%), but not 2-(6-methoxy-2-naphthyl)propionamide (the amide form of naproxen) (ee = 0%). The characteristics of the *P. chlororaphis* B23 amidase are the same as the characteristics of enantioselective amidases described by Mayaux et al. (J. F. Mayaux, E. Cerbelaud, F. Soubrier, D. Faucher, and D. Petre, J. Bacteriol. 172:6764–6773, 1990; J. F. Mayaux, E. Cerbelaud, F. Soubrier, P. Yeh, F. Blanche, and D. Petre, J. Bacteriol. 173:6694–6704, 1991) and Kobayashi et al. (M. Kobayashi, H. Komeda, T. Nagasawa, M. Nishiyama, S. Horinouchi, T. Beppu, H. Yamada, and S. Shimizu, Eur. J. Biochem. 217:327–336, 1993).

Nitriles are converted to their corresponding acids by two biological pathways. In one pathway, nitrilases (EC 3.5.5.1) hydrolyze nitriles directly to the corresponding carboxylic acids and ammonia:

$$RCN + 2\,H_2O \rightarrow RCOOH + NH_3 \quad (1)$$

The second pathway is a two-step pathway. Nitriles are hydrated and converted into the corresponding amides by nitrile hydratase (EC 4.2.1.84), and amides are hydrolyzed to the corresponding carboxylic acids by amidases (EC 3.5.1.4):

$$RCN + H_2O \rightarrow RCONH_2 \quad (2)$$

$$RCONH_2 + H_2O \rightarrow RCOOH + NH_3 \quad (3)$$

These pathways can be used to produce useful chemicals. For example, acrylamide is being produced from acrylonitrile by using the *Rhodococcus rhodochrous* J1 nitrile hydratase (4). Biological routes can be more efficient than chemical routes, and they can be regio- or stereospecific as well. Several amidases have been shown to be enantioselective (3, 4, 7, 8) and may be useful in the production of higher-value acids [e.g., (*S*)-2-arylpropionic acids, which are nonsteroidal anti-inflammatory drugs].

Although the genes which encode *Pseudomonas chlororaphis* B23 nitrile hydratase and amidase have been cloned (12), few characteristics of this amidase have been described previously (9, 11). In this paper we describe the purification and characteristics of this amidase, including the results of several enantioselective reactions.

MATERIALS AND METHODS

Materials. The following chemicals were purchased: (D,L)-phenylalaninamide and (D)- and (L)-phenylalanine were obtained from Sigma Chemical Co., St. Louis, Mo., and (*R*,*S*)-2-phenylpropionitrile and (*R*)- and (*S*)-2-phenylpropionic acids were obtained from Aldrich Chemical Co., Milwaukee, Wis. (*R*,*S*)-(2-(4-Chlorophenyl)-3-methylbutyramide) [(*R*,*S*)-CPIAm] was supplied by W. J. Linn (DuPont Agricultural Products Department). (*R*,*S*)-2-(6-Methoxy-2-naphthyl) propionamide (the amide form of naproxen) [(*R*,*S*)-NPAm] was supplied by J. S. Thompson (DuPont Central Research and Development Department).

Microorganism and fermentation conditions. *P. chlororaphis* B23 (= FERM-BP187) was obtained from the Fermentation Research Institute, Tokyo, Japan. Frozen stock solutions were prepared upon receipt following overnight growth in 4Y medium containing 10 mM propionitrile. 4Y medium contained (per liter) 8.6 g of KH_2PO_4, 0.01 g of yeast extract, 10 g of glucose, 0.24 g of citric acid (trisodium salt dihydrate), 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $FeSO_4 \cdot H_2O$, 1 ml of SL-7 trace metal solution (2), and 2.2 ml of butyronitrile (Fluka). Glycerol was added to a final concentration of 15% before the stock solutions were quickly frozen at −60°C. Laboratory cultures were generally maintained in medium containing 10 mM propionitrile. Subcultures were prepared by incubating organisms for 20 h at 21°C and then for 4 h at 26°C in two 10-ml test tubes containing the medium described above. The resulting subcultures were inoculated into two 100-ml volumes of the same medium and incubated with rotary shaking (200 rpm) for 21.5 h at 26°C. Next, the 100-ml cultures were transferred to two 500-ml volumes of the same medium and incubated with rotary shaking (200 rpm) for 6 h at 26°C. Finally, these cultures were used to inoculate a 50-liter fermentor (Chemap, Männedorf, Switzerland) containing standard medium supplemented with 0.5 ml of polyethylene glycol (Aldrich) per liter, and the preparation was incubated for 17.5 h at 25°C with agitation (180 to 500 rpm) and aeration (20 to 40 liters/min; pressure, 4 to 14 lb/in^2). The pH of the culture was maintained at pH 7. Cells were harvested in the late log phase by centrifugation for 25 min at 15,000 × *g* with a model AS-26P continuous-discharge centrifuge (Sharples, Warminster, Pa.) at a flow rate of 4 liters/min. The yield was 4.9 g (wet/weight) of cells per liter.

Purification of amidase. All purification procedures were performed at 4°C. Cells (516 g, wet weight) were resuspended in 3 liters of standard buffer, which contained 50 mM potassium phosphate (pH 7.2), 1 mM EDTA, and 1 mM dithiothreitol (DTT). The cells were disrupted by two passes through a homogenizer (Gaulin, Everett, Mass.) at 8,000 to 12,000 lb/in^2. The cell debris was removed by centrifugation for 30 min at 40,000 × *g* with a Sharples model T-1 continuous-discharge centrifuge at a flow rate of 200 ml/min. The resulting supernatant was the cell extract.

Ammonium sulfate fractionation was performed by adding solid ammonium sulfate to the cell extract. The active protein fractions precipitated at ammonium sulfate saturation values between 30 and 55%. The protein pellet was collected by centrifugation at 27,500 × *g* for 60 min, dissolved in standard buffer supplemented with 20% ammonium sulfate, and dialyzed against the same buffer.

The dialyzed protein sample was applied to a 500-ml Phenyl TSK 650M column (Tosohaas, Montgomeryville, Pa.) that had been equilibrated with standard buffer supplemented with 20% ammonium sulfate. The sample was eluted

* Corresponding author. Phone: (302) 695-4008. Fax: (302) 695-8114.

with an exponential gradient (500 ml of 20% ammonium sulfate in standard buffer to 2.5 liters of standard buffer). The active fractions were precipitated with 55% ammonium sulfate. After centrifugation at 27,000 × *g* for 15 min, the pellet was dissolved and dialyzed against standard buffer.

The protein was applied to a 250-ml DEAE 650M TSK column (Tosohaas) that had been equilibrated with standard buffer. After the column was washed with the same buffer, amidase was eluted from the column with a linear 0 to 0.2 M NaCl gradient in standard buffer. The active fractions were concentrated by precipitation with 55% ammonium sulfate. After centrifugation at 27,000 × *g* for 15 min, the pellet was dissolved in a minimum volume of standard buffer.

The resulting concentrated fraction was loaded onto a gel filtration column (TSK HW55F; 5 by 50 cm; Tosohaas), equilibrated, and eluted with 50 mM Tris (pH 7.8)–1 mM DTT at a flow rate 3 ml/min. The active fractions were pooled and applied to a ceramic hydroxyapatite column (60 to 100 μm; 100 ml; American International Chemical, Natick, Mass.) that had been equilibrated with 50 mM Tris (pH 7.8). Amidase was eluted with a linear 0 to 40 mM potassium phosphate gradient. The amidase fraction (16.2 ml) was concentrated by ultrafiltration through a PM-30 membrane (Amicon, Beverly, Mass.).

The resulting concentrated material was applied to a 250-ml of DEAE 650M TSK column (Tosohaas) that had been equilibrated with Tris (pH 7.8)–1 mM EDTA–1 mM DTT. The material was eluted with a 0 to 0.2 M NaCl gradient in the same buffer. The active fractions were concentrated by ultrafiltration through a PM-30 membrane and used for characterization studies.

Enzyme assay for amidase activity. Unless indicated otherwise, amidase-catalyzed turnover of propionamide to propionic acid and ammonia was measured by using a coupled spectrophotometric assay. The amount of product formed was determined with a coupling enzyme, glutamate dehydrogenase (EC 1.4.1.2), which converts ammonia, 2-oxoglutarate, and the cofactor NADH to L-glutamate and NAD^+. The turnover of NADH to NAD^+ was measured by determining the decrease in A_{340} and was stoichiometric to the turnover of propionamide. Each reaction mixture was preincubated at 30°C for 15 min and contained 100 mM Tris (pH 7.8), 100 mM propionamide, 10 mM 2-oxoglutarate, 0.56 mM NADH, 2 mM ADP, 15 U of glutamate dehydrogenase, and up to 0.1 U of amidase. This procedure resulted in a convenient maximal rate of complete NADH turnover in 5 min. In this study 1 U of activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of product per min.

Under some circumstances the coupled enzyme assay was not suitable, and it was necessary to determine propionic acid content directly by gas chromatography (GC). Each GC assay reaction mixture contained 100 mM Tris (pH 7.8), 10 mM propionamide, and an appropriate amount of amidase (purified or in cells) and was preincubated at 25°C. At different times, 0.5-ml aliquots were removed, and their pH values were adjusted to 2.5 to 3 with phosphoric acid. The GC analysis was performed with a model HP5880 GC equipped with a Nukol column (0.58 mm by 15 m; Supelco, Bellefonte, Pa.) under the following conditions: a helium flow rate of 2.5 ml/min; an initial oven temperature of 80°C, with the temperature increased at a rate of 25°C/min to 220°C and then kept at 220°C for 3.5 min; an injector temperature of 230°C; and a flame ionization detector temperature of 240°C. Our conclusions were based on previously determined retention times for authentic standards.

Protein determination. Protein content was determined by measuring the A_{280} and by the bicinchoninic method (14), using the bicinchoninic acid reagent supplied by Pierce Chemical Co., Rockford, Ill., and crystalline bovine serum albumin as the standard.

Analytical determinations. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the subunit molecular weight; 10 to 15% PhastGel gradient gels were used with the Pharmacia Phast System. Proteins from a Pharmacia low-molecular-weight calibration kit were used as the molecular weight markers. The native molecular size was determined by gel filtration chromatography, using a Zorbax GF-250 column (pore size, 15 nm; 9.4 by 250 mm; DuPont, Wilmington, Del.) and chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), lactate dehydrogenase (140 kDa), aldolase (158 kDa), catalase (232 kDa), and β-galactosidase (440 kDa) as the standard proteins. For the N-terminal sequence analysis, automated gas phase Edman degradations were performed with a model LF 3000 protein sequencer (Beckman, Emeryville, Calif.). Samples were dialyzed against 50 mM ammonium bicarbonate.

Effects of inhibitors on activity. The effects of inhibitors (each at a final concentration of 1 mM) were determined after 7.4 μg of amidase in 0.1 M Tris (pH 7.8) was preincubated at 25°C. Residual activities were determined after 100 mM propionamide (final volume, 1 ml) was added and the preparation was incubated for 30 min at 25°C. The amount of propionic acid produced was determined by GC.

Substrate specificity. The standard amidase assay was modified to measure amidase activity against various amides. Amides (10 mM) were added to the standard reaction mixture, and the reaction was started with 1.85 μg of amidase. Controls were included to check for inhibition of the coupling enzyme by any substrate. In these control preparations 100 mM ammonia (as ammonium sulfate) was added directly (no amidase was present). A GC assay was performed with *N*-methyl-substituted compounds and benzonitrile.

Stereoselectivity. (*R*,*S*)-CPIAm and (*R*,*S*)-NPAm assays were developed previously (16). Since both of these amides are soluble only at concentrations up to about 1 mM, they were dissolved in acetone, added to vials, and dried down. Each 1-ml reaction mixture contained 2.1 μg of (*R*,*S*)-CPIAm or 0.46 μg of (*R*,*S*)-NPAm (only a portion in solution) in 100 mM Tris (pH 7.8) containing 0.37 mg of amidase. At different times between 4 and 40 h individual reactions were stopped by acidifying the reaction mixtures to pH 3 with 20 μl of 3 M sulfuric acid. Controls revealed that amidase activity was maintained. (*R*,*S*)-2-Phenylpropionamide was synthesized by a method suggested by Nagasawa and coworkers (3, 10). For the (*R*,*S*)-2-phenylpropionamide assays we used individual time points, and each reaction mixture contained 0.2 ml of 3.1 mM amide (each enantiomer) in 100 mM Tris (pH 7.8); the reactions were started by adding 29.6, 2.96, or 0.296 μg of amidase. After 20 min and 1, 4, and 24 h the reactions were stopped by heating the reaction mixtures at 95°C for 3 min. (D,L)-Phenylalanine amide assay mixtures (1 ml) contained 2.0 mg of (D,L)-amide in 100 mM Tris (pH 7.8), and the reactions were started by adding 3.7 μg of amidase. At different times between 5 and 120 min, individual reactions were stopped by acidifying the preparations to pH 3 with 30 μl of 1.5 M phosphoric acid. After chiral high-performance liquid chromatography (HPLC) analysis, the enantiometric excess [ee] was determined:

$$ee_{S\text{-acid}\,(\%)} = [(S_{acid} - R_{acid})/(S_{acid} + R_{acid})] \times 100 \quad (4)$$

Chiral HPLC analysis. We analyzed (*R*,*S*)-CPIAm and (*R*,*S*)-(2-(4-chlorophenyl)-3-methylbutyrate) as well as (*R*,*S*)-NPAm and (*R*,*S*)-2-(6-methoxy-2-naphthyl)propionate with a CHIRAL-AGP column (4.6 by 100 mm; ASTEC, Inc., Whippany, N.J.) and a model HP1090M HPLC (Hewlett Packard, Avondale, Pa.); compounds were eluted with a 95% 0.01 M Na_2HPO_4–5% pure ethanol (pH 6.0) mobile phase at a flow rate of 0.9 ml/min and were detected at 220 nm [(*R*,*S*)-CPIAm and (*R*,*S*)-(2-(4-chlorophenyl)-3-methylbutyrate)] or 234 nm [(*R*,*S*)-NPAm and (*R*,*S*)-2-(6-methoxy-2-naphthyl)propionate]. The configuration analysis for these compounds was based on previously determined retention times obtained with authentic standard enantiomers or racemic mixtures (16). We also performed an analysis with (*R*,*S*)-2-phenylpropionamide and (*R*,*S*)-2-phenylpropionate, in which we used the same system and column but a 0.01 M Na_2HPO_4 (pH 4.3) mobile phase and detection at 220 nm (8); the configuration analysis for these compounds was based on the retention times of enantiomeric acid standards. And finally, we also performed an analysis with (D,L)-phenylalanine amide. After modification with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent; Pierce), diastereomers were separated on an octyldecylsilane (C_{18}) column (Zorbax; DuPont) by using a 34% acetonitrile–66% 0.1% H_3PO_4 (pH 2.1) mobile phase and were detected at 340 nm (6).

Induction of amidase activity. Shake flask cultures (20 ml; 250 rpm) of *P. chlororaphis* B23 were grown overnight in the presence of inducers (concentration, 10 mM) in medium containing (per 100 ml) 1 g of sucrose, 0.2 g of L-cysteine, 0.2 g of L-glutamate, 0.2 g of L-proline, 0.05 g of KH_2PO_4, 0.05 g of K_2HPO_4, 0.05 g of $MgSO_4 \cdot 7H_2O$, and 0.001 g of $FeSO_4 \cdot 7H_2O$. After centrifugation for 10 min at 15,000 × *g* to harvest cells, 20 mg of cells was suspended in 100 mM Tris (pH 7.8)–10 mM propionamide. Amidase activity was determined after 120 min of incubation at 25°C by detecting propionic acid formation by GC. Controls in which cells were grown in the absence of amide were included.

RESULTS

Purification. The *P. chlororaphis* B-23 amidase was purified as described in Materials and Methods. After the cell extract was prepared, amidase was purified to near homogeneity by ammonium sulfate (30 to 55%) fractionation followed by hydrophobic, anion-exchange, gel filtration, and ceramic hydroxyapatite chromatography steps. DTT was required throughout the purification procedure to maintain enzymatic activity. When either propionamide or 2-(4-chlorophenyl)-3-methylbutyramide was used as the substrate, only one activity peak was found with each column. The extinction coefficient for amidase at 280 nm was 1.0 $(mg/ml)^{-1} \cdot cm^{-1}$. The overall level of purification was 114-fold, and the recovery rate was 21% (Table 1). The pure enzyme turned over propionamide to propionate and ammonia at a rate of 11.7 μmol/min/mg of protein under our standard reaction conditions.

Protein structure. The protein structure and mass that we found agree with the data published previously, including estimates based on DNA sequences (9, 12). Analytical gel filtration HPLC revealed that the molecular mass of the native enzyme is about 105 kDa. The subunit molecular mass was determined by SDS-PAGE; a single protein band at 54 kDa was obtained. Therefore, the native form of amidase is a dimer consisting of identical subunits. The NH_2-terminal sequence

TABLE 1. Purification of *P. chlororaphis* B23 amidase

Step	Total amt of protein (mg)[a]	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Cell extract	71,920	7,400	0.10	100	1
Ammonium sulfate fractionation (30–55%)	20,125	4,500	0.22	61	2
Phenyl TSK	3,370	3,783	1.1	51	11
First DEAE TSK column	1,090	3,270	3.0	44	29
HW55F filtration	445	2,303	5.2	31	50
Hydroxyapatite	155	1,597	10.3	22	100
Second DEAE TSK column	133	1,560	11.7	21	114

[a] Determined by measuring the A_{280}.

that we determined was Asp-Ile-Thr-Arg-Pro-Thr-Leu-Asp-Gln-Val-Leu-Asp-Ile-Ala-Thr-Gln-Leu-His-Met-Gln-Leu-Thr-His-Glu. This sequence was very similar to the sequence reported previously (12); the only discrepancy was in cycle 14 (Ala instead of the previously reported Arg).

Effect of pH on activity and stability. Enzyme activity was determined between pH 3.4 and 11.0 by the standard coupled assay. The amidase exhibited a broad range of maximal activity against the substrate propionamide at pH values between 7.0 and 8.6 (Fig. 1A). The stability of the amidase was determined after the purified enzyme was preincubated in various buffers having pH values ranging from 3.9 to 9.9. The amidase exhibited 100% activity at pH values between 5.9 and 9.9 (data not shown).

Effect of temperature on activity and stability. Enzyme activity was determined at temperatures between 20 and 60°C by the standard assay. We found that the amidase activity increased to the maximum value at 50°C and then decreased rapidly at higher temperatures (Fig. 1B). The stability of the amidase was determined by measuring the residual activity after 30 min of preincubation at temperatures between 25 and 60°C. The amidase was found to be stable at temperatures up to 50°C (data not shown).

Inhibitors. The effects of several inhibitors were determined by studying turnover of propionamide by amidase. The substrate and product concentrations were determined by GC after the amidase was preincubated for 30 min with inhibitors (each at a concentration of 1 mM) (Table 2). Propionamide was converted to propionic acid at a rate of 7.7 μmol/min/mg of amidase, and this value was defined as 100%. Amidase activity was significantly affected by sulfhydryl reagents, such as Ag^{+}, Cu^{2+}, and 5,5′-dithiobis(2-nitrobenzoic acid) but was not affected by iodoacetic acid. Inhibition was counteracted by the presence of 1 mM DTT. This finding indicates that thiol groups are involved in the conformation or active site of the amidase, as proposed previously (9). Some other metal ions, such as Fe^{2+} and Cd^{2+}, were also somewhat inhibitory. The carbonyl reagent cysteamine did not have any significant effect. Two chelating reagents, EDTA and *o*-phenanthroline, also had no inhibitory effect.

Substrate specificity. The activity of the amidase was determined with a variety of substrates (Table 3). The amidase appeared to have a wide substrate specificity. Conversion of propionamide to propionic acid at a rate of 11.7 μmol/min/mg of amidase was defined as 100%. Excellent activity was observed with aliphatic amides, such as propionamide, *n*-butyramide, and isobutyramide (highest activity observed). In general, lower levels of activity were observed with unsaturated aliphatic amides, including acrylamide, methacrylamide, and crotonamide. Excellent activity was also observed with several aromatic amides, including nicotinamide and D,L-phenylalanine amide. No activity was observed with the amides form-

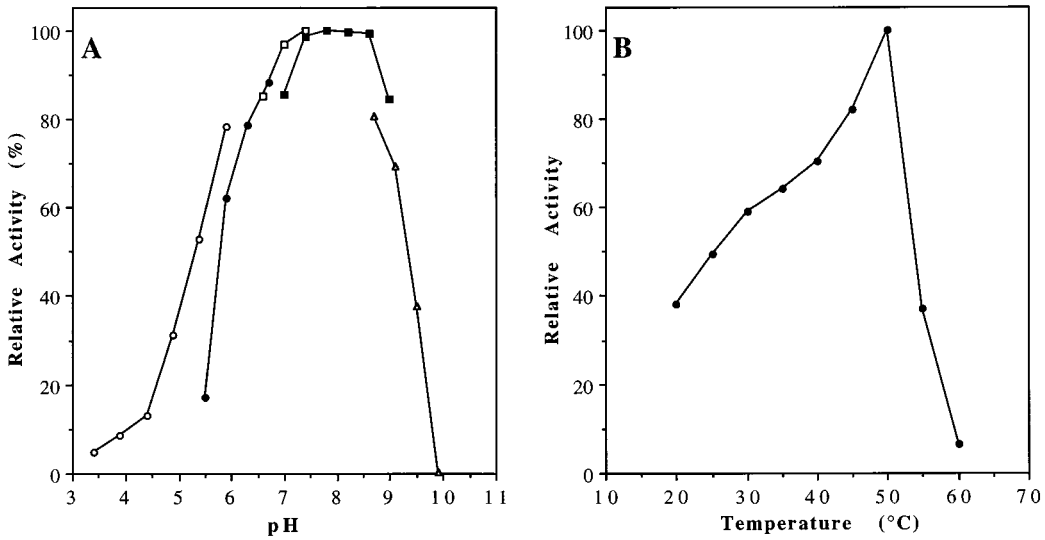


FIG. 1. Optimum pH (A) and optimum temperature (B) for *P. chlororaphis* B23 amidase activity. (A) Levels of activity were determined by using the standard assay and the following buffers (each at a final concentration of 0.1 M): acetate (○), 2-(*N*-morpholino)ethanesulfonic acid (MES) (●), 3-(*N*-morpholino)propanesulfonic acid (MOPS) (□), Tris (■), and 3-(cyclohexylamino)ethanesulfonic acid (CHES) (△). (B) Levels of activity were determined at temperatures between 20 and 60°C by the standard assay.

TABLE 2. Effects of inhibitors on amidase purified from *P. chlororaphis* B23

Compound	% Remaining activity[a]	% Remaining activity in the presence of 1 mM DTT
$AgNO_3$	25	93
$CuSO_4$	51	87
DTNB[b]	57	106
Iodoacetic acid	92	93
Cysteamine	88	
$FeCl_2$	57	
$CdCl_2$	69	
EDTA	91	
o-Phenanthroline	99	

[a] Amidase activity was measured by GC in the presence of 1 mM inhibitor. The level of activity observed in the absence of inhibitor was defined as 100% (7.7 $\mu mol \cdot min^{-1} \cdot mg^{-1}$).
[b] DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

amide, urea, *N*-methylacetamide, and *N*-methylbenzamide or with benzonitrile.

Kinetic properties. Results from our steady-state kinetic plots for the strain B23 amidase were consistent with an allosteric enzyme that exhibits positive cooperativity (13). The Lineweaver-Burk plot curve approached a horizontal line at the $1/v$ axis, while the Eadie-Scatchard plot was curved (Fig. 2A). An apparent n (n_{app}) value was determined at the peak of the curve:

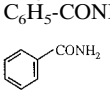
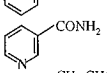
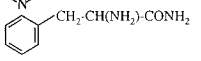
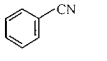
$$v = [(n_{app} - 1)/n_{app}]\, V_{max} \quad (5)$$

Replotting $1/v$ versus $1/S^{(n_{app})}$ (Fig. 2B) yielded an intrinsic binding constant (K') for propionamide of 0.25 mM, a V_{max} of 11.7 μmol/min/mg, and a turnover number (K_{cat}) of 10.5 s^{-1}.

Stereoselectivity. The purified strain B23 amidase was examined for stereoselectivity by studying hydrolysis of several racemic amides (Table 4). This enzyme exhibited S-specific amidase activity for phenylalaninamide (enantiomeric excess [ee] = 55%), 2-phenylpropionamide (ee = 100%), and 2-(4-chlorophenyl)-3-methylbutyramide (ee = 96%) but not for the amide form of naproxen (ee = 0%).

Induction of amidase activity. The effects of several inducers on amidase production in *P. chlororaphis* B23 were also determined. Substrate and product concentrations were determined by GC after amidase was incubated with 10 mM propionamide for 120 min (data not shown). Several compounds induced amidase production; these compounds included (in decreasing order of activity) isobutyramide, *n*-propionamide, *n*-butyramide, isovaleramide, methacrylamide, formamide, and acrylamide. Compounds which did not induce amidase synthesis included crotonamide, benzamide, nicotinamide, *N*-methylacetamide, *N*-methylformamide, acetamide, sodium propionate, sodium *n*-butyrate, urea, and ammonium. Controls consisting of cultures grown without inducers exhibited no amidase activity and produced similar growth in the presence and in the absence of inducers.

TABLE 3. Substrate specificity of the amidase purified from *P. chlororaphis* B23

Substrate	Chemical formula	Relative activity (%)[a]
Formamide	H-$CONH_2$	0
Acetamide	CH_3-$CONH_2$	21
Propionamide	CH_3-CH_2-$CONH_2$	100
n-Butyramide	CH_3-CH_2-CH_2-$CONH_2$	62
Isobutyramide	$(CH_3)_2$-CH-$CONH_2$	468
Acrylamide	CH_2═CH-$CONH_2$	29
Methacrylamide	CH_2═C(CH_3)-$CONH_2$	52
Crotonamide	CH_3-CH═CH-$CONH_2$	4
Urea	NH_2-$CONH_2$	0
Fluoroacetamide	F-CH_2-$CONH_2$	144
N-Methylacetamide	CH_3-CONH-CH_3	0[b]
N-Methylbenzamide	C_6H_5-CONH-CH_3	0[b]
Benzamide		44
Nicotinamide		106
DL-Phenylalanine amide		360
Benzonitrile		0[b]

[a] A level of activity of 11.7 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ or 10.5 turnovers $\cdot s^{-1}$ was defined as 100%.
[b] As determined by the GC assay.

DISCUSSION

In this paper, we describe purification and characteristics of a *P. chlororaphis* B23 amidase. A combination of ammonium sulfate precipitation and hydrophobic, anion-exchange, gel filtration, and ceramic hydroxyapatite chromatography steps resulted in a homogeneous preparation.

The strain B23 amidase is a homodimer with a native molecular mass of 105 kDa and a subunit molecular mass of 54 kDa. Our Lineweaver-Burk and Eadie-Scatchard plots (Fig. 2) were typical of the plots obtained with allosteric enzymes that exhibit positive cooperativity. The n_{app} value determined from the Eadie-Scatchard plot indicates only a minimum value for the interacting substrate binding sites of the enzyme (and can be nonintegral). This value was used to determine a V_{max} of 11.7 μmol/min/mg and a K_{cat} value of 10.5 s^{-1} (or a molecular activity value of 1.26 kmol/min/mol of amidase) at 30°C for the conversion of propionamide to propionate. The enzyme has a fairly high temperature optimum (50°C) and a broad pH optimum (pH 7.0 to 8.6) and is stable over wide pH and temperature ranges.

The strain B23 amidase does not require metal ions since no inhibition was observed in the presence of EDTA. Analysis of the purified enzyme by inductively coupled plasma discharge also revealed no trace of metals (data not shown). The amidase was inhibited by several thiol reagents, indicating that a cysteine residue is required for activity. The amidase activity was protected and not inhibited by DTT, indicating that disulfide bonds are not critical for enzyme activity. The enzyme was also not inhibited by a carbonyl reagent, cysteamine.

The strain B23 enzyme exhibited activity with a broad range of amides, including aliphatic, unsaturated, and aromatic amides. Only *N*-methyl-substituted amides or the smallest amides (formamide and urea) were not suitable as substrates. Acyltransferase activity was also detected with amides and hydroxylamine (data not shown).

The enantioselective properties of the *P. chlororaphis* B23 amidase are similar to those of several other amidases. Mayaux et al. described a common consensus region in the predicted amino acid sequences for eight genes of different amidases, including enantioselective amidases from a *Rhodococcus* species (8) and *Brevibacterium* sp. strain R312 (7). Later, it was reported that *R. rhodochrous* J1 (4) and *P. chlororaphis* B23 (12) amidases also contain this consensus region. These amidases are all homodimers that are similar in size. Three of the

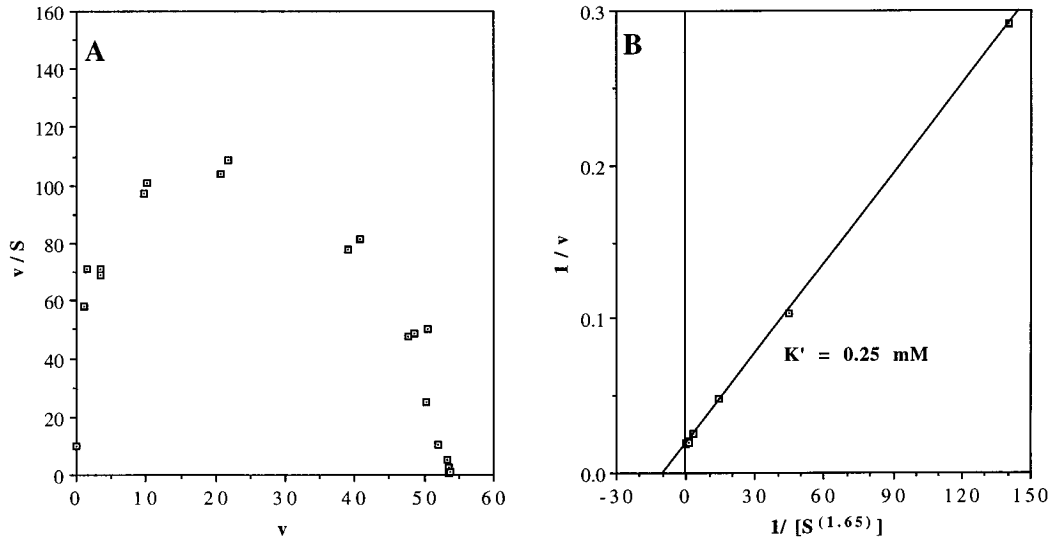
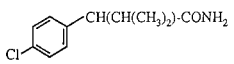
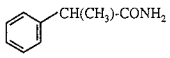
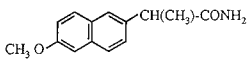
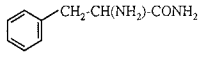


FIG. 2. Kinetics of *P. chlororaphis* B23 amidase activity when propionamide is the substrate. (A) Eadie-Scatchard plot. (B) Lineweaver-Burk plot adjusted by using the n_{app} value (1.65).

four have been shown to be sensitive to sulfhydryl reagents, and one cysteine is present in the consensus region and is strictly conserved. The *P. chlororaphis* B23 amidase does exhibit enantioselectivity for three aromatic amides, including (*S*)-2-phenylpropionamide (as do the *R. rhodochrous* J1 amidase [4], the *Brevibacterium* sp. strain R312 amidase [7], and the *Rhodococcus* species amidase [8]) and (*S*)-2-(4-chlorophenyl)-3-methylbutyramide, but not for 2-(6-methoxy-2-naphthyl)propionamide (the amide form of naproxen). In addition, an intermediate level of enantioselectivity was observed for another aromatic substrate, phenylalaninamide (ee = 55%). At this point, we can say that the enzyme can accept substrates with an aryl group α or β to the amide carbon. The change in the structure of the naproxen amide may seem small compared with 2-phenylpropionamide, but the (*R*) and (*S*) forms are both turned over (enantioselectivity is completely lost), perhaps reflecting a structural constraint of the enzyme. This amidase was specific for the (*S*) form of three substrates, but this may not always be the case. Examples such as the *Brevibacterium* sp. strain R312 amidase (7) and the *Rhodococcus* species amidase (8) exhibit specificity for (*S*)-2-phenylpropionamide and also for (*R*)-2-(4-hydroxy-phenoxy)propionamide. In the consensus region of the four enzymes mentioned above, 28 of 56 amino acid residues are strictly conserved. There are now four amidases which are known to have this amidase consensus sequence and carry out stereoselective hydrolysis of 2-aryl propionamides. In addition to these four enzymes, there are several other enzymes which have been shown to have significant portions of this consensus region, but enantioselectivity has not been characterized in these enzymes; these enzymes include the *Aspergillus nidulans* acetamidase, the *Flavobacterium* sp. aminohexanoate cyclic dimer hydrolase, the *Agrobacterium tumefaciens* indole acetamide hydrolase, the *Pseudomonas syringae* (*Pseudomonas savastanoi*) indole acetamide hydrolase, and the *Bradyrhizobium japonicum* indole acetamide hydrolase (8). Another common feature of the enantioselective amidases is the close proximity of the amidase gene to the gene for nitrile hydratase (4, 7, 8). In contrast, the enantioselective amidases have been shown to exhibit no significant sequence homology with the aliphatic amidases of *Pseudomonas aeruginosa* (1) and the wide-spectrum amidases of *Brevibacterium* sp. strain R312 (15). It appears that these two types of amidases can be classified as Mayaux et al. suggested, not only on the basis of the consensus sequence, but also on the basis of enantioselective hydrolysis of 2-aryl propionamides (8). Even though it is likely that the consensus region is involved in the catalytic function of the enzyme, a more thorough study will be required to link the region and the

TABLE 4. Enantioselectivity of the amidase purified from *P. chlororaphis* B23

Substrate	Chemical formula	ee (%)[a]	Relative activity (%)[b]
(*R*,*S*)-CPIAm		96	0.2
(*R*,*S*)-2-Phenylpropionamide		100	2.3
(*R*,*S*)-NPAm		0	0.025
(D,L)-Phenylalaninamide		55	100

[a] See equation 4.
[b] A level of activity of 12 μmol of phenylalaninamide · min^{-1} · mg of $amidase^{-1}$ was defined as 100%.

amino acid sequence to the enantioselectivity toward 2-aryl propionamides.

Several factors have made *P. chlororaphis* B23 a good second-generation choice for acrylamide production in Japan. Even though the nitrile hydratase is linked genetically to the enantioselective amidase which we studied (5), nitrile hydratase activity was optimized for acrylamide production by methacrylamide induction, a temperature of 10°C, and acrylonitrile inhibition of amidase. Amidase activity was barely detected (5, 9). It now appears that the enantioselective properties of the *P. chlororaphis* B23 amidase may make it useful in the production of higher-value acids on its own or in combination with a nitrile hydratase.

ACKNOWLEDGMENTS

We thank J. Simonds and W. Wagner for fermentation and cell disruption, J. Lacey for HPLC analysis and evaluation of enantioselectivity, T. Miller for N-terminal amino acid analysis, P. Folsom for GC evaluations, and M. Odom for valuable discussions.

REFERENCES

1. **Ambler, R. P., A. D. Auffret, and P. H. Clarke.** 1987. The amino acid sequence of the aliphatic amidase from *Pseudomonas aeruginosa*. FEBS Lett. **215:**285–290.
2. **Gherna, R., P. Pienta, and R. Cote (ed.).** 1989. Bacteria and bacteriophages, 17th ed., p. 341. American Type Culture Collection, Rockville, Md.
3. **Gilligan, T., H. Yamada, and T. Nagasawa.** 1993. Production of S-(+)-2-phenylpropionic acid from (*R*,*S*)-2-phenylpropionitrile by the combination of nitrile hydratase and stereoselective amidase in *Rhodococcus equi* TG328. Appl. Microbiol. Biotechnol. **39:**720–725.
4. **Kobayashi, M., H. Komeda, T. Nagasawa, M. Nishiyama, S. Horinouchi, T. Beppu, H. Yamada, and S. Shimizu.** 1993. Amidase coupled with low-molecular-mass nitrile hydratase from *Rhodococcus rhodochrous* J1. Eur. J. Biochem. **217:**327–336.
5. **Kobayashi, M., T. Nagasawa, and H. Yamada.** 1992. Enzymatic synthesis of acrylamide: a success story not yet over. Trends Biotechnol. **10:**402–408.
6. **Marfey, P.** 1984. Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. Carlsberg Res. Commun. **49:**591–596.
7. **Mayaux, J. F., E. Cerbelaud, F. Soubrier, D. Faucher, and D. Petre.** 1990. Purification, cloning, and primary structure of a new enantiomer-selective amidase from *Brevibacterium* sp. strain R312: structural evidence for genetic coupling with nitrile hydratase. J. Bacteriol. **172:**6764–6773.
8. **Mayaux, J. F., E. Cerbelaud, F. Soubrier, P. Yeh, F. Blanche, and D. Petre.** 1991. Purification, cloning, and primary structure of a new enantiomer-selective amidase from a *Rhodococcus* strain: structural evidence for a conserved genetic coupling with nitrile hydratase. J. Bacteriol. **173:**6694–6704.
9. **Nagasawa, T., K. Ryuno, and H. Yamada.** 1989. Superiority of *Pseudomonas chlororaphis* B23 nitrile hydratase as a catalyst for the enzymatic production of acrylamide. Experientia **45:**1066–1070.
10. **Nagasawa, T., K. Takeuchi, V. Nardi-Dei, Y. Mihara, and H. Yamada.** 1991. Optimum culture conditions for the production of cobalt-containing nitrile hydratase by *Rhodococcus rhodochrous* J1. Appl. Microbiol. Biotechnol. **34:** 783–788.
11. **Nagasawa, T., and H. Yamada.** 1989. Microbial transformations of nitriles. Trends Biotechnol. **7:**153–158.
12. **Nishiyama, M., S. Horinouchi, M. Kobayashi, T. Nagasawa, H. Yamada, and T. Beppu.** 1991. Cloning and characterization of genes responsible for metabolism of nitrile compounds from *Pseudomonas chlororaphis* B23. J. Bacteriol. **173:**2465–2472.
13. **Segel, I.** 1975. Enzyme kinetics, p. 365. John Wiley & Sons, New York.
14. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150:**76–85.
15. **Soubrier, F., S. Levy-Schil, J. F. Mayaux, D. Petre, A. Arnaud, and J. Crouzet.** 1992. Cloning and primary structure of the wide-spectrum amidase from *Brevibacterium* sp. strain R312: high homology to the *amiE* product from *Pseudomonas aeruginosa*. Gene **116:**99–104.
16. **Stieglitz, B., W. J. Linn, W. Jobst, K. M. Fried, R. D. Fallon, K. Ingvorsen, and B. Yde.** January 1992. International patent WO 92/01062. A process for producing enantiomers of 2-aryl-2-alkanoic acids. DuPont/Novo Nordisk patent application DK1616/90.