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Production of D-Aminoacylase from *Alcaligenes denitrificans* subsp. *xylosoxydans* MI-4

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A bacterial strain that produces D-aminoacylase was isolated from soil and identified as *Alcaligenes denitrificans* subsp. *xylosoxydans* MI-4. L-Aminoacylase activity in this strain was only 1 to 2% of D-aminoacylase activity. D-Aminoacylase was inducibly produced. *N*-Acetyl-DL-leucine was the best inducer, and the D-isomer had the ability to induce the enzyme. Enzymatic resolution of *N*-acetyl-DL-methionine with the crude enzyme was carried out, and the D/L ratio in the resolved methionine was approximately 100/7, suggesting that resolution with crude enzymes may become possible by removing small amounts of the contaminated L-form with L-amino acid oxidase.

D-Amino acids are used widely as useful intermediate materials for synthesis of various compounds such as bioactive peptides and antibiotics. Several methods have been developed for the preparation of D-amino acids, e.g., optical resolution of the racemate (1), use of hydantoinase (14, 17), and coenzyme pyridoxal 5′-phosphate-dependent enzymes (11, 15).

Resolution of DL-amino acids has commercial importance and has been performed by physicochemical, chemical, and enzymatic methods (1). The physicochemical and chemical methods are not suitable for industrial resolution. At present, enzymatic methods are most the useful and convenient methods. One of these enzymes, L-aminoacylase, has been immobilized on DEAE-Sephadex and is now utilized in industry (2).

The occurrence of microbial D-aminoacylase was demonstrated in the cell extracts from some *Pseudomonas* species (5–7), *Streptomyces* species (12, 13), and, quite recently, *Alcaligenes* species (16), but the coexistence of L-aminoacylase was also confirmed. To produce D-amino acids from *N*-acetyl-DL-amino acids with crude enzymes or intact cells containing D-aminoacylase, we screened to obtain D-aminoacylase-producing bacteria in which L-aminoacylase activity is weaker or not present at all. We isolated a strain, MI-4, which was identified as *Alcaligenes denitrificans* subsp. *xylosoxydans*. In this paper, we report the culture conditions for producing D-aminoacylase from *A. denitrificans* subsp. *xylosoxydans* MI-4.

MATERIALS AND METHODS

Media and culture conditions. For the isolation of D-aminoacylase-producing bacteria, the following medium was used (medium A): *N*-acetyl-D-2-amino-4-chloro-4-pentenoic acid, 0.1%; glycerol, 0.5%; KH_2PO_4, 0.1%; K_2HPO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.01%; and yeast extract, 0.01% (pH 7.0), as reported previously (10). Medium B for the production of D-aminoacylase contained the following: carbon source, 1%; nitrogen source, 1%; inducer, 0.5%; KH_2PO_4, 0.1%; K_2HPO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.01%; and yeast extract, 0.05% (pH 7.0). Cultures in 2-liter Sakaguchi flasks containing 500 ml of the medium were incubated at 30°C for 22 h on a reciprocal shaker at 125 rpm. Experiments were also done with 1.2-liter cultures grown in a 2-liter fermentor (Mitsuwa Rikagaku Kogyo Co., Ltd., Osaka, Japan). Stirring was set at 200 rpm, and the temperature was adjusted to 30°C. Aeration was set to 1.2 liters/min after inoculation.

Isolation of D-aminoacylase-producing bacteria. A 1-g sample of soil was suspended in 5.0 ml of sterile distilled water by vigorous mixing. A 0.1-ml portion of the soil suspension was added to 1.0 ml of medium A in a test tube and cultivated at 30°C. After a 24-h cultivation, one loopful of the culture was transferred to another test tube containing 1.0 ml of fresh medium A. After this procedure was repeated several times, the cultures (0.1 ml) were streaked on nutrient agar plates. After 48 h, colonies were picked and purified by streaking three times on the nutrient agar plates. Each colony was tested for D-aminoacylase activity.

Assay of D-aminoacylase activity. The standard assay mixture for D-aminoacylase contained 100 mM potassium phosphate buffer (pH 7.8), 10 mM *N*-acetyl-D-methionine, and an appropriate amount of enzyme in a final volume of 1.0 ml. The reaction was started by the addition of the substrate. After incubation at 30°C for 20 min, the reaction was stopped by the addition of 0.1 ml of 50% trichloroacetic acid and the solution was centrifuged at 1,400 × *g* for 10 min. The supernatant solution was extracted with ethyl ether to remove trichloroacetic acid. D-Methionine liberated was analyzed by high-performance liquid chromatography. In a blank, the substrate was added after the enzyme was denatured with trichloroacetic acid. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of D-methionine per min. Specific activity is expressed as units per milligram of protein.

Resolution of *N*-acetyl-DL-methionine. The reaction mixture contained 600 mM potassium phosphate buffer (pH 7.8), 300 mM *N*-acetyl-DL-methionine, and 19.4 mg of cell extract in a final volume of 6.0 ml. The reaction mixture was incubated at 30°C. Methionine resolved was assayed by high-performance liquid chromatography. To isolate D-methionine, we adjusted the pH of the reaction mixture to 7.8 with ammonia instead of potassium phosphate buffer (pH 7.8).

Analytical methods. The optical activity of methionine formed by resolution was determined by L- or D-amino acid oxidases. The reaction mixture contained 10 mM potassium phosphate buffer (pH 8.0), 0.074 U of L-amino acid oxidase (or 0.056 U of D-amino acid oxidase), and an appropriate

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amount of methionine in a final volume of 0.1 ml. After the reaction was completed, the residual methionine was assayed by high-performance liquid chromatography, which was performed with a chromatograph (Japan Spectroscopic Co., Tokyo) equipped with an FP-110 fluorescence detector (excitation at 365 nm, emission at 455 nm). The buffer was 0.2 M sodium citrate buffer (pH 4.25). *o*-Phthalaldehyde was used as a fluorescent reagent. For the identification of methionine, the thin-layer chromatography method was also used. Cellulose thin-layer chromatography plates (10 by 10 cm; Funakoshi Co., Tokyo, Japan) were used with the following solvent system; *n*-butanol-acetic acid-water (63:10:27 [vol/vol/vol]) and phenol-water (100:36 [wt/wt in NH_3 vapor]). Protein was estimated by the method of Lowry et al. (8) with crystalline egg albumin as the standard.

Chemicals. *N*-Acetyl-D-, -L-, and -DL-amino acids, L-amino acid oxidase (*Crotalus adamanteus* venom, type IV), and D-amino acid oxidase (porcine kidney, type X) were purchased from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals used were of reagent grade from commercial sources.

RESULTS

Characteristics of *A. denitrificans* subsp. *xylosoxydans* MI-4. Strain MI-4 is an aerobic, nonsporeforming, oxidase (Kovacs)-positive, catalase-positive, gram-negative motile rod. Colonies which were 2 mm in diameter after 3 days at 30°C were round, regular, entire, semitranslucent, smooth, low convex, and pale brownish buff. This strain did not produce pyocyanin and fluorescence. It produced neither acid nor gas from glucose. In the medium of Hugh and Leifson (O-F test), acid was produced from xylose, but no change was seen with glucose. Nitrate was reduced to gaseous nitrogen. H_2S (TSI agar) was produced, but no indole was seen. Growth factor was not required. Urease, arginine dehydrolase, DNase, lecithinase, lipase, β-galactosidase, and decarboxylase (arginine, lysine, and ornithine) were not produced. It did not hydrolyze starch, casein, Tween 80, and esculin. Gelatin was not liquefied. It utilized as a carbon source glucose, gluconate, xylose, lactate, acetate, malate, caprate, adipate, citrate, and phenylacetate but not arabinose, mannose, mannitol, maltose, *N*-acetylglucosamine, L-arginine, and betaine. Strain MI-4 grew at 37 and 41°C but not at 45°C. These characteristics identify strain MI-4 as *A. denitrificans* subsp. *xylosoxydans* MI-4 according to *Bergey's Manual of Determinative Bacteriology* (3, 4).

Aminoacylase in cell extracts from *A. denitrificans* subsp. *xylosoxydans* MI-4. The activities of L- and D-aminoacylases were assayed with cell extracts from cells cultivated at 30°C for 22 h in glucose-$(NH_4)_2SO_4$ medium B containing 0.5% *N*-acetyl-DL-methionine as the inducer. The specific activity of L-aminoacylase (0.0076 U/mg of protein) was very low, about 1 to 2% of that of D-aminoacylase (0.440 U/mg of protein).

Culture conditions for D-aminoacylase production. To determine the conditions for maximizing the production of D-aminoacylase, we investigated the effect of the concentration of inducer. Increasing the concentration of *N*-acetyl-DL-methionine as an inducer increased D-aminoacylase activity, although D-aminoacylase activity was present even in the absence of an inducer (Fig. 1). L-Aminoacylase resulted in very little induction (specific activity, 0.0019 to 0.0028 U/mg of protein). An optimal concentration of 0.5% *N*-acetyl-DL-methionine was chosen for further study.

Various *N*-acetyl-DL-amino acids were tested for their ability to induce D-aminoacylase (Table 1). The best inducer was *N*-acetyl-DL-leucine, showing 63-fold induction. *N*-Acetyl-DL-methionine had a 16-fold induction and *N*-acetyl-DL-valine had only a 6-fold induction, whereas neither *N*-acetylglycine nor *N*-acetyl-DL-tryptophan served as inducers. Induction was also observed when DL-methionine or DL-leucine was used, although D-aminoacylase activity was about 50% of that found in cells induced with *N*-acetyl-DL-methionine or *N*-acetyl-DL-leucine. *N*-Acetyl-DL-methionine was substituted for *N*-acetyl-DL-leucine as an inducer for economic reasons in additional studies.

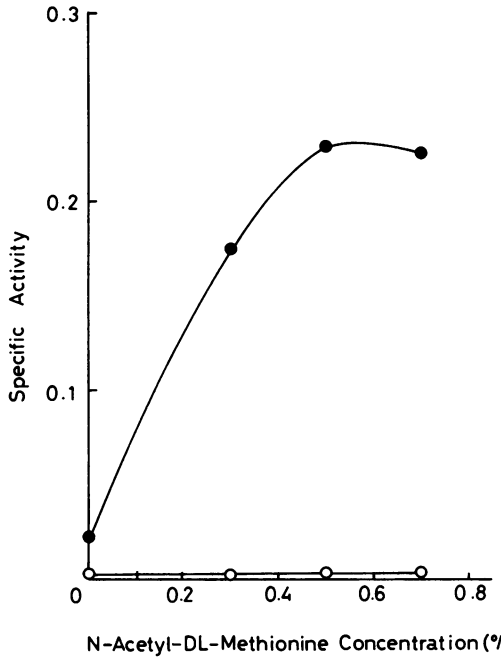

FIG. 1. Effect of *N*-acetyl-DL-methionine concentration on enzyme production. The cells were grown at 30°C for 22 h in 2-liter Sakaguchi flasks containing 500 ml of medium B supplemented with 1% glucose and 1% ammonium sulfate as the carbon and nitrogen sources, respectively. Symbols: ●, D-aminoacylase; ○, L-aminoacylase.

To examine which optical isomer of *N*-acetyl-amino acids induces D-aminoacylase, we grew the cells in the presence of 0.5% of each isomer of *N*-acetylmethionine. The L-isomer did not induce D-aminoacylase. This result suggests that the induction of D-aminoacylase is specific for the D-stereoisomer. D-Methionine completely inhibited growth. L-Methionine did not inhibit growth but failed to induce D-aminoacylase. Therefore, DL-methionine induction was caused by D-methionine, whose growth inhibition was reversed by L-methionine.

TABLE 1. Effect of inducers on production of D-aminoacylase

Inducer (0.5%)[a]	Total protein (mg)	Sp act (U/mg of protein)	Total units
None	150	0.019	2.8
N-Acetyl-DL-leucine	71	1.21	85.9
N-Acetyl-DL-methionine	68	0.317	21.5
N-Acetyl-DL-valine	41	0.120	4.9
N-Acetylglycine	90	0.015	1.3
N-Acetyl-DL-tryptophan	85	0.011	0.9
DL-Methionine	17	0.141	2.3

[a] The experimental conditions are described in the legend to Fig. 1.

TABLE 2. Effect of carbon and nitrogen sources on production of D-aminoacylase[a]

Carbon source (1%)	Nitrogen source (1%)	Total protein (mg)	Sp act (U/mg of protein)	Total units
Glucose	$(NH_4)_2SO_4$	154	0.309	47.5
Glycerol	$(NH_4)_2SO_4$	15	0.514	7.7
Citrate	$(NH_4)_2SO_4$	325	0.054	17.5
L-Glutamate	$(NH_4)_2SO_4$	279	0.041	11.4
Peptone	$(NH_4)_2SO_4$	311	0.174	54.1
Glucose	Peptone	549	0.149	81.8
Glucose	Corn steep liquor	267	0.153	40.8
Glucose	L-Glutamate	291	0.013	3.7

[a] The cells were grown in a 2-liter Sakaguchi flask containing 500 ml of medium B at 30°C for 22 h. The concentration of corn steep liquor was 0.5%. *N*-Acetyl-DL-methionine (0.5%) was used as the inducer.

The effects of various carbon and nitrogen sources on the production of D-aminoacylase were tested (Table 2). Glycerol as a carbon source resulted in the highest specific enzyme activity compared with the other carbon sources, but growth with glycerol was very poor. High total units of D-aminoacylase were obtained in glucose-$(NH_4)_2SO_4$, peptone-$(NH_4)_2SO_4$, glucose-peptone, and glucose-corn steep liquor media.

The time courses of induction of D-aminoacylase is given in Table 3. When *N*-acetyl-DL-methionine was used as an inducer, the cell yields were low because of the growth inhibition of the D-methionine liberated. Therefore, an inducer was added to a culture after the cells grew into the stationary phase. In the glucose-$(NH_4)_2SO_4$ medium (Table 3), total protein and total units of D-aminoacylase after 12 h of induction from the stationary phase were three to four times higher than those observed after 22 h of cultivation to which an inducer was added at the start of the experiment. In glucose-peptone medium (data not shown) and glucose-corn steep liquor medium (data not shown), on the other hand, D-aminoacylase was not fully induced. The combination of glucose and $(NH_4)_2SO_4$ as carbon and nitrogen sources was suitable for D-aminoacylase production. Washed cells from a culture grown in glucose-$(NH_4)_2SO_4$ medium were incubated with shaking in 10 mM potassium phosphate buffer (pH 7.0) containing 0.5% *N*-acetyl-DL-methionine as an inducer. D-Aminoacylase, however, was not induced (data not shown). Total units and specific activity of D-aminoacylase reached a maximum 18 and 24 h, respectively, after the addition of the best inducer, *N*-acetyl-DL-leucine (data not shown). However, the specific activity of the enzyme was about 45% of the value measured in cells grown in Sakaguchi flasks (Table 1).

TABLE 3. Time course of induction of D-aminoacylase[a]

Cultivation time (h)	Total protein (mg)	Sp act (U/mg of protein)	Total units
(A) After start of expt			
16	189	0.129	24.3
22	331	0.196	64.8
25	487	0.152	74.0
29	1,001	0.073	73.0
33	643	0.095	61.0
(B) After stationary phase			
0	208	0.012	2.4
4	512	0.117	59.9
8	1,046	0.203	212.3
12	1,094	0.279	305.2

[a] The cells were grown in a 2-liter jar fermentor containing 1.2 liters of glucose-$(NH_4)_2SO_4$ medium B. *N*-acetyl-DL-methionine (0.5%) was added at the start of the cultivation (A) or at the stationary phase (21 h) (B).

Resolution of *N*-acetyl-DL-methionine. Resolution of *N*-acetyl-DL-methionine by the cell extracts was performed. The cell extracts contained no detectable methionine racemase and methionine-degrading activities. After 2 h of incubation, nearly 100% of 25 mM *N*-acetyl-D-methionine was hydrolyzed with 3.2 mg of the cell extracts. The D/L ratio in the resolved methionine was approximately 100/7. To isolate D-methionine, we performed a large-scale reaction (100 ml). After 2 h (80% hydrolysis), the reaction mixtures were applied to a Dowex 50 × 8 (H^+) column (2 by 15 cm). After the column was washed with water, methionine was eluted with 2 N ammonia. Methionine was several times crystallized from ethanol. L-Methionine in crystalline methionine (132 mg; D-form, 122.8 mg; L-form, 9.2 mg) could be decomposed by 0.148 U of L-amino acid oxidase. Methionine was identified by high-performance liquid chromatography, thin-layer chromatography, and nuclear magnetic resonance and the D-form was demonstrated by D-amino acid oxidase.

DISCUSSION

In the course of the enzymatic resolution of *N*-acetyl-DL-2-amino-4-chloro-4-pentenoic acid, D-aminoacylase from *Streptomyces tuirus* (13) did not show activity toward this compound. In this study, to isolate new D-aminoacylase-producing bacteria with lower substrate specificity from the soil, we used *N*-acetyl-D-2-amino-4-chloro-4-pentenoic acid as the screening substrate and nitrogen source. L-2-Amino-4-chloro-4-pentenoic acid isolated from fruit bodies of *Amanita pseudoporphyria* Hongo has strong antibacterial activity (9). Since D-2-amino-4-chloro-4-pentenoic acid liberated from *N*-acetyl-D-2-amino-4-chloro-4-pentenoic acid also has antibacterial activity, the isolates could easily be purified by repeated inoculation on medium A. We isolated *A. denitrificans* subsp. *xylosoxydans* MI-4. This strain is the second example of a D-aminoacylase-producing *Alcaligenes* species.

D-Aminoacylase activities in the cell extracts from *A. denitrificans* were higher than those from *Streptomyces* (12) and *Pseudomonas* (5, 7) species. The specific activity of D-aminoacylase from *A. denitrificans* subsp. *denitrificans* DA181 (16) was 5.2 μmol/min per mg of protein, about 4.3-fold higher than that from *A. denitrificans* subsp. *xylosoxydans* MI-4 (Table 1), but the temperature conditions for the enzyme assay were different: 37°C for the DA181 enzyme and 30°C for the MI-4 enzyme. The activities of L-aminoacylase from the MI-4 enzyme, DA181 enzyme, and *Streptomyces* enzyme (12, 13) were about 1 to 2, 7, and 50%, respectively, of those of D-aminoacylase from the respective enzymes. D-Aminoacylases from *A. denitrificans* subsp. *xylosoxydans* MI-4, *A. denitrificans* subsp. *denitrificans* DA181, and *Streptomyces* species (13) were inducible. Detailed studies of induction for D-aminoacylase have not been reported for several different *Pseudomonas* species (5–7). *N*-Acetyl-DL-leucine and *N*-acetyl-DL-valine were the common inducers for the *Streptomyces* (13), *A. denitrificans* subsp. *denitrificans* DA181 (16), and *A. denitrificans* subsp. *xylosoxydans* MI-4 enzymes. For both DA181 and MI-4 enzymes, among the *N*-acetyl-DL-amino acids tested, *N*-acetyl-DL-leucine was the most effective inducer and *N*-acetyl-DL-tryptophan was inert as an inducer. On the other

hand, most of the *N*-acetyl-D- or -DL- amino acids have the ability to stimulate cell growth in strain DA181, whereas most of the *N*-acetyl-D-amino acids inhibit rather than stimulate growth in strain MI-4. Therefore, we added an inducer to the medium in the stationary phase of growth. DL-Methionine and DL-leucine also induced strain MI-4 enzyme, as observed with the *Streptomyces* enzyme (13). The use of peptone or corn steep liquor in the cultures repressed the induction of the MI-4 enzyme, whereas the *Streptomyces* and DA181 enzymes were induced regardless of the presence of peptone.

Enzymatic resolution of *N*-acetyl-DL-phenylglycine was carried out by the purified D-aminoacylase from *Streptomyces olivaceus* 62-3 (12). However, we tried to resolve *N*-acetyl-DL-methionine with the cell extracts from *A. denitrificans* subsp. *xylosoxydans* MI-4. Since L-methionine was resolved in small amounts, the removal of L-methionine by L-amino acid oxidase was easy. The present work revealed that resolution by crude enzymes can be achieved.

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