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A NEW D-STEREOSPECIFIC AMINO ACID AMIDASE FROM *OCHROBACTRUM ANTHROPI*

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SUMMARY: A new D-stereospecific amino acid amidase has been partially purified from *Ochrobactrum anthropi* SCRC SV3, which had been isolated and selected from soil. The *Mr* of the enzyme was estimated to be about 38,000, and its isoelectric point was 5.3. The enzyme catalyzes the stereospecific hydrolysis of D-amino acid amide to yield D-amino acid and ammonia. The major substrates included D-phenylalanine amide, D-tyrosine amide, D-tryptophan amide, D-leucine amide, and D-alanine amide.

The occurrence and application of three types of hydrolases acting on amino acid derivatives have been reported. They include L-specific aminoacylase (1), L-specific aminopeptidase (2), and D-specific aminoacylase (3,4). It is wellknown that two processes for the kinetic resolution of amino acid derivatives have been industrialized utilizing them. One is the process which employs L-specific aminoacylase (1) as a catalyst for the resolution of racemic *N*-acyl amino acid, and the other is the one with L-specific aminopeptidase (2) for amino acid amides. Much attention is being paid to the D-stereospecific amino acid amide hydrolase, because it is only recently that the group of the enzymes become known to occur. In the previous paper, we have discovered and characterized a new enzyme "D-aminopeptidase" from a bacterial isolate, *Ochrobactrum anthropi* (formerly *Achromobacter* sp.) SCRC C1-38 (5). Exploitation of such an unusual enzyme would provide a new target to study the structure and function of the enzyme, and to speculate, for example, from what an ancestral enzyme it has evolved. The use of such an enzyme could also bring about a new methodologies in the kinetic resolution of amino acid amides to yield D-amino acid in water, and in the synthesis of D-amino acid *N*-alkylamides in organic solvents (5). We here report an occurrence and characterization of a new D-stereospecific amino acid amidase in another bacterial isolate from soil, *Ochrobactrum anthropi* SCRC SV3.

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Abbreviation used: Boc, *tert*-butoxycarbonyl.

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MATERIALS AND METHODS

Chemicals: The chemical synthesis of amino acid amides and amino acid esters will be reported elsewhere.

Screening of Microorganisms and Cultivation: Microorganisms which have an ability to grow on D-valine amide as a nitrogen source were isolated from soil by an acclimation culture technique, using a medium composed of 0.5% sucrose, 0.2% D-valine amide HCl, 0.2% K_2HPO_4, 0.1% NaCl, 0.02% $MgSO_4$ $7H_2O$, 0.05% yeast extract, and vitamin mixture at a concentration described previously (6), in tap water, pH 7. Soil samples were aerobically incubated with the medium at 30°C. Half of the medium was replaced by the fresh medium every other day. After about three months' acclimation, when valine was begun to be detected in the culture fluid by thin layer chromatography, we could isolate a bacterial strain SCRC SV3 from the mixed culture as a D-valine amide degrader. The strain was identified as *O. anthropi* according to a new classification proposed by Holmes *et al.* (7). It was formerly classified in the genus *Achromobacter* (8).

Partial Purification of D-Amino Acid Amidase from *O. anthropi* SCRC SV3: The enzyme activity was estimated either by the determination of D-phenylalanine from D-phenylalanine amide, or by measuring ammonia from various amino acid amides. The reaction mixture contained 50 μmol Tris-HCl, pH 8.5, 5 μmol D-amino acid amide HCl, and the enzyme sample in a total volume of 0.5 ml. The reaction was terminated by boiling for 3 min after incubating at 30°C for 10 min. The hydrogen peroxide formed from D-phenylalanine by the action of D-amino acid oxidase was determined by the oxidative coupling with 4-aminoantipyrine and phenol in the presence of peroxidase (9). Ammonia was measured by an assay kit from Kyowa Medex (Tokyo, Japan), which utilizes NAD^+-dependent glutamate dehydrogenase. One unit of the enzyme activity was defined as the amount of enzyme which catalyzed the formation of 1 μmol of D-phenylalanine or ammonia per min. *O. anthropi* SCRC SV3 was aerobically grown for 20 hr at 30°C in a medium called TGY medium (6). All the purification procedures were performed at a temperature lower than 5°C, unless otherwise noted. The buffer used throughout this study was potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol. The cells obtained from 40 liters of culture (280 g wet weight) was suspended in 0.1 M buffer, and disrupted for 20 min by a 9 kHz sonic oscillator. To the cell extract was added 5% protamine sulfate to a concentration of 0.1 g protamine to 1 g protein. The precipitate formed after stirring for 30 min was removed by centrifugation. To the supernatant was added solid ammonium sulfate to 60% saturation. The active precipitate was dialyzed and applied to the 1st DEAE-Toyopearl (Tosoh, Tokyo, Japan) column (6.5 x 30 cm) and eluted with 0.1 M buffer. The 2nd DEAE-Toyopearl column (2.2 x 13 cm) chromatography was carried out in the same condition. Dialyzed enzyme solution was applied to a hydroxyapatite column (6.5 x 4.5 cm), equilibrated with 0.01 M buffer, and eluted with a linear gradient of 0.01-0.2 M buffer. The active fractions were dialyzed and concentrated by ultrafiltration (Amicon, PM 30) and applied to a Sephadex G-200 column (2.2 x 116 cm) equilibrated with 0.05 M buffer containing 0.1 M NaCl. The active fractions were dialyzed, concentrated and further applied to a Tosoh HPLC column G-3000 SW (0.75 x 60 cm), eluted as described previously (10). The active fractions were collected and concentrated by ultrafiltration.

Other methods: Protein was estimated, and isoelectric focusing was carried out as described previously (10).

RESULTS

Partial Purification of D-Amino Acid Amidase from *O. anthropi* SCRC SV3: *O. anthropi* SCRC SV3 which had been isolated from soil was chosen as a likely source of the enzyme. The enzyme was partially purified about 250-fold up to a specific activity of 24.1 units/mg, following D-phenylalanine amide hydrolyzing activity. L-Phenylalanine amide hydrolyzing activity was removed at the step of 1st DEAE-Toyopearl column chromatography, when eluted by 0.05 M buffer.

Table 1. Substrate Specificity of D-Amino Acid Amidase

Substrate[a]	Relative activity (%)	Substrate	Relative activity (%)
D-Phenylalanine amide	100	D-Phenylglycine amide	15
D-Tyrosine amide	97	D-Proline amide	9.7
D-Tryptophan amide	96	D-Lysine amide	2.5
D-Leucine amide	46	D-Histidine amide	1.5
D-Norleucine amide	40	D-Asparagine	1.4
D-Alanine amide	33	D-Glutamine amide	1.1
D-Methionine amide	28	D-Phenylalanine methylester	192
D-Norvaline amide	15		

[a] D-Threonine amide, glycine amide, D-glutamine, D-asparagine amide, D-α-amino butyric acid amide, D-isoglutamine, D-arginine amide, D-valine amide, and D-isoleucine amide were hydrolyzed by 0.1 to 0.6 % the rate for D-phenylalanine amide. The following compounds were not the substrate for the enzyme: 2-Phenylacetamide, acetamide, propionamide, Boc-D-alanine amide, D-isoasparagine, D-alanine-*p*-nitroanilide, D-alanylglycine, D-alanyl-D-alanine, D-alanyl-D-alanyl-D-alanine, D-alanyl-L-alanine, D-alanyl-L-alanyl-L-alanine, L-alanyl-D-alanine, L-phenylalanine amide, L-tyrosine amide, L-tryptophan amide, L-leucine amide, L-alanine amide, L-methionine amide, L-phenylglycine amide, L-proline amide, L-lysine amide, L-histidine amide, L-alanine-*p*-nitroanilide, L-alanyl-L-alanine, and L-phenylalanine methylester.

Properties of the Enzyme: The *Mr* of the enzyme was estimated to be about 38,000 by gel filtration on HPLC with the G-3000 SW column, in the condition described previously (10).

The substrate specificity of the enzyme was examined with various amides, peptides and amino acid esters. As shown in Table 1, the enzyme acted on various aromatic D-amino acid amides including D-phenylalanine amide, D-tyrosine amide, D-tryptophan amide, and D-phenylglycine amide, *etc.* However, it did not act on L-amino acid amides at rates faster than 0.1% that for their D-enantiomers. Peptides with D-alanine at the *N*-terminal were inactive as a substrate, while D-alanine amide was relatively well hydrolyzed (by 30% the rate for D-phenylalanine amide). Boc-D-alanine amide and aliphatic amino acid amides such as 2-phenylacetamide, acetamide and propionamide were inactive as a substrate, indicating that the enzyme prefers D-amino acid amide with a free α-amino group. The apparent *Km* values for D-phenylalanine amide, D-tyrosine amide, D-leucine amide and D-alanine amide were calculated to be 0.089, 0.18, 0.057 and 0.54 mM, respectively.

Effects of pH and temperature on the enzyme activity were investigated. The enzyme showed maximal activity at pH 7.5 to 8.0. When the enzyme was kept at 30°C for 1 hr with 0.05 M buffers of various pHs, more than 90% of the initial activity was retained at pH 7.5 to pH 10.0. Almost no loss of activity was observed when the enzyme was incubated in 0.1 M Tris-HCl, pH 8.0, up to 35°C for 10 min, whereas at 40°C, about 80 % of the activity was lost. Effects of various metal ions and inhibitors were next investigated. The enzyme was inhibited to 80-95% after incubation at 30°C for 30 min by serine protease inhibitors such as phenylmethane sulfonyl fluoride and diisopropyl fluorophosphate at a concentration of 0.1 mM, while carbonyl reagents (KCN, semicarbazide and NaN_3 at 5 mM), chelating reagents (EDTA at 50 mM, α , α' -dipyridyl at 5mM, and 8-hydroxyquinoline at 1 mM), sulfhydryl reagents (5,5'-dithiobis(2-nitrobenzoic acid) and PCMB (at 0.2 mM)), and *N*-ethylmaleimide (at 5 mM) showed no inhibitory effect.

DISCUSSION

A number of amidases which act on amino acid amides have been characterized. They include acylamide amidohydrolase (EC 3.5.1.4) and aminopeptidase. Acylamide amidohydrolase from a nitrile-hydrolyzing *Brevibacterium* sp. R312 catalyzes the hydrolysis of D-alanine amide, much slower than low *Mr* aliphatic carboxylic acid amides (11). An aminopeptidase from *Trichosporon cutaneum* catalyzes the hydrolysis of amino acid amides such as L-tryptophan amide, as well as various peptide substrates with L-tryptophan at the *N*-terminal (12). Aminopeptidases from human liver (13), swine kidney (14), fungi (15,16), and bacteria (17-19) also catalyze the hydrolysis of L-amino acid amides, together with peptide substrates. We have discovered and characterized a new enzyme "D-aminopeptidase" from *Ochrobactrum anthropi* (formerly *Achromobacter* sp.) SCRC C1-38 (3). The enzyme prefers peptides with a free D-amino acid at the *N*-terminal as substrates, to D-amino acid *N*-alkylamides and D-amino acid amides. It has a *Mr* of about 122,000 and composed of two identical subunits (*Mr* = 59,000), and appears to be a thiol-peptidase. However, there has been no report which dealt with an enzyme, the substrate- and stereospecificities of which are highly restricted to hydrophobic D-amino acid amides. The enzyme could be called a "D-amino acid amidase", rather than a D-aminopeptidase (3). Considering that amino acid esters are easily hydrolyzed non-enzymatically, the role of the enzyme would be to hydrolyze D-amino acid amide to yield ammonia, which was lacking in the medium and set to be essential for the growth of the bacterium during the three months' acclimation period. The reason for the slow rate of the hydrolysis of D-valine amide by the enzyme could not explained.

The enzyme appeared to have a serine residue at the active site, rather than cysteine or metal. Known acylamide amidohydrolases and aminopeptidases are either thiol- (11,12) or metal- (13-19) enzymes, except for an instance that alkylamidase from sheep liver is potently inhibited by paraoxon, a serine-peptidase inhibitor (20).

Comparison of the properties and the primary structure of the enzyme with the D-aminopeptidase (3) and other amidases would pose an interest in enzymology, because the enzyme has a marked differences in the substrate specificity, *Mr*, and possible active center, from other amidases hitherto known. Intensive characterization of the enzyme and molecular cloning of the gene for the enzyme are in progress.

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