Tetrahedron Vol. 45, No. 18, pp. 5743 to 5754, 1989
Printed in Great Britain.

0040-4020/89 $3.00+.00

FIRST STEREOSELECTIVE SYNTHESIS OF D-AMINO ACID N-ALKYL AMIDE CATALYZED BY D-AMINOPEPTIDASE

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(*Received in Japan* 16 *March* 1989)

Abstract: The first stereoselective synthesis of D-amino acid N-alkyl amides was achieved by the use of D-aminopeptidase from Ochrobactrum anthropi. The enzyme immobilized by urethane prepolymer PU-6 catalyzed aminolysis reaction of racemic amino acid esters in organic solvents.

Enzymes are useful as catalysts for the preparation of optically active compounds[1], because they are chiral at the active center, which distinguishes stereochemical differences of reactants. Conventional chemical synthesis generally lacks stereoselectivity and requires expensive catalysts and substrates for a large scale production of an optically active compound[2].

The use of proteases in the formation of a peptide bond is an alternative to the chemical methods[3]. D-amino acid containing peptides have recently been noticed for their interesting biological activity[4]. Only a limited knowledge has been available about enzymes specific for D-amino acid containing peptides, and none of them has been used for peptide or amide synthesis, although nonstereospecific enzymatic syntheses of such peptides have been reported[5]. These synthetic procedures require expensive D-amino acid derivatives because of the inherent nonstereoselectivity. Recently, we aimed to synthesize an amide bond D-stereoselectively by the use of an enzyme, and started a screening program for a new enzyme. We succeeded in isolating a bacterium Ochrobactrum anthropi (formerly Achromobacter sp.)

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SCRC C1-38 producing a desirable D-specific aminopeptidase. The enzyme was purified to homogeneity and its properties have been intensively characterized[6]. The enzyme stereospecifically hydrolyzes D-amino acid amides, and D-amino acid containing peptides to give D-amino acid in a highly D-stereoselective manner, and thus can be used in the preparation of D-amino acids from racemic amino acid amides. In this paper, we describe the first stereospecific synthesis of D-amino acid N-alkylamides by the use of an enzyme in organic media[7].

Results and Discussion

Enzymatic Aminolysis Reaction of D-Alanine Methylester With 3-Aminopentane In Water. The enzymatic synthesis of peptides are categorized either in the thermodynamic or the kinetic controlled systems[3]. In the former cases, a peptide bond is formed by the reverse process of hydrolysis. In the latter cases, ester or amide substrate forms acyl-enzyme complex with serine or thiol protease, and then deacylated by a nucleophile such as an amine, forming a peptide bond. In water, the acyl-enzyme complex is competitively hydrolyzed. Since the novel D-aminopeptidase has been characterized to be a thiol-peptidase[6], the kinetically controlled system was expected to be applied for an amide bond formation. The enzyme stereospecifically hydrolyzes D-amino acid amides and peptides whose N-terminal have not been protected, releasing a D-amino acid. If racemic alanine esters or amides were used for an acyl donor, D-aminoacyl-enzyme complex will be formed and then deacylated by amines, yielding D-alanine N-alkylamides in high optical purity. After examining some reaction conditions, we succeeded in an aminolysis reaction of D-alanine methylester hydrochloride with 5 equivalents of 3-aminopentane using D-aminopeptidase (13.2 units/mL reaction system) in water, as shown in Fig. 1. D-Alanine-3-aminopentane amide was formed in 78 % yield after 7.5 min. However, the amide formed was successively hydrolyzed yielding D-alanine as the final product[8]. D-Alanine polymer was not formed in this system.

Immobilization of D-Aminopeptidase. We next investigated the aminolysis reaction in non-aqueous organic media. No product was formed in polar or non-polar media such as butylacetate, trichloroethane, isooctane, dimethylformamide, dimethylsulfoxide and methanol, etc., presumably due to an inactivation of the enzyme. We examined several immobilization resins[9] such

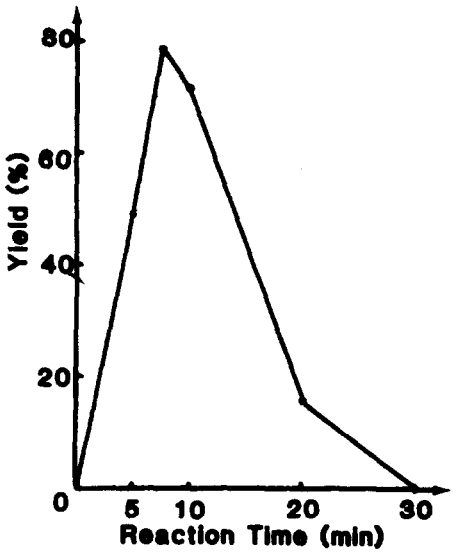


Figure 1. Enzymatic aminolysis of D-alanine methylester hydrochloride by 3-aminopentane in water. A reaction mixture (1 mL) containing 100 mM D-alanine methyl ester hydrochloride, 500 mM 3-aminopentane, 13.2 units of enzyme and water was incubated at 30°C. Yields were calculated by ninhydrin method.

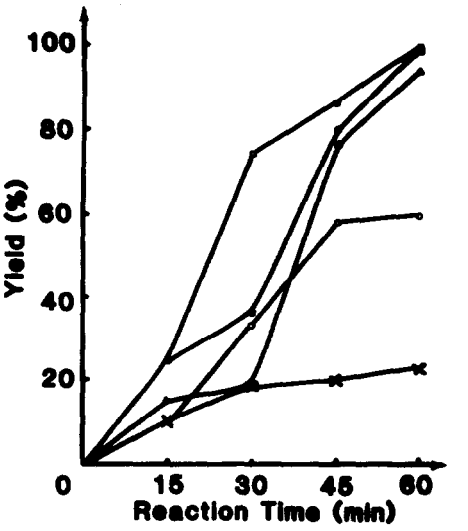


Figure 2. Effect of organic solvents on the enzymatic aminolysis reaction. Reaction mixtures (1 mL) containing 100 mM D-alanine methylester hydrochloride, 500 mM of 3-aminopentane, 100 mg of PU-6 immobilized enzyme (1.6 units) and various water-saturated organic solvents were incubated at 30°C. Yields were calculated by ninhydrin method. The solvents used were: (□) butylacetate; (●) benzene; (▲) 1,1,1-trichloroethane; (○) toluene; (×) di-isopropylether.

as cross-linked polyacrylamide, Amberlite XAD, photo-crosslinkable resin prepolymers ENT and urethane-prepolymers PU, to stabilize the enzyme in organic solvents. Photo-crosslinkable resin-prepolymers ENT and urethane-prepolymers PU[10] were selected to be suitable for the immobilization of the enzyme. Yields of the total activity of the enzyme after immobilization by several prepolymers were 5 to 12 %. The low recovery of the enzyme activity after the immobilization would probably due to lower permeability of the substrates and the product into the resin, as compared with the intact enzyme system. Urethane prepolymer PU-6 was found to be the most efficient immobilizing resin. The immobilized enzymes were applied for the aminolysis reaction of D-alanine methylester hydrochloride with 3-aminopentane to give D-alanine 3-aminopentane amide in organic media. Water saturated 1,1,1-trichloroethane was chosen as a typical solvent. One hundred mM of D-alanine methylester hydrochloride and 500 mM of 3-aminopentane were suspended in water saturated 1,1,1-trichloroethane (1 mL). To this was added small pieces of the immobilized enzyme (100 mg/mL reaction medium; 1 unit/mL medium), and the mixture was vigorously shaken at 30°C. Only PU-6 immobilized enzyme catalyzed the aminolysis reaction in the solvent in high rate, while a little product was formed with ENTP-entrapped enzyme, and none with ENTG- and PU-3-entrapped enzymes. The effect of several organic solvents was

investigated using PU-6 immobilized enzyme as shown in Fig. 2. When butylacetate and benzene were used, the aminolysis reaction progressed to a completion, and the secondary hydrolysis of D-alanine 3-aminopentane amide was suppressed as expected. 1,1,1-Trichloroethane, toluene and di-isopropylether were found to be favorable solvents. On the other hand, non-polar isooctane, and polar-solvents such as methanol, acetone, dimethylsulfoxide, N,N-dimethylformamide, tetrahydrofuran, dioxane and tert-butanol were found to be unsuitable for the reaction.

Enzymatic Aminolysis Using Immobilized Whole Cell System. We next investigated the use of immobilized whole-cell system for the aminolysis reaction. The reactions were also performed using ENTG and PU-6 immobilized wet or acetone-dried O. anthropi SCRC C1-38 cells[11] in butylacetate with excess amount of the nucleophile. D-Alanine 3-aminopentane amide was synthesized in 37 % yield (with wet cells), and 16% (with dry cells) entrapped with ENTG-3800; and 12 % (wet and dry cells) entrapped with PU-6 in 6 hours. The immobilized cells were also shown to be applicable for the reaction, although the the yields were lower because only a limited amount of the enzyme could be charged in the reaction mixture. Our current studies on the molecular cloning of the gene for the enzyme and its overproduction will solve this problem.

Stereoselective Aminolysis of Racemic Alanine Derivatives. Figure 3 shows courses of the aminolysis reaction of racemic alanine methylester hydrochloride by 3-aminopentane using immobilized D-aminopeptidase in butylacetate. High D-stereospecific aminolysis was again achieved as has been observed in the hydrolysis of racemic alanine amide[12]. D-Alanine amide hydrochloride also served well as a substrate in this aminolysis reaction to give optically pure D-alanine-3-aminopentane amide. The reason for the lower velocity observed with the racemic alanine methylester would probably be due to an inhibition of enzyme activity by L-form

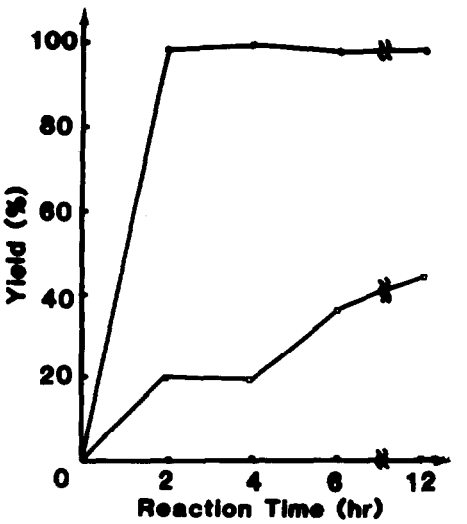


Figure 3. Stereoselectivity in the enzymatic aminolysis reaction. Reaction mixtures (1 mL) containing 100 mM various acyl donors, 500 mM 3-aminopentane, 100 mg of PU-6 immobilized enzyme (1.6 units) and water-saturated butyl acetate were incubated at 30°C. Yields were calculated by ninhydrin method. The acyl donors used were: (●) D-alanine methylester; (□) DL-alanine methylester; (■) L-alanine methylester.

of the ester. D-Alanine 3-aminopentane amide was obtained in 45.2 % yield (theoretical yield was 90.4 %) in 6 hr in a preparative scale synthesis using DL-alanine methylester hydrochloride as an acyl donor. The optical purity of the amide was more than 99 % e.e., as analyzed by HPLC with a CROWNPAK CR(+) column. The product was N-tert-butoxycarbonylated by di-tert-butoxycarbonate/triethylamine in an aqueous dioxane. Spectrum data of the resulting compound was assigned to be N-Boc-D-alanine-3-aminopentane amide, with reference to those of the authentic sample (see experimental part). L-Stereospecific aminolysis reaction using thermolysin, papain and thiosubtilisin has been known[13]. Recently, enzymatic non-stereoselective syntheses of D-amino acid containing amides and peptides have also been reported, utilizing D-amino acid derivative as an acyl donor or an acyl acceptor[5]. The aminolysis reaction of this study is clearly distinct from the above methods in that it is strictly D-stereospecific, although it could not be applied to the peptide synthesis in the reaction conditions employed in this study.

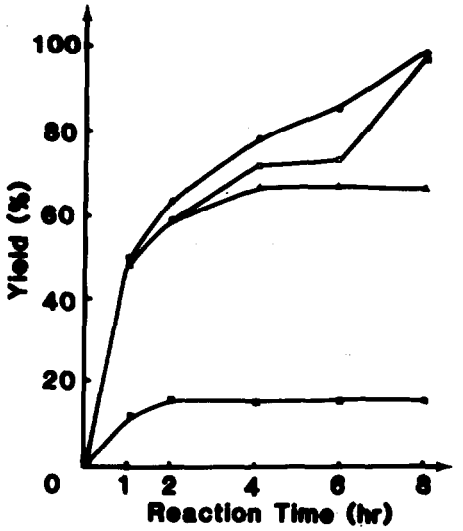


Figure 4. Effect of acyl-acceptors on the enzymatic aminolysis reaction. Reaction mixtures (1 mL) containing 100 mM D-alanine methylester hydrochloride, 500 mM of various acyl accepters, 100 mg PU-6 immobilized enzyme (1.6 units) and water-saturated 1,1,1-trichloroethane were incubated at 30°C. Yields were calculated by dansylation method (see experimental part). The amines used were: (●) 3-aminopentane; (□) *neo*-pentylamine; (▲) n-butylamine; (■) benzylamine.

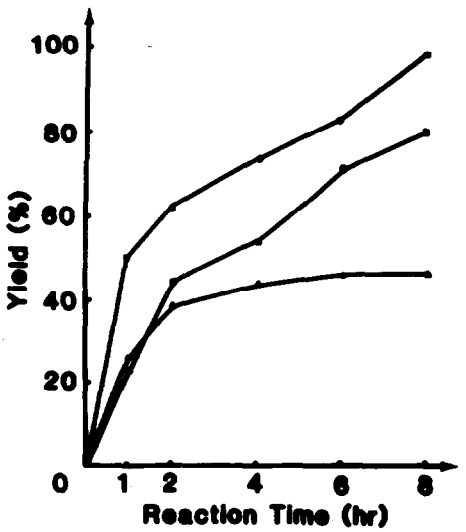


Figure 5. Effect of acyl-donors on the enzymatic aminolysis reaction. Reaction mixtures (1 mL) containing 100 mM of various acyl donors, 500 mM 3-aminopentane, 100 mg of PU-6 immobilized enzyme (1.6 units) and water-saturated butyl acetate were incubated at 30°C. Yields were calculated by ninhydrin method. The acyl donors used were: (●) D-alanine methylester; (■) glycine methylester; (▲) D-α-amino butyric acid methylester.

Effect of Amine Component on the Enzymatic Aminolysis. Figure 4 shows courses of the enzymatic aminolysis reactions of D-alanine methylester hydrochloride with several alkyl amines. These results shows that not only a primary amine, but also bulky and aromatic-ring containing amines are good substrate for this synthesis. Laurylamine and aniline did not serve as a substrate, probably due to their low solubility and low nucleophilicity.

Enzymatic Aminolysis of Esters of Several Amino Acids. Several D-amino acid esters were used as acyl-donor with 3-aminopentane as a fixed nucleophile. Figure 5 shows that the enzyme accepts esters of D-alanine, glycine and D-α-amino butyric acid as acyl donors, while methylesters of 19 kinds of D-amino acids[14], β-alanine, and carboxylic acids such as acetic acid, n-butyric acid, benzoic acid and phenylacetic acid are not substrate for this reaction. Similar to the hydrolytic reaction, the enzyme required α-amino group as an acyl donor for the N-alkylamidation, as evidenced by the fact that Boc-D-alanine amide and Z-D-alanine methylester were inactive as a substrate[6]. Methylesters of D-valine, D-leucine, D-serine, D-threonine, and D-methionine did not serve as a substrate in the aminolysis reaction, possibly due to the low affinity to form the acyl-enzyme intermediate[15]. The

Table I Catalytic Center Activity (kcat) of the Aminolysis Reaction Compared with Other Non-Selective Enzymatic Peptide Syntheses

Reaction Substrate	Enzyme	kcat(min^{-1})
Z-Tyr-OMe + D-Met-OMe	α-Chymotrypsin	20 - 40[a]
N-F-D-Ala-OEtCl + L-Phe-NH_2	Subtilisin	0.19[b]
D-Ala-OMe + 3-aminopentane	D-Aminopeptidase	7700[c]

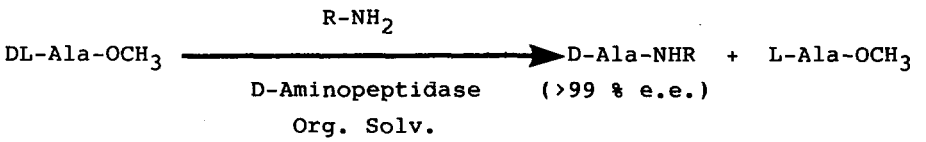
[a] See ref. 5a). [b] See ref. 5d). [c] This study.

enzyme thus showed rather limited acyl-donor specificity, in contrast to the wider substrate specificity in the hydrolytic reaction[6].

Table I compares the catalytic center activity (kcat, min^{-1}) observed in this aminolysis reaction with other enzymatic D-amino acid containing peptide formations. The kcat value of this aminolysis reaction was found to be several hundred to ten thousand times higher than those calculated from the values in the literatures[5]. This D-stereospecific aminolysis reaction is highly effective for the synthesis of D-amino acid N-alkyl amides.

Conclusions

D-Aminopeptidase from a bacterium O. anthropi SCRC C1-38, was utilized for a stereoselective synthesis of D-alanine N-alkylamide from an amine and D-amino acid amide or D-amino acid methylester. In water, D-alanine N-alkylamide once formed was successively hydrolyzed to yield D-alanine. When the aminolysis reaction was performed in water-saturated organic solvents such as butylacetate, benzene and 1,1,1-trichloroethane with immobilized enzyme as a catalyst, it progressed quantitatively well in a highly D-stereoselective manner, to give optically pure D-alanine N-alkylamides. D-Alanine polymer was not synthesized.



R= 3-pentyl-, neopentyl-, benzyl-, n-butyl-

The acyl-donor specificity of this reaction was rather limited with D-alanine, glycine and D-α-amino butyric acid as substrates, while the acyl-acceptor specificity was relatively wide with bulky, aromatic-containing and straight-chain amines. The kcat of this reaction was much higher than those of the non-stereoselective synthesis of D-amino acid containing peptides. The enzyme provides a novel method in the stereospecific synthesis of D-amino acid N-alkylamide from an inexpensive racemic amino acid derivative, without any protection of the amino group of the amino acid.

Experimental Section

Materials. Ultraviolet absorbance was measured by a Hitachi 228A double-beam spectrophotometer. Optical rotations were measured at 20°C with a Horiba SEPA-200 polarimeter. NMR spectra were measured by a Varian EM-390 using tetramethylsilane as a reference. IR spectra were measured with a JASCO A-202 infrared spectrophotometer. Filter paper (No. 51-A, Advantec

Ltd., Tokyo, Japan) was used for paper chromatography. High performance liquid chromatography was carried out by a Tosoh (Tokyo, Japan) HLC-803D at 200 nm, equipped with a CROWNPAK CR(+) (Daicel Chem. Ind. Ltd., Tokyo, Japan).

Synthesis. All D-amino acid methylesters were prepared from D-amino acids with thionylchloride in methanol at -20°C. D-amino acid amides were obtained by ammonolysis of the corresponding methylesters with dry NH_3 gas in methanol at room temperature. Authentic D-alanine N-alkylamides were synthesized in 3 steps: 1) N-tert-butoxycarbonylation of D-alanine by di-tert-butoxycarbonate/triethylamine in an aqueous dioxane. 2) aminolysis of Boc-D-alanine by acid-anhydride procedure using isobutylchloroformate/triethylamine/amines in THF. 3) Deprotection of amino group using 4N HCl/ethylacetate[16]. Glycine and D-α-aminobutyric acid alkylamides were also synthesized by the same procedure (vide supra).

Purification of D-aminopeptidase from O. anthropi SCRC C1-38. D-aminopeptidase was purified from O. anthropi SCRC C1-38 by a procedure involving protamine sulfate treatment, ammonium sulfate fractionation, column chromatographies on DEAE-Toyopearl and Butyl-Toyopearl, up to a specific activity of 210 units/mg protein, according to a procedure described elsewhere[6]. Protein was determined by the method of Lowry et al[17]. One unit of enzyme activity was defined as described previously.

Immobilization of D-Aminopeptidase by Photo-crosslinkable- and Urethane-prepolymers. 1) Photo-crosslinkable prepolymer ENTG-3800 - To a solution of D-aminopeptidase in 2.75 mL of 10 mM potassium phosphate buffer (pH 7) were added ENTG-3800 (4.6 g) and benzoin ethylether (37 μL) as a photo-sensitizer. The mixture was poured into a space (ca. 1mm) between two sheets of transparent polyester, which were then illuminated with near-ultraviolet light for 5 min. The gel formed (thickness, ca. 1 mm) was cut into a small pieces (ca. 3 x 3 mm). 2) Photo-crosslinkable prepolymer ENTP-2000 - To a mixture of the enzyme in 1.6 mL of 10 mM potassium phosphate buffer (pH 7), ENTP-2000 (3.2 g) and benzoin ethylether (32 μL), 1.6 mL of benzene/isooctane = 1/1 was added. The mixture was polymerized by the illumination of ultraviolet light as described above. The gel was cut into small pieces. 3) Urethane prepolymer PU-6 - Urethane prepolymer PU-6 (0.7 g) which had been melted at 60°C and cooled to room temperature was mixed quickly with 1 mL of 10 mM buffer (pH 7) solution of the enzyme, and the mixture was kept at 4°C for 1 hr. The gel formed was cut into small pieces (ca. 3 x 3 x 3

mm). 4) Urethane prepolymer PU-3- Urethane prepolymer PU-3 (1 g), the enzyme solution (1mL) and one drop of ethanol were mixed vigorously. The mixture was polymerized and the gel was cut as described above.

Determination of Yields of the Enzymatic Aminolysis of D-Alanine Methylester by Several Amines. An aliquot of the reaction mixture (50 μL) was dansylated by 10 mM acetone solution of dansylchloride (1 mL) and 10 mM Na_2CO_3-$NaHCO_3$ buffer (pH 10, 500 μL) at room temperature for 1 hr. Ethylacetate extracts of the mixture was applied on a descending paper chromatography with n-hexane. A light-blue fluorescence band, made visible by an ultraviolet light of wavelength 250-290 nm, was cut off and extracted with 5 mL of methanol. The concentration of the product was calculated from the absorbance at 205 nm, with the synthetic authentic product as a standard.

Preparative Scale Synthesis of D-Alanine 3-Aminopentane Amide Using Immobilized D-Aminopeptidase. DL-Alanine methylester (or amide) hydrochloride (1 mmol) and 3-aminopentane (5 mmol) were suspended in water-saturated butylacetate (10 mL). The pH of the reaction medium was about 10. To this was added PU-6 immobilized enzyme(1.6 units) and incubated at 30°C for six hours. The immobilized enzyme was filtered off and the solvent evaporated. The product was treated with 4N HCl/ethylacetate[16] and dried over anhydrous magnesium sulfate. The solvent was evaporated to dryness to give 87.9 mg (45.2 % yield) of the product (>99 % e.e. by HPLC), which was then dissolved in 65 % aqueous dioxane and N-tert-butoxycarbonylated by adding triethylamine and di-tert-butoxycarbonate at 0°C. The resulting solution was stirred overnight at room temperature, and quenched by an addition of aqueous solution of $NaHCO_3$. Aqueous solution obtained after an evaporation was extracted with 50 mL portions of ethylacetate for several times, and the combined extracts were washed with 5% $NaHCO_3$, 5% citric acid and brine. The ethylacetate layer was dried over anhydrous $MgSO_4$, concentrated and purified by column chromatography on silica gel (hexane/ethylacetate = 1/1), to give N-tert-butoxycarbonyl-D-alanine 3-aminopentane amide.: ^{1}HNMR ($CDCl_3$) δ 0.88 (t, 6H), 1.35 (d, 3H), 1.4-1.52 (m, 4H), 1.46 (s, 9H), 3.70(m, 1H), 4.13 (5th, 4H), 5.23 (br, 1H), 6.10 (br, 1H); IR νmax (KBr) 3330, 2980, 1692, 1658, 1526, 1453, 1369, 1252, 1169, 1069, 1002, 609 cm^{-1}; $[\alpha]_D$ +50.6° (c1.25 $CHCl_3$); authentic data of Boc-D-alanine 3-aminopentane amide was +50.9°

Acknowledgment.

We thank Professor A. Tanaka and Dr. K. Sonomoto of Kyoto University for their kind supply of urethane-prepolymers PU-3 and PU-6.

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(11) a) Immobilized wet cells were prepared as follows. O. anthropii SCRC C1-38 cells were collected by centrifugation and washed by 0.1 M potassium phosphate buffer (pH 7). The washed cells were suspended in the same buffer to the cell concentration of 0.8 g (dry cell weight)/mL and immobilized by several resins (see experimental part). b) Immobilized acetone-dried cells were prepared as follows. To the washed cells in 0.1 M buffer (0.8 g/mL) was added cold acetone (-20°C), and the mixture was vigorously mixed at -20°C. The cells were filtered and washed by cold acetone for several times. The filtrate was dried under vacuum and suspended in 0.01 M potassium phosphate buffer (pH 7) to a concentration of 0.1 g(dry cell weight)/mL and then immobilized.

(12) D-aminopeptidase shows lower stereospecificity toward amino acid esters: D-alanine methylester and L-alanine methylesters are hydrolyzed by 75 % and 53 %, respectively, of the velocity toward D-alanine amide[6]. In contrast to the hydrolytic reaction, L-alanine methylester was inacive as a substrate in this aminolysis reaction.

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(14) The following methylesters of D-amino acids were inactive as a substrate: D-valine, D-leucine, D-isoleucine, D-serine, D-threonine, D-aspartic acid, D-asparagine, D-glutamic acid, D-glutamine, D-methionine, D-lysine, D-histidine, D-phenylalanine, D-tyrosine, D-tryptophan, D-proline, D-phenylglycine, D-norvaline, and D-norleucine.

(15) The relative rates for hydrolysis of D-valine amide, D-leucine amide, D-serine amide, D-threonine amide, and D-methionine amide were 1.8, 0.8, 29, 9.0, and 2.0 %, respectively, to that for D-alanine amide[6].

(16) Obtained from Kokusan kagaku Co. Ltd., Tokyo, Japan.

(17) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. J. Biol. Chem. **1951**, 193, 265-275.