J. Biochem. 131, 247–254 (2002)

A New Type of Aminoacyltransferase from *Saccharothrix* sp. AS-2 Favorable for the Synthesis of D-Amino Acid–Containing Peptides

Akio Sugihara,*[,1] Yuji Shimada,* Shigeo Sugihara,† Takahisa Nakai,‡ Tomisaburo Kakuno,§ Toshihiro Nagao,* Yomi Watanabe,* and Yoshio Tominaga*

Osaka Municipal Technical Research Institute, 1-6-50 Morinomiya, Joto-ku, Osaka 536-8553; †Tokushima Bunri University, Shido-cho, Okawa-gun, Kagawa 769-2101; ‡Kaneka Corporation, 1-8, Miyamae-cho, Takasago 676-8688; and §M&S Instruments Inc., 2-12-4 Mikunihonmachi, Yodogawa-ku, Osaka 532-0005

Received November 5, 2001; accepted November 28, 2001

A unique enzyme with some properties favorable for the synthesis of D-amino acid–containing peptides has been purified from the culture broth of *Saccharothrix* sp. AS-2. The purification steps included ammonium sulfate fractionation, chromatographies on CM-Toyopearl 650M and ProtEx Butyl, and sucrose density-gradient isoelectric focusing. The enzyme, consisting of four subunits of 56 kDa, showed its maximum transfer activity at around pH 8.2 and 35°C, and had an isoelectric point of 5.8. The enzyme yielded homooligomers from methyl esters of D-Asp(OMe), D-Met, D-Phe, D-Trp, D-Tyr, and L-Glu(OMe), but showed no hydrolytic activity toward any of the D- or L-amino acid methyl esters tested. The homooligomers were not formed from the corresponding free amino acids. The reaction of Ac-D-Phe-OMe with DL-Ala-NH_2, DL-Leu-NH_2, DL-Phe-NH_2, or DL-Trp-NH_2 was effectively catalyzed by the enzyme, both the DD- and DL-stereoisomers of the expected *N*-acetyldipeptide being yielded. The resulting dipeptides remained unhydrolyzed even after 48 h incubation. Also, it showed no detectable hydrolytic activity toward casein, diastereomers of diAla, diMet, and diPhe, D-/L-amino acid amides, or D-/L-amino acid *p*-nitroanilides, indicating that the enzyme had no peptidase activity leading to secondary hydrolysis of the growing peptide. The enzyme activity was strongly depressed by phenylmethanesulfonyl fluoride, but not by penicillin G or ampicillin, suggesting that the protein is a serine enzyme lacking penicillin-binding ability. These observations lead us to the conclusion that the enzyme from *Saccharothrix* sp. AS-2 characterized in this study is a new type of aminoacyltransferase with an amino acid ester as the acyl donor, and has potential utility as a catalyst for the synthesis of D-amino acid-containing peptides.

Key words: aminoacyltransferase, D-amino acid–containing peptides, enzymatic peptide synthesis, *Saccharothrix* sp.

There is increasing evidence that oligopeptides play important roles in physiological, immunological, and sensory responses (*1–4*). As a result, the development of methods suitable for their production on a large scale has attracted much interest in recent years. The application of enzymes to oligopeptide synthesis has a number of well-documented advantages over conventional chemical methods (*5–11*). In particular, enzymatic synthesis avoids racemization, dispenses with the requirement for side chain protection, and allows the use of mild, nonhazardous reaction conditions. Actually a number of oligopeptides, such as angiotensin, dynorphin, and enkephalins, have been prepared using a combination of enzymatic and chemical methods (*12–15*). However, the enzymatic synthesis, in which proteases have exclusively been used, suffers from some drawbacks including an unfavorable thermodynamic equilibrium, narrow substrate- and stereospecificities, and undesirable proteolysis of the growing peptide. Although the first disadvantage has been alleviated by using proteases in biphasic aqueous organic mixtures, reverse micelles, or nonaqueous media, the other problems remain, due to the physiological function of proteolytic enzymes. Also, the number of biologically important D-amino acid–containing peptides, in which a single D-amino acid is a requisite for the biological activity, has increased in the last decade (*16–22*). Great efforts have consequently been made to solve the problems mentioned above (*23–25*). The employment of high concentrations of organic solvents in the reaction system, sometimes an anhydrous environment, has been found to relax the stereospecificity and to suppress the secondary hydrolysis. However, proteases are generally unstable in such an environment, and are not always adopted for practical peptide synthesis.

Recently, several D-amino acid–specific peptidases were isolated and characterized (*26–36*). Although some of them were shown to form peptides of the DD-configuration, no examples have been reported of the synthesis of peptides of the DL-configuration using these enzymes. Although some reports have appeared on the isolation of bacterial α-amino-acid-esterases (*37–40*), no data are available for their use in peptide synthesis. In our previous paper we

[1] To whom correspondence should be addressed. Tel: +81-6-6963-8073, Fax: +81-6-6963-8079, E-mail: sugihara@omtri.city.osaka.jp

reported a novel α-amino-acid-esterase from *Bacillus* (*B.*) *mycoides*, and showed that the enzyme, although devoid of peptidase activity, was capable of synthesizing peptides of the DD- and DL-configurations (*41*). These properties are highly desirable for peptide synthesis, but there remains the drawback that the enzyme shows high hydrolytic activity toward amino acid esters used as substrates. We report here the purification and characterization of a unique aminoacyltransferase from *Saccharothrix* sp. AS-2 lacking hydrolytic activity, and its use in the synthesis of D-amino acid–containing peptides.

MATERIALS AND METHODS

Materials—Peptone and casein were purchased from Nissui Pharmaceuticals (Tokyo) and Merck (Darmstadt, Germany), respectively. Phenylmethanesulfonyl fluoride (PMSF), trichloroacetic acid (TCA), and Quick CBB (Coomassie Brilliant Blue solution) were obtained from Wako Pure Chemical Industries (Osaka). A protein assay kit was obtained from Bio-Rad (Hercules, USA). D-/L-Ala, D-/L-Arg(NO_2), D-/L-Asp(OMe), L-Cys(Bzl), D-/L-Glu(OMe), D-/L-His, L-Ile, D-/L-Leu, D-/L-Lys(Z), D-/L-Met, D-/L-Phe, Ac-D-/L-Phe, D-/L-Pro, D-/L-Ser, D-/L-Thr, D-/L-Trp, D-/L-Tyr, and D-/L-Val, their methyl esters, and amides of D-/L-Ala, L-Arg, L-Asp(NH_2), D-/L-Gln, L-Glu(NH_2), L-His, L-Ile, D-/L-Leu, L-Lys, L-Met, D-/L-Phe, D-/L-Pro, L-Ser, L-Thr, L-Trp, L-Tyr, L-Val, and Gly were obtained from Bachem (Bubendorf, Switzerland). Amides of D-Arg, D-Asn, D-Gln, D-His, D-Ile, D-Lys, D-Met, D-Ser, D-Thr, D-Trp, D-Tyr, and D-Val were prepared by the method of Wang and McMurray (*42*). The *p*-nitroanilides of D-/L-Ala, D-/L-Leu, and D-/L-Phe, and the diastereomers of diAla, diMet, and diPhe were also from Bachem. Pharmalyte 3-10, and the molecular mass standards for gel filtration and SDS-PAGE were products of Amersham Pharmacia Biotech (Uppsala, Sweden). All other reagents were of analytical grade.

Aminoacyltransferase Assay—Aminoacyltransferase activity was routinely assayed at pH 7.0 using a mixture of 200 mM Ac-D-Phe-OMe and 200 mM L-Phe-NH_2 dissolved in 50 mM phosphate, pH 7.0. The reaction was started by the addition of 5 μl of the enzyme (0.4 unit) to 95 μl of the substrate, allowed to proceed for 5 min at 25°C, and stopped by the addition of 5 μl of twice-distilled 2 N HCl. The decrease in the amount of the substrate was monitored on a Cadenza CD-C18 column (4.6 × 75 mm; Imtakt, Kyoto) connected to a LC-9A HPLC system equipped with a SPD-6A uv detector set at 220 nm (Shimadzu, Kyoto). Samples were eluted with a mobile phase of 5–49% CH_3CN containing 0.05% TFA at the flow rate of 1.0 ml/min. One unit of aminoacyltransferase was defined as the activity producing 1 μmol of Ac-D-Phe-L-Phe-NH_2 per min under the above conditions. The above HPLC system was also used in other assays.

Other Enzyme Assays—Activities of the enzyme other than proteolysis were assayed at 25°C with 95 μl of amino acids, their methyl esters, amides, *p*-nitroanilides, or diastereomers of diAla, diMet, and diPhe dissolved in 50 mM phosphate, pH 7.0, to which 5 μl (0.4 unit) of the enzyme was added. Esterase activity was assayed with either 1 mM fatty acid esters of *p*-nitrophenol according to the method of Somorin and Skorepa (*43*), or 200 mM amino acid methyl ester, which had been passed through a column of Dowex 1-X2 anion-exchange resin (Dow Chemical, MI, USA) to remove remaining free amino acids. Methanol released from the ester was quantified on a Daisopak SP-120-5-ODS-BP column maintained at 40°C, which was connected to a Hitachi HPLC system equipped with a L-7100 pump and a L-7490 differential refractometer (Hitachi, Tokyo). A mobile phase of 100 mM phosphate (pH 3.8) was used at the flow rate of 0.7 ml/min. One unit of esterase activity was defined as the activity producing 1 μmol of *p*-nitrophenol or methanol per min under the above assay conditions. In the case of homooligomer formation, esterase activity was assayed by measuring the resulting free amino acid with an ALC-1000 amino acid analyzer (Shimadzu) according to the method of Ishida *et al.* (*44*). Amidase activity was measured with 200 mM amino acid amide under the same conditions as those for the esterase assay. Ammonia released from an amide was determined by the method of Weatherburn (*45*). One unit of amidase activity was defined as the activity releasing 1 μmol of ammonia per min under the above conditions. Peptidase activity was assayed with 10 mM dipeptide in the same manner as above, and hydrolysis was monitored with the LC-9A HPLC system or the ALC-1000 amino acid analyzer. When necessary, D-/L-amino acid analysis was performed with the LC-9A HPLC system. Samples were hydrolyzed with twice-distilled HCl for 16 h at 105°C, and then derivatized with *o*-phthalaldehyde and *N*-isobutyryl-L-cysteine (*46*). Proteolytic activity was measured according to the method of Wilcox with some modification using casein as the substrate (*47*). One milliliter of the enzyme solution was added to 5 ml of 0.6% (w/v) casein in 50 mM Tris-HCl, pH 7.2. After incubation at 25°C for an appropriate time, the reaction was stopped by the addition of 5 ml of a mixture composed of 36 ml of 50% TCA, 220 ml of M CH_3COONa, and 330 ml of M CH_3COOH. The reaction mixture was allowed to stand for a further 20 min. The resulting precipitate was removed by filtration through a filter paper No. 131 (Advantec Toyo, Tokyo), and then the absorbance of the filtrate at 275 nm was measured with a UV-1600 spectrophotometer (Shimadzu) against a control prepared by mixing the enzyme, TCA and the substrate in that order. One unit of proteolytic activity was defined as the activity yielding soluble peptides equivalent to 1 μmol of L-tyrosine per min under the above conditions.

Cultivation of the Microorganism—*Saccharothrix* sp. AS-2 was isolated from a soil sample and then aerobically cultivated in 500-ml flasks, each containing 150 ml medium composed of 2% peptone, 1% corn steep liquor, 0.2% KH_2PO_4, and 0.2% NaCl, pH 7.0, for 3 days at 27°C.

Purification of Aminoacyltransferase—All operations during the enzyme purification were carried out at 15°C unless otherwise indicated. A total of 7.2 liters of the culture broth was centrifuged at 10,000 ×*g* for 20 min. To the supernatant was added solid $(NH_4)_2SO_4$ to 70% saturation. The resulting precipitate was allowed to settle overnight and then recovered by filtration through Celite. The crude enzyme was dissolved in a minimal amount of water, dialyzed against 5 mM acetate, pH 5.0, and then put on a CM-Toyopearl 650M column (2.5 × 22 cm; Tosoh) equilibrated with the same buffer. After the column had been washed with 1,000 ml of the acetate buffer, proteins were eluted with a linear gradient of 0–800 mM NaCl at the flow rate of 25 ml/h. The active fractions were collected and concentrated 10-fold by ultrafiltration with a membrane with a nominal

molecular mass cutoff of 10 kDa. The concentrated enzyme solution was then subjected to sucrose density-gradient isoelectric focusing in a 110 ml–volume column with Pharmalyte 3-10 as the carrier ampholytes. The electrophoresis was performed at 400 V for 40 h at 2°C, and proteins were collected with a fraction collector at 1 ml per tube. The active fractions were pooled, concentrated and then subjected to hydrophobic interaction chromatography on a ProtEx Butyl column (8 × 10 cm, especially prepared by Mitsubishi Chemicals, Tokyo) preequilibrated with 50 mM acetate, pH 5.6, containing 20% saturated $(NH_4)_2SO_4$. Proteins were eluted by linearly decreasing the salt concentration to 0% saturation.

Determination of the Molecular Mass of the Enzyme—The molecular mass of the native enzyme was estimated by gel filtration on a Superdex 200 HR 10/30 column (1 × 30 cm; Amersham Pharmacia Biotech) using 50 mM phosphate, pH 7.0, containing 150 mM NaCl. Ferritin (470 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa) were used for calibration. The molecular mass of the denatured enzyme was estimated by SDS-PAGE according to the method of Laemmli on a 9% polyacrylamide gel (*48*). The marker proteins used were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (20.1 kDa). After the electrophoresis, the gel was stained with Quick CBB.

Protein Determination—The protein concentrations of the samples were determined using a protein assay kit from Bio-Rad (Hercules, USA) with bovine serum albumin as the standard. Chromatographic elution patterns were monitored in terms of the absorbance at 280 nm.

Inhibitory Assay—An enzyme solution was mixed with each inhibitor, and then the mixture was incubated for 1 h at 25°C in 50 mM phosphate, pH 7.0, before the enzyme assay. The reagents and their final concentrations were 1 mM PMSF, 1 mM *N*-ethylmaleimide (NEM), 1 mM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E-64), 1 mM pepstatin, 1 mM $HgCl_2$, 5 mM EDTA, 10 mM penicillin G, and 10 mM ampicillin. The action of the enzyme on penicillin G and ampicillin was also checked by HPLC at 220 nm by monitoring the generation of 4-aminopenicillanic acid.

Enzymatic Peptide Synthesis—Homooligopeptide synthesis was carried out with 200 mM amino acid or the methyl ester as the substrate. Ninety-five microliters of the substrate was incubated with 5 μl (0.4 unit) of the enzyme at 25°C for 10 min in 50 mM phosphate, pH 7.0. The reaction was followed by monitoring the decrease in the amount of the substrate. The ability of the enzyme to form diastereomers of Ac-Phe-Ala-NH_2, Ac-Phe-Leu-NH_2, Ac-Phe-Phe-NH_2, and Ac-Phe-Trp-NH_2 was examined as above, using a mixture of 200 mM Ac-D-/L-Phe-OMe and 400 mM DL-Ala-NH_2, DL-Leu-NH_2, DL-Phe-NH_2 or DL-Trp-NH_2. The mixture was dissolved in 50 mM phosphate, pH 7.0, containing 20% dimethylformamide (DMF) to dissolve the acylated amino acid ester. The reaction products were analyzed with the LC-9A HPLC system using a mobile phase of 5–49% CH_3CN containing 0.05% TFA at the flow rate of 1.0 ml/min, as described under "*Aminoacyltransferase Assay.*"

Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry—Enzymatically synthesized peptides were isolated by HPLC, and then their masses were measured with a Voyager linear DE-SY MALDI-TOF mass spectrometer (PerSeptive Biosystems, CA, USA) using α-cyano-4-hydroxycinnamic acid as the matrix.

RESULTS

Purification of the Enzyme—Table I summarizes the purification of the aminoacyltransferase. The enzyme was purified approximately 152-fold with a yield of 2% of the initial activity. CM-Toyopearl 650M chromatography eliminated most of the colored materials, and increased the specific activity of the enzyme effectively. Subsequent electrofocusing was also effective in removing acidic contaminants (Fig. 1). The enzyme preparation after the hydrophobic interaction chromatography was homogeneous, as judged on SDS-PAGE, which gave a single protein band corresponding to molecular mass of 56 kDa (Fig. 2). Gel filtration on a Superdex 200 column gave a value of 230 kDa as the molecular weight of the enzyme, suggesting that the enzyme is a tetrameric protein.

Properties of the Enzyme—The isoelectric focusing of the enzyme after the cation-exchange chromatography afforded

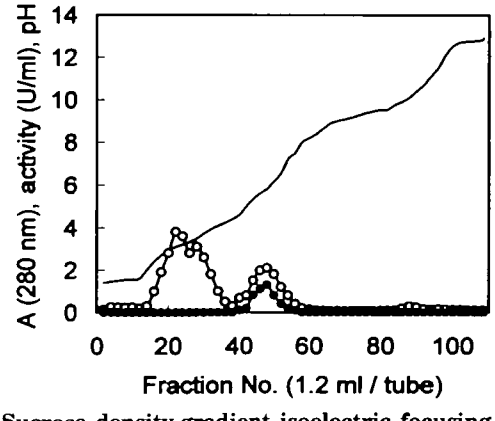

Fig. 1. **Sucrose density-gradient isoelectric focusing of aminoacyltransferase.** The enzyme preparation after cation-exchange chromatography was subjected to electrofocusing on a 110 ml-column with Pharmalyte 3-10 as the carrier ampholytes. The electrofocusing was performed at 400 V for 40 h at 2°C. ○, absorbance at 280 nm; ●, aminoacyltransferase activity measured with a mixture of 200 mM Ac-D-Phe-OMe and 200 mM L-Phe-NH_2 at pH 7.0; —, pH.

TABLE I. **Purification of aminoacyltransferase from *Saccharothrix* sp. AS-2.**

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Culture supernatant	96,100	53,200	0.55	100
10–70% $(NH_4)_2SO_4$	10,800	20,200	1.87	38
CM-Toyopearl 650M	520	8,500	16.3	16
Isoelectric focusing	98	4,800	49.0	9
ProtEx Butyl	7	1,064	152	2

a single active peak corresponding to a pI of 5.8 (Fig. 1). The optimum pH and temperature of the enzyme estimated with a mixture of Ac-D-Phe-OMe and L-Phe-NH_2 as substrates were around 8.2 and 35°C, respectively. The effects of some typical inhibitors and two β-lactams were also investigated (Table II). Out of these chemicals, only PMSF at 1 mM had a drastic inhibitory effect, suggesting that the enzyme was a serine hydrolase. Incubation of the enzyme with 10 mM penicillin G or ampicillin caused neither en-zyme inhibition nor substrate hydrolysis.

The enzyme exhibited aminoacyltransfer activity toward selected D- and L-amino acid methyl esters (Table III), L-Glu(OMe)-OMe being the only L-amino acid ester recognized by the enzyme. However, it did not act on any of the free amino acids tested. In addition, the enzyme exhibited

Fig. 2. **SDS-PAGE of the purified aminoacyltransferase.** SDS-PAGE was performed on a 12% gel. Lane 1, molecular weight marker proteins consisting of phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20.1 kDa); lane 2, purified enzyme. The gel was stained with Quick CBB.

TABLE II. **Effects of inhibitors on aminoacyltransferase.**

Inhibitor	Remaining activity (%)[a]
None	100
1 mM PMSF	2
1 mM NEM	94
1 mM E-64	96
1 mM pepstatin	90
1 mM $HgCl_2$	85
5 mM EDTA	105
10 mM penicillin G	101
10 mM ampicillin	96

[a]After incubation for 1 h at 25°C, pH 7.0. Remaining activity was expressed as a percentage of the activity without an inhibitor.

TABLE III. **Aminoacyltransferase activity toward α-amino acid methyl esters.**

Substrate	Activity (%)[a]
D-Phe-OMe	100
L-Phe-OMe	0
D-Asp(OMe)-OMe	91
L-Asp(OMe)-OMe	0
D-Trp-OMe	14
L-Trp-OMe	0
D-Tyr-OMe	12
L-Tyr-OMe	0
D-Met-OMe	5
L-Met-OMe	0
D-Glu(OMe)-OMe	0
L-Glu(OMe)-OMe	33

[a]Activity was measured as described under "*Enzymatic Peptide Synthesis*," and was expressed as a percentage of the activity obtained with D-Phe-OMe as the substrate. Other amino acid methyl esters, including D-/L-Ala, D-/L-Arg(NO_2), L-Cys(Bzl), Gly, D-/L-His, L-Ile, D-/L-Leu, D-/L-Lys(Z), D-/L-Pro, D-/L-Ser, D-/L-Thr, and D-/L-Val, were inert as substrates.

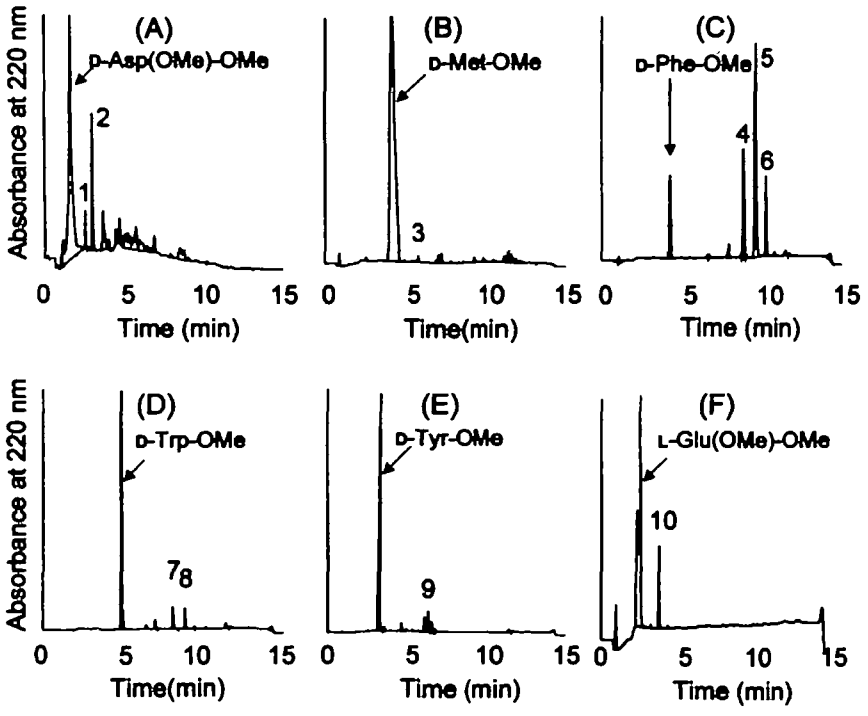

Fig. 3. **HPLC of reaction products derived from D-amino acid esters.** Reaction products were derived from (A) D-Asp(OMe)-OMe, (B) D-Met-OMe, (C) D-Phe-OMe, (D) D-Trp-OMe, (E) D-Tyr-OMe, and (F) L-Glu(OMe)-OMe. The reaction was conducted by incubating 95 μl of 200 mM D-amino acid methyl ester with 5 μl (0.4 unit) of the enzyme for 10 min at pH 7.0 and 25°C. The numbered peaks indicate those subjected to amino acid analysis and MALDI-TOF mass spectrometry.

no detectable hydrolytic activity toward *p*-nitrophenyl acetate, butyrate, caproate, caprylate, laurate, or palmitate, *p*-nitroanilides of D-/L-Ala, D-/L-Leu, and D-/L-Phe, or amino acid amides of D-/L-Ala, D-/L-Arg, D-/L-Asp(NH_2), D-/L-Gln, D-/L-Glu(NH_2), D-/L-His, D-/L-Ile, D-/L-Leu, D-/L-Lys, D-/L-Met, L-Phe, D-/L-Pro, D-/L-Ser, D-/L-Thr, D-/L-Trp, D-/L-Tyr, D-/L-Val, and Gly. None of the dipeptides including DD-, DL-, LD-, LL-diPhe, and DD-, or LL-diAla were hydrolyzed by the enzyme. Similarly, casein did not undergo enzyme-catalyzed hydrolysis even after 16 h incubation.

Enzymatic Peptide Synthesis—Of the free amino acids, amino acid esters and amino acid amides tested, six methyl esters, *i.e.* those of D-Asp(OMe), D-Met, D-Phe, D-Trp, D-Tyr, and L-Glu(OMe), gave some products on incubation with the enzyme (Fig. 3). The structures of the products were confirmed by amino acid analysis and mass spectrometry (Table IV). As can be seen, the enzyme synthesized methyl esters of oligopeptides under the reaction conditions. The products derived from D-Asp(OMe)-OMe were separated into at least 20 peaks on HPLC, indicating the formation of many different species of oligopeptides. In contrast, only one product was obtained from L-Glu(OMe)-OMe. Its mass spectrum exhibited two extra signals besides those appearing for the control (Fig. 4). Interpretation of the two signals as those derived through the addition of H^+ (M = 287.3) or Na^+ (M = 309.3) to the product led to the conclusion that the product is a cyclic dimer of the amino acid ester.

When a mixture of Ac-D-Phe-OMe and DL-Ala-NH_2, DL-Leu-NH_2, DL-Phe-NH_2, or DL-Trp-NH_2 was incubated with the enzyme, two new peaks appeared on HPLC. Parallel to these experiments, each enantiomer of these amino acid amides was used instead of the racemic mixture to assist the identification of the dipeptides obtained above. All the new peaks were then isolated, and subjected to amino acid analysis and mass spectroscopy. They were confirmed to be the expected Ac-DD- and Ac-DL-stereoisomers. Table V summarizes the retention time, mass, yield after 10 min, and proposed structure of each product, and the ratio of the initial rates of formation of the two stereoisomers. The ratio varied appreciably with the amide used, ranging from 0.74 for Ac-D-Phe-D-/L-Leu-NH_2 to 8.12 for Ac-D-Phe-D-/L-Trp-NH_2. On the other hand, when Ac-L-Phe-OMe was used as the acyl donor, it did not react with any of the amino acid amides tested.

Figure 5 shows HPLC of the reaction product from Ac-D-Phe-OMe and DL-Trp-NH_2. It indicates that the DL-stereoisomer was synthesized much faster than the DD-isomer. With DL-Trp-NH_2 as the amine component, crystals appeared after 15 min-reaction, which were collected, dissolved in DMF, and then subjected to HPLC (Fig. 6). They were identified as Ac-D-Phe-L-Trp-NH_2.

TABLE IV. **Retention times, molecular masses, and proposed structures of the reaction products from D-Asp(OMe)-OMe, D-Met-OMe, D-Phe-OMe, D-Trp-OMe, D-Tyr-OMe, and L-Glu-(OMe)-OMe.**

Peak No.[a]	Retention time[b] (min)	Molecular mass[c] (Da)	Yield[d] (%)	Structure[e]
1	2.7	290.3		(D-Asp(OMe))$_2$-OMe
2	3.1	419.4		(D-Asp(OMe))$_3$-OMe
3	5.7	230.3		(D-Met)$_2$-OMe
4	8.8	644.9	15	(D-Phe)$_4$-OMe
5	9.6	798.2	24	(D-Phe)$_5$-OMe
6	10.2	915.4	7.1	(D-Phe)$_6$-OMe
7	8.1	809.2	2.1	(D-Trp)$_4$-OMe
8	8.9	1,003.4	1.6	(D-Trp)$_5$-OMe
9	6.3	1,216.6	0.9	(D-Tyr)$_7$-OMe
10	4.1	286.3		(L-Glu(OMe))$_2$-OMe

[a]Peak number shown in Fig. 3. [b]Retention time in Fig. 3. [c]Measured by MALDI-TOF mass spectrometry. [d]Estimated from the absorbance at 220 nm in Fig. 3 for aromatic peptides. [e]From results of amino acid analysis and MALDI-TOF mass spectrometry.

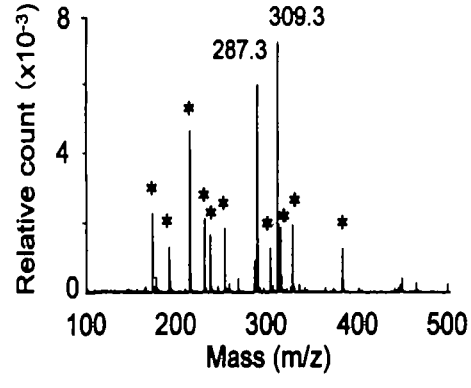

Fig. 4. **MALDI-TOF mass spectrum of the product obtained from L-Glu(OMe)-OMe.** Ninety-five microliters of 200 mM L-Glu-(OMe)-OMe was incubated with 5 μl (0.4 unit) of the enzyme for 10 min at pH 7.0 and 25°C. The product was isolated by HPLC, and then subjected to TOF mass spectrometry. The signals with asterisks are due to the matrix used.

TABLE V. **Retention times on HPLC, masses, yields, and yield ratios of the products obtained from mixtures of Ac-D-Phe-OMe (acyl donor), and DL-Ala-NH_2, DL-Leu-NH_2, DL-Phe-NH_2, DL-Trp-NH_2 (acyl acceptors).[a]**

N-component	Product[b]	Retention time[c] (min)	Mass (Da)	Yield[d] (%)	Yield ratio[e] (DL/DD)
DL-Ala-NH_2	Ac-D-Phe-L-Ala-NH_2	5.5	353	15.4	4.05
	Ac-D-Phe-D-Ala-NH_2	5.0	353	3.8	
DL-Leu-NH_2	Ac-D-Phe-L-Leu-NH_2	8.1	319	10.2	0.74
	Ac-D-Phe-D-Leu-NH_2	7.4	319	13.8	
DL-Phe-NH_2	Ac-D-Phe-L-Phe-NH_2	8.6	353	26.7	1.33
	Ac-D-Phe-D-Phe-NH_2	8.0	353	20.1	
DL-Trp-NH_2	Ac-D-Phe-L-Trp-NH_2	8.5	392	27.6	8.12
	Ac-D-Phe-D-Trp-NH_2	8.2	392	3.4	

[a][Ac-D-Phe-OMe] = 200 mM, [amino acid amide] = 400 mM, with 0.4 unit of enzyme for 10 min at pH 7.0 and 25°C in the presence of 20% DMF. [b]Structure determined from the results of amino acid analysis and mass spectrometry. [c]Obtained on HPLC. [d]Estimated by HPLC on the basis of the amount of Ac-D-Phe-OMe invested. [e]The ratio of the initial rates of formation.

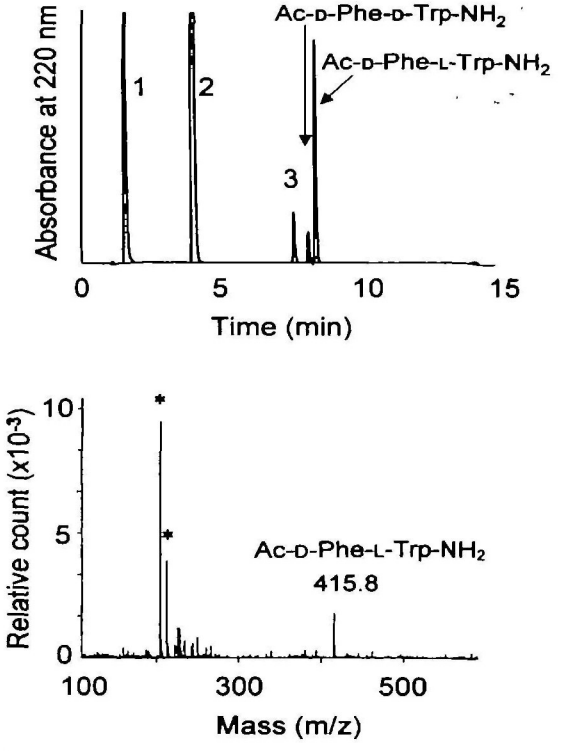

Fig. 5. **HPLC (upper) and mass spectrum (lower) of the reaction product derived from Ac-D-Phe-OMe and DL-Trp-NH_2.** A mixture of 200 mM Ac-D-Phe-OMe and 400 mM DL-Trp-NH_2 was incubated with 5 μl (0.4 unit) of the enzyme for 10 min pH 7.0 and 25°C in the presence of 20% DMF. After the reaction, the products were separated from the substrates by HPLC, and then subjected to MALDI-TOF mass spectrometry. The signals with asterisks are due to the matrix used. Peaks 1, 2, and 3 are DMF, L-Trp-NH_2, and Ac-D-Phe-OMe, respectively.

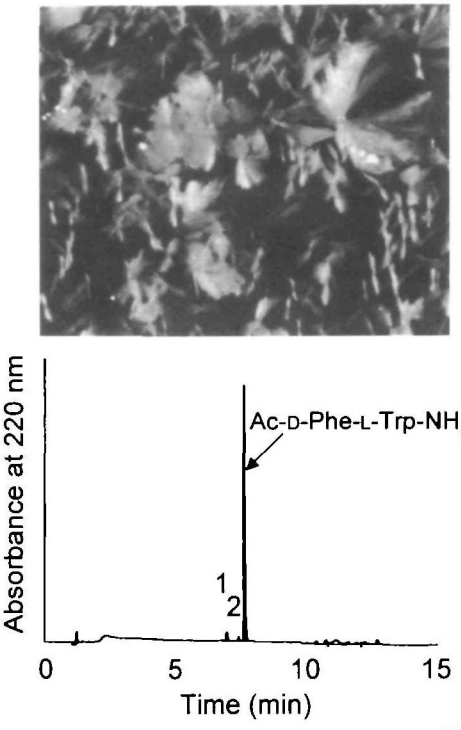

Fig. 6. **Crystals (upper) obtained from a mixture of Ac-D-Phe-OMe and DL-Trp-NH_2, and their HPLC (lower).** Crystals were obtained by incubating 95 μl of a mixture of 200 mM Ac-D-Phe-OMe and 400 mM DL-Trp-NH_2 with 5 μl (0.4 unit) of the enzyme for 30 min at pH 7.0 and 25°C in the presence of 20% DMF. They were separated from the supernatant, dissolved in 50% CH_3CN, and then subjected to HPLC. Peaks 1 and 2 are Ac-D-Phe-OMe and Ac-D-Phe-D-Trp-NH_2, respectively.

DISCUSSION

We have isolated a *Saccharothrix* sp. from soil that extracellularly produces a novel enzyme acting on several D- and L-amino acid esters to yield their oligomers. Since the culture broth showed hydrolytic activity toward both D- and L-amino acid esters, complete enzyme purification was necessary to determine if the activity was due to a single enzyme. The purification work demonstrated that the homo-oligopeptide-forming activity in the culture broth was attributable to a single enzyme. As far as the activity toward α-amino acid ester is concerned, hydrolase and aminoacyltransferase should be taken into account. Actually, beef liver esterase (*49*), leucine aminopeptidase (*50*), trypsin (*51*), chymotrypsin (*52*), *Ochrobactrum anthropi* aminopeptidase (*53*), and *B. mycoides* α-amino-acid-esterase (*41*) have been reported to form oligopeptides from α-amino acid esters. However, unlike the enzymes cited above, the enzyme from *Saccharothrix* sp. AS-2 should not be referred to as a hydrolase because it lacks hydrolytic activity toward a variety of α-amino acids, their esters, amides, several dipeptides, casein, and carboxylic acid esters of *p*-nitrophenol. From the results of inhibition experiments involving β-lactam antibiotics, the enzyme was considered not to be a penicillin-binding protein, as many of the D-amino acid-specific proteases so far isolated are. The catalytic function and substrate specificity of the *Saccharothrix* sp. AS-2 enzyme led to the idea that it is a member of the aminoacyltransferase [EC 2.3.2] family. At present there are fifteen aminoacyltransferases registered (*54*). Out of them, D-glutamyltransferase, γ-glutamyltransferase, lysyltransferase, γ-glutamylcyclotransferase, aspartyltransferase, agaritine γ-glutamyltransferase, protein-glutamine γ-glutamyltransferase, D-alanine γ-glutamyltransferase, and glutathione γ-glutamylcysteinyltransferase accept free amino acids as substrates, while the remaining aminoacyltransferases accept aminoacyl-tRNAs. Hence we conclude that the enzyme from *Saccharothrix* sp. AS-2 is a new type of aminoacyltransferase, by which amino acid esters are recognized as acyl donors.

The fact that the enzyme was strongly inhibited by 1 mM PMSF suggested an important role of serine residue(s) in the catalysis. As is well-known, serine and cysteine peptidases have the ability to synthesize peptides from amino acid esters or amides through a kinetic approach (*5, 6, 11*). We thus examined if the enzyme from *Saccharothrix* sp. AS-2 could catalyze the peptide synthesis. As was expected, it formed oligopeptides from some of the D-amino acid esters although it did not act on free D- or L-amino acids. Out of the methyl esters tested, those of D-Asp(OMe), D-Met, D-Phe, D-Trp, D-Tyr, and L-Glu(OMe) were accepted for homooligopeptide formation (Fig. 3 and Table III). As regards D-Asp and L-Glu esters, not the α- or β(γ)-monoester but only the α-,β(γ)-diester was accepted. The active

site of the enzyme may not accommodate negatively charged amino acid esters.

It has been well documented that there are many biologically important peptides containing a single D-amino acid, which is a requisite for activity. In conventional protease-catalyzed peptide synthesis, the enzymes are very specific for L-amino acids as acyl donors. The amine component is more flexible, both L- and D-amino acids being acceptable, although D-amino acids usually react about one-tenth as fast as the L-counterparts (*20–22*). Thus, the synthesis of a peptide of the LD-configuration can be accomplished without much difficulty. On the contrary, peptides of the DL-configuration are much more difficult to synthesize. Although the enantioselectivity of the enzyme catalysis can be altered by solvent engineering, as typically shown for the synthesis of Ac-D-Phe-L-Phe-NH_2 involving subtilisin in anhydrous *tert*-amyl alcohol (*23*), the approach is not always applicable to all proteases owing to their general instability or ineffectiveness in such an environment.

In the last decade, an increasing number of enzymes acting on D-amino acid–containing peptides has been isolated and their mode of action clarified (*55*). Some of these enzymes were shown to hydrolyze peptides of the DL- as well as the DD-configuration. On the other hand, no examples have been reported of the synthesis of peptides of the DL-configuration using these enzymes, although the synthesis of peptides of the DD-configuration has been reported. Quite recently we found that an α-amino-acid-esterase from *B. mycoides* could easily form peptides of the DL-configuration (*41*). However, the enzyme showed high hydrolytic activity toward amino acid esters, which led to a limited peptide yield. In contrast, the enzyme in this study showed no hydrolytic activity toward any of the amino acid esters tested. We attempted to use Ac-D-Phe-OMe as the acyl donor, and D/L-Ala-NH_2, D/L-Leu-NH_2, D/L-Phe-NH_2, or D/L-Trp-NH_2 as the amine component. Although the methyl esters of L-Ala, L-Leu, L-Phe, L-Trp, and L-Tyr were all unacceptable for homooligopeptide formation, we expected they could be used as amine components. In fact, the enzyme synthesized both DD- and DL-dipeptides without secondary hydrolysis of the products. The fact that the enzyme produces a crystalline precipitate of Ac-D-Phe-L-Trp-NH_2 from a mixture of Ac-D-Phe-OMe and DL-Trp-NH_2 suggests the usefulness of the enzymatic synthesis and separation of a particular diastereomer.

All these observations, together with the finding that several oligopeptides were formed only from D- and L-amino acid methyl esters, indicate that the enzyme has a broader specificity as compared with other known aminoacyltransferases, and indicate its potential usefulness for the synthesis of D-amino acid–containing peptides. Further studies on the stereospecificity and structure–function relationship of the enzyme are now in progress for a better understanding of the reaction mechanism.

REFERENCES

1. Lipmann, F. (1973) Nonribosomal polypeptide synthesis on polyenzyme templates. *Acc. Chem. Res.* **6**, 361–367
2. Steward, M.W. and Howard, C.R. (1987) Synthetic peptides: A next generation of vaccines? *Immunol. Today* **8**, 51
3. Eipper, B.A., Stoffers, D.A., and Mains, R.E. (1992) The biosynthesis neuropeptides: Peptide amidation. *Annu. Rev. Neurosci.* **15**, 57–85
4. Isowa, Y., Ohmori, M., Ichikawa, T., Kurita, H., Sato, M., and Kaoru, M. (1977) The synthesis of peptides by means of proteolytic en-zymes. *Bull. Chem. Soc. Jpn.* **50**, 2762–2765
5. Kullman, W. (1987) *Enzymatic Peptide Synthesis*, CRC Press, Florida
6. Morihara, K. (1987) Using proteases in peptide synthesis. *TIBTECH* **5**, 164–170
7. Wong, C.-H. and Wang, K.-T. (1991) New development in enzymatic peptide synthesis. *Experimentia* **47**, 1123–1129
8. Bongers, J. and Heimer, E.P. (1994) Recent applications of enzymatic peptide synthesis. *Peptides* **15**, 183–193
9. Wong, C.-H. and Whitesides, G.M. (1994) *Enzymes in Synthetic Organic Chemistry*, Elsevier, Oxford
10. Bordusa, F., Ullman, D., and Jakubke, H.D. (1997) Peptide synthesis from N- to C-terminus: an advantageous strategy using protease catalysis. *Angew. Chem. Int. Ed. Engl.* **109**, 1099–1101
11. Kasche, V. (2001) Proteases in peptide synthesis in *Proteolytic Enzymes* (Beynon, J.R. and Bond, J.S., eds.) pp. 265–292, Oxford University Press, Oxford, UK
12. Isowa, Y., Ohmori, M., Sato, M., and Mori, K. (1977) The enzymatic synthesis of protected valine-5 angiotensin II amide-1. *Bull. Chem. Soc. Jpn.* **50**, 2766–2772
13. Kullman, W. (1982) Protease-catalyzed peptide bond formation: Application to synthesis of the carboxy-terminal octapeptide of cholecystokinin. *Proc. Natl. Acad. Sci. USA* **79**, 2840–2844
14. Kayahara, H., Ohhashi, A., Tadasa, K., and Kato, S. (1990) N-Protected tripeptide inhibitors of angiotensin converting enzyme. *Agric. Biol. Chem.* **54**, 1325–1326
15. Clapes, P., Mata-Alvarez, J., Valencia, G., Reig, F., Torres, J.L., and Garcia-Anton, J.M. (1989) Continuous enzyme synthesis of Z-kyotorphin amide in an enzyme-immobilized fixed-bed reactor. *J. Chem. Tech. Biotechnol.* **45**, 191–202
16. Mor, A., Delfour, A., Sagan, S., Amiche, M., Pradelles, P., Rossier, J., and Nicolas, P. (1989) Isolation of dermenkphalin from amphibian skin, a high-affinity delta-selective opioid heptapeptide containing a D-amino acid residue. *FEBS Lett.* **25**, 269–274
17. Yasuda-Kamatani, Y., Nakamura, M., Minakata, H., Nomoto, K., and Sakiyama, F. (1995) A novel cDNA sequence encoding the precursor of the D-amino acid-containing neuropeptide fulicin and multiple alpha-amidated neuropeptides from *Achatina fulica*. *J. Neurochem.* **64**, 2248–2255
18. Jimenez, E.C., Olivera, B.M., Gray, W.R., and Cruz, L.J. (1996) Contryphan is a D-tryptophan-containing conus peptide. *J. Biol. Chem.* **271**, 28002–28005
19. Kreil, G. (1997) D-Amino acids in animal peptides. *Annu. Rev. Biochem.* **66**, 337–345
20. Mignogna, G., Simmaco, M., and Barra, D. (1998) Occurrence and function of D-amino acid-containing peptides and proteins: antimicrobial peptides in *D-Amino Acids in Sequences of Secreted Peptides of Multicellular Organisms* (Jolles, P., ed.) pp. 29–36, Birkhauser Verlag, Basel
21. Pierre, T.N., Seon, A.A., Amiche, M., and Nicolas, P. (2000) Phylloxin: A novel peptide antibiotic of the dermaseptin family of antimicrobial/opioid peptide precursors. *Eur. J. Biochem.* **267**, 370–378
22. Petkov, D.D. and Stoineva, I.B. (1984) Enzyme peptide synthesis by an iterative procedure in a nucleophile pool. *Tetrahedron Lett.* **25**, 3751–3754
23. West, J.B. and Wong, C.-H. (1986) Enzyme-catalyzed irreversible formation of peptide containing D-amino acids. *J. Org. Chem.* **51**, 2728–2735
24. Barbas, C.F., Matos, J.R., West, J.B., and Wong, C.-H. (1988) A search for peptide ligase: cosolvent-mediated conversion of proteases to esterases for irreversible synthesis of peptides. *J. Am. Chem. Soc.* **110**, 5162–5166
25. Margolin, A.L., Tai, D.-F., and Klibanov, A.M. (1987) Incorporation of D-amino acids into peptides via enzymatic condensation in organic solvents. *J. Am. Chem. Soc.* **109**, 7885–7887
26. Tamura, T., Yasuo, I., and Strominger, J.L. (1976) Purification to homogeneity and properties of two D-alanine carboxypeptidases from *Escherichia coli*. *J. Biol. Chem.* **251**, 414–423

27. Romeis, T. and Holtje, J.-V. (1994) Penicillin-binding protein 7/8 of *Escherichia coli* is a DD-endopeptidase. *Eur. J. Biochem.* **224**, 597–604
28. Nguyen-Disteche, M., Leyh-Bouille, M., Pirlot, S., Frere, J.-M., and Ghuysen, J.-M. (1986) *Streptomyces* K15 DD-peptidase-catalysed reactions with ester and amide carbonyl donors. *Biochem. J.* **235**, 167–176
29. Frere, J.-M., Ghuysen, J.-M., Perkins, H.R., and Nieto, M. (1973) Kinetics of concomitant transfer and hydrolysis reactions catalysed by the exocellular DD-carboxypeptidase transpeptidase of *Streptomyces* R61. *Biochem. J.* **135**, 483–492
30. Asano, Y., Ito, H., Dairi, T., and Kato, Y. (1996) An alkaline D-stereospecific endopeptidase with β-lactamase activity from *Bacillus cereus*. *J. Biol. Chem.* **271**, 30256–30262
31. Asano, Y. and Komeda, H. (2000) Gene cloning, nucleotide sequencing, and purification and characterization of the D-stereospecific amino acid amidase from *Ochrobactrum anthropi* SV3. *Eur. J. Biochem.* **267**, 2028–2036
32. Frere, J.-M., Leyh-Bouille, M., Ghuysen, J.-M., Nieto, M., and Perkins, H.R. (1976) Exocellular D-carboxypeptidases-transpeptidases from *Streptomyces*. *Methods Enzymol.* **45**, 610–636
33. Kempe, M., Ekberg, B., and Mosbach, K. (1989) Enzymatic synthesis of dipeptide units of the D-D-configuration in aqueous media. *Int. J. Pept. Protein Res.* **34**, 147–152
34. Kato, Y., Asano, Y., Nakazawa, A., and Kondo, K. (1990) Synthesis of D-alanine oligopeptides catalyzed by D-aminopeptidase in nonaqueous media. *Biocatalysis* **3**, 207–215
35. Komeda, H. and Asano, Y. (1999) Synthesis of D-alanine oligopeptides catalyzed by alkaline D-peptidase from *Bacillus cereus* DF4-B. *J. Mol. Catalysis B: Enzymatic* **6**, 379–386
36. Wang, W. and McMurray, J.S. (1999) A selective method for the preparation of primary amides: synthesis of Fmoc-L-4-carboxamidophenylalanine and other compounds. *Tetrahedron Lett.* **40**, 2501–2504
37. Takahashi, T., Yamazaki, Y., and Kato, K. (1974) Substrate specificity of an α-amino acid ester hydrolase produced by *Acetobacter turbidans* A.T.C.C.9325. *Biochem. J.* **137**, 497–503
38. Kato, K., Kawahara, K., Takahashi, T., and Kakinuma, A. (1980) Purification of α-amino acid ester hydrolase from *Xanthomonas citri*. *Agric. Biol. Chem.* **44**, 1069–1074
39. Kato, K., Kawahara, K., Takahashi, T., and Kakinuma, A. (1980) Substrate specificity of α-amino acid ester hydrolase from *Xanthomonas citri*. *Agric. Biol. Chem.* **44**, 1075–1082
40. Kato, K. (1980) Kinetics of acyl transfer by α-amino acid ester hydrolase from *Xanthomonas citri*. *Agric. Biol. Chem.* **44**, 1083–1088
41. Sugihara, A., Shimada, Y., Sugihara, S., Nagao, T., Watanabe, Y., and Tominaga, Y. (2001) A novel α-amino-acid-esterase from *Bacillus mycoides* capable of forming peptides of DD- and DL-configurations. *J. Biochem.* **130**, 119–126
42. Wang, W. and McMurray, J.S. (1999) Synthesis of D-alanine oligopeptides catalyzed by alkaline D-peptidase from *Bacillus cereus* DF4-B. *J. Mol. Catalysis B: Enzymatic* **6**, 379–386
43. Somorin, O. and Skorepa, J. (1978) Aliesterase activity in normal and postheparin human blood sera. *J. Biochem.* **83**, 617–623
44. Ishida, Y., Fujita, T., and Asai, K. (1981) New detection and separation method for amino acid analysis by high-performance liquid chromatography. *J. Chromatogr.* **204**, 143–148
45. Weatherburn, M.W. (1967) Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem.* **39**, 971–974
46. Bruckner, H., Haasmann, S., Langer, M., Westhauser, T., Wittner, R., and Godel, H. (1994) Liquid chromatographic determination of D- and L-amino acids by derivatization with *o*-phthalaldehyde and chiral thiols. *J. Chromatogr.* **666**, 259–273
47. Wilcox, P.E. (1970) Chymotrypsinogens—trypsinogens in *Methods in Enzymology* (Perlman, G.E. and Lorand, L., eds.) Vol. 19, pp. 64–80, Academic Press, New York
48. Laemmli, U.K. (1969) Cleavage of structure proteins during assembly of the head of bacteriophage T4. *Nature* **277**, 680–685
49. Goldberg, M.I. and Fruton, J.S. (1969) Beef liver esterase as a catalyst of acyl transfer to amino acid esters. *Biochemistry* **8**, 86–97
50. Shippey, S.S. and Binkley, F. (1958) Amino acid esterase activities of swine kidney. *J. Biol. Chem.* **230**, 699–705
51. Inagami, T. and Sturtevant, J.M. (1960) Nonspecific catalyses by chymotrypsin and trypsin. *J. Biol. Chem.* **235**, 1019–1023
52. Bender, M.L. and Kezdy, F.J. (1965) Mechanism of action of proteolytic enzymes. *Annu. Rev. Biochem.* **34**, 49–76
53. Asano, Y., Nakazawa, A., Kato, Y., and Kondo, K. (1989) Properties of a novel D-stereospecific aminopeptidase from *Ochrobactrum anthropi*. *J. Biol. Chem.* **264**, 14233–14239
54. *Enzyme Nomenclature* (1992) pp. 199–201, Academic Press, New York. Update on Internet: http//www.chem..qmw.ac.uk/iubmb/enzyme/EC23/
55. Asano, Y. and Lubbehüsen, T. (2000) Enzymes acting on D-amino acid containing peptides. *J. Biosci. Bioeng.* **89**, 295–306