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Synthesis of D-phenylalanine oligopeptides catalyzed by alkaline D-peptidase from *Bacillus cereus* DF4-B [1]

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Abstract

Synthesis of D-phenylalanine oligopeptides from D-phenylalanine methylester has been demonstrated by use of alkaline D-peptidase (ADP) from *Bacillus cereus*. An expression plasmid pKADP was constructed by placing the PCR-amplified ADP gene (*adp*) under the *tac* promoter of pKK223-3. Oligomerization of D-phenylalanine methylester by use of the purified ADP from the transformant *Escherichia coli* was investigated under several conditions. D-Phenylalanine dimer, $(D\text{-Phe})_2$, and trimer, $(D\text{-Phe})_3$, were produced in 25.4% and 8.6% yield, respectively, when 50 mM of the substrate was incubated for 8 h with ADP (2.0 U/ml and 0.4 U/ml, respectively) in 100 mM triethylamine–HCl (pH 11.5). Addition of dimethylsulfoxide to the reaction mixture resulted in the production of tetramer, $(D\text{-Phe})_4$ in 6.7% yield with the decrease of the $(D\text{-Phe})_2$ and $(D\text{-Phe})_3$ production. This is the first study on the synthesis of D-phenylalanine oligomers by use of a D-stereospecific endopeptidase.

Keywords: Alkaline D-peptidase; D-Phenylalanine; Peptide

1. Introduction

Enzyme-catalyzed peptide bond formation represents a promising alternative to chemical synthesis procedures [1–3]. Several attempts have been reported on the in vitro synthesis of the D-amino acid containing peptides and amides [4–10]. Naturally, however, these enzymatic reactions have an inevitable drawback as they are not stereospecific for substrates with D-configurations. A D-stereospecific peptidase, D-aminopeptidase was isolated from *Ochrobactrum anthropi* and characterized [11,12]. The enzyme can be applied for kinetic resolution of racemic amino acid amides to yield D-amino acids [11,13], synthesis of *N*-alkylamides from DL-amino acid esters and amines [14], and synthesis of D-alanine oligopeptides from D-alanine methylester [15]. We also characterized a novel extracellular D-stereospecific endopeptidase 'Alkaline D-peptidase (ADP)' from a bacterium *Bacillus cereus* strain DF4-B, isolated with a synthetic substrate $(D\text{-Phe})_4$ [16]. The enzyme (Mr: 37,952, monomer) acts as a dipeptidyl endopeptidase strictly D-stereospecific toward oligopeptides such as $(D\text{-Phe})_3$ and $(D\text{-Phe})_4$.

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[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

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The ADP gene (*adp*) was isolated and its deduced amino acid sequence revealed that the ADP could be classified as a member of penicillin-recognizing enzymes.

Since the ADP was found to be a serine peptidase [16], we attempted to use it for kinetically controlled peptide synthesis. In this study, we describe for the first time the synthesis of D-phenylalanine oligomers by use of the ADP as catalyst in an alkaline aqueous medium.

2. Materials and methods

2.1. Materials

D-Phenylalanine methylester as a substrate and authentic $(D\text{-Phe})_2$, $(D\text{-Phe})_3$, and $(D\text{-Phe})_4$ were chemically synthesized from D-phenylalanine by the procedure as described previously [16]. ^{1}H-NMR spectrum was recorded in CD_3OD using a JEOL JNM-EX400 spectrometer (Tokyo, Japan), with tetramethylsilane as the internal standard. Mass spectrum was recorded on a JEOL JMS-AX500 mass spectrometer (Tokyo, Japan) under fast atom bombardment (FAB) conditions. DEAE-Toyopearl 650 M and Butyl-Toyopearl 650 M were purchased from Tosoh (Tokyo, Japan). Centriprep-10 concentrator was obtained from Amicon, (Beverly, MA). Cosmosil 5C18-MS was from Nacalai Tesque (Kyoto, Japan). An expression vector pKK223-3 was obtained from Pharmacia (Uppsala). All other chemicals used in this work were of analytical grade and commercially available.

2.2. Construction of expression plasmid pKADP

In order to construct an expression plasmid, *Eco*RI and a *Pst*I restriction enzyme sites were generated at the upstream and downstream from *adp*, respectively, by PCR with pADP1 [16] as a template and two oligonucleotide primers (Fig. 1). The nucleotide sequence of the DNA fragment amplified by PCR was determined and found not to contain any point mutations. The plasmid designated as pKADP was constructed by placing the PCR-amplified *adp* under the *tac* promoter of pKK223-3. In this construction, the ribosome binding sequence on the pKK223-3 locates 14 nucleotides upstream of the putative translational initiation codon (ATG) of the *adp* [16]. The plasmid pKADP was used to transform *Escherichia coli* JM109.

2.3. Purification of ADP from E. coli JM109/pKADP

E. coli JM109/pKADP was subcultured at 37°C for 12 h in a test tube containing 5 ml of LB medium with 80 μg/ml ampicillin; the subculture was then inoculated into a 2–1 shaking flask containing 500 ml of the above medium. After a 7 h incubation at 37°C with reciprocal shaking, isopropyl β-D-thiogalactopyranoside was added to the medium to a final concentration of 1 mM to induce the *tac* promoter, followed by a further incubation at 30°C for 5 h. Then the cells were harvested by centrifugation for 20 min at $9000 \times g$ at 4°C.

All purification steps were performed at temperatures lower than 4°C. Potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA was used throughout the purification.

Step 1: The harvested cells from 10 l culture were suspended in 100 ml 0.1 M buffer, and disrupted by sonication at 19 kHz for 10 min with an insonator model 201 M (Kubota, Japan).

Step 2: After centrifugation, the resulting supernatant was fractionated with solid ammonium sulfate. The precipitate obtained at 45–80% saturation was collected by centrifugation and dissolved in 10 mM buffer. The resultant enzyme solution was dialyzed against 5 l of the same buffer for 24 h.

Step 3: The dialyzed solution was applied to a DEAE-Toyopearl 650 M column (3.0×15 cm) equilibrated with 10 mM buffer. The enzyme was eluted with the same buffer and the fractions containing enzyme activity were combined.

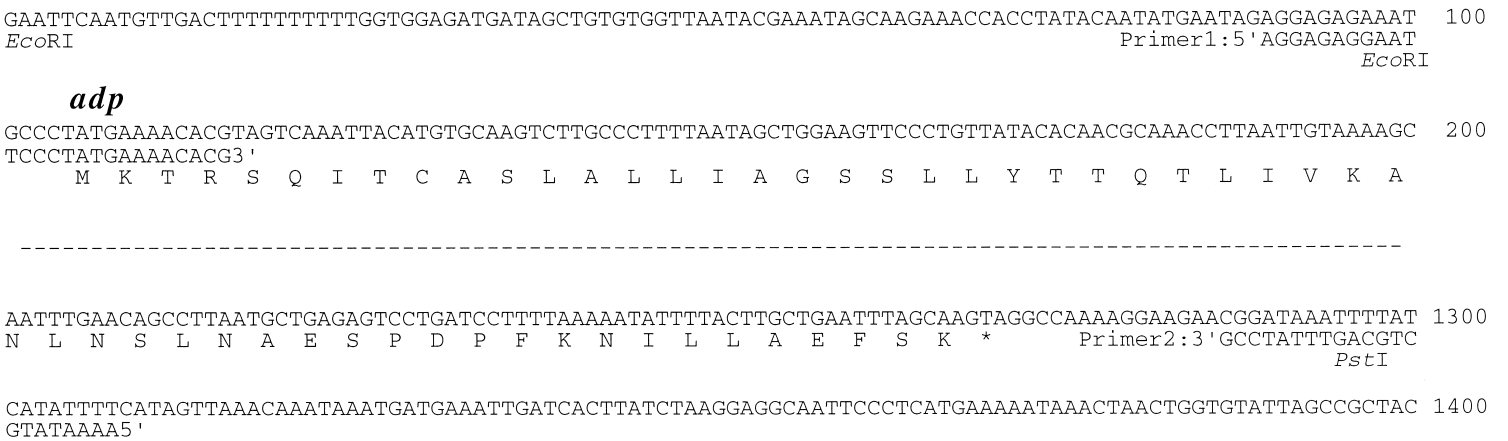

Fig. 1. Sequences of oligonucleotide primers for the expression of *adp*. Part of the nucleotide sequence of pADP1 insert [16] corresponding to the primers was also shown.

Step 4: The enzyme solution was then brought to 30% ammonium sulfate saturation and applied to a Butyl-Toyopearl 650 M column (3.0 × 15 cm) equilibrated with 10 mM buffer saturated with 30% ammonium sulfate. The enzyme was eluted with a linear gradient of ammonium sulfate (30 to 0% saturation) in 10 mM buffer. The active fractions were combined and concentrated with Centriprep-10.

Protein was determined by the Coomassie brilliant blue-R250 dye-binding method of Bradford [17] using dye reagent supplied by Bio-Rad (USA). The protein content of the eluate obtained on column chromatography was monitored at 280 mm. One unit of enzyme activity was defined as described previously [16].

2.4. General procedure for the enzymatic oligomerization of D-phenylalanine methylester by use of alkaline D-peptidase

The reaction mixture was composed of 50 μmol of D-phenylalanine methylester hydrochloride, 50 μl of dimethyl sulfoxide, 100 μmol of triethylamine–HCl, pH 11.5, and 2 μmol of $MgSO_4$, and the polymerization was started by addition of the enzyme in a total volume of 1 ml. The mixture was incubated at 30°C and the reaction was terminated with 1/10 volume of 3 M phosphoric acid. The amount of $(D\text{-Phe})_2$ formed was estimated with a Waters 600E HPLC apparatus equipped with a Cosmosil 5C18-MS reverse-phase column (4.6 × 150 mm) at a flow rate of 1.0 ml/min using 35% methanol in 5 mM KH_2PO_4/H_3PO_4 buffer, pH 2.9. The retention time for $(D\text{-Phe})_2$ was 8.3 min. The amount of $(D\text{-Phe})_3$ and $(D\text{-Phe})_4$ formed was estimated with the same HPLC system using 55% methanol in 5 mM KH_2PO_4/H_3PO_4 buffer, pH 2.9. The retention times for $(D\text{-Phe})_3$ and $(D\text{-Phe})_4$ were 4.4 and 10.6 min, respectively. Absorbance of the eluate was monitored at 254 nm. A control incubation of the reaction mixture with a preinactivated enzyme revealed no or negligible oligomerization of the substrate.

2.5. Characterization of $(D\text{-Phe})_2$

The enzymatically synthesized $(D\text{-Phe})_2$ was purified by preparative thin layer chromatography on silica gel (developing solvents: chloroform/methanol/acetic acid = 8/2/1) and identified.: ^{1}H-NMR (CD_3OD) δ_{ppm} 7.066–7.199 (m, 10 H), 4.465 (m, 2H), 3.125 (m, 2H), 2.935(m, 2H); FABMS: m/z 335 (rel. int. 22%, M + Na), 313 (11, M + 1), 223 (34), 131 (61), 120 (100). The synthesized $(D\text{-Phe})_2$ and an authentic $(D\text{-Phe})_2$ had the same retention time (8.3 min) in HPLC analysis under the condition described above.

3. Results and discussion

3.1. Enzymatic oligomerization in aqueous solution

We have previously described the characterization of the alkaline D-peptidase (ADP) purified from *B. cereus* DF4-B [16]. The enzyme not only hydrolyzed $(D\text{-Phe})_3$ and $(D\text{-Phe})_4$ to form $(D\text{-Phe})_2$ and D-phenylalanine, but also acted on D-phenylalanine methylester and D-phenylalanine amide to form $(D\text{-Phe})_2$. This finding gave us the opportunity to further investigate the synthesis of D-phenylalanine oligopeptides from D-phenylalanine methylester by use of the ADP.

ADP was purified from *E. coli* JM109/pKADP to a specific activity of 2.0 units/mg. We investigated the oligomerization of D-phenylalanine methylester with the ADP under the condition as described in Section 2. D-Phenylalanine oligopeptides were analyzed qualitatively by using HPLC as described in Section 2. Identification of one of the products ^{1}H-NMR and mass spectrometry indicated that one of the enzymatically synthesized D-phenylalanine oligopeptides was the dimer (see Section 2).

Alkaline conditions are usually essential for peptide bond formation in kinetically controlled systems [18,19]. We investigated the oligomerization reaction under various buffer conditions using the ADP (Fig. 2). Increased syntheses of (D-Phe)$_2$ and (D-Phe)$_3$ were observed over a range of alkaline conditions (pH 10–12). Therefore, the pH of the reaction mixture is thought to be an important factor for the enzymatic oligomerization of D-phenylalanine methylester. Of the buffer conditions tested in this study, triethylamine–HCl (pH 11.5) was selected for the oligomerization reactions.

A small part of the substrate, D-phenylalanine methylester was spontaneously degraded to free D-phenylalanine at the above alkaline conditions without the enzyme. Then, we investigated whether the ADP acts on the free D-phenyl-

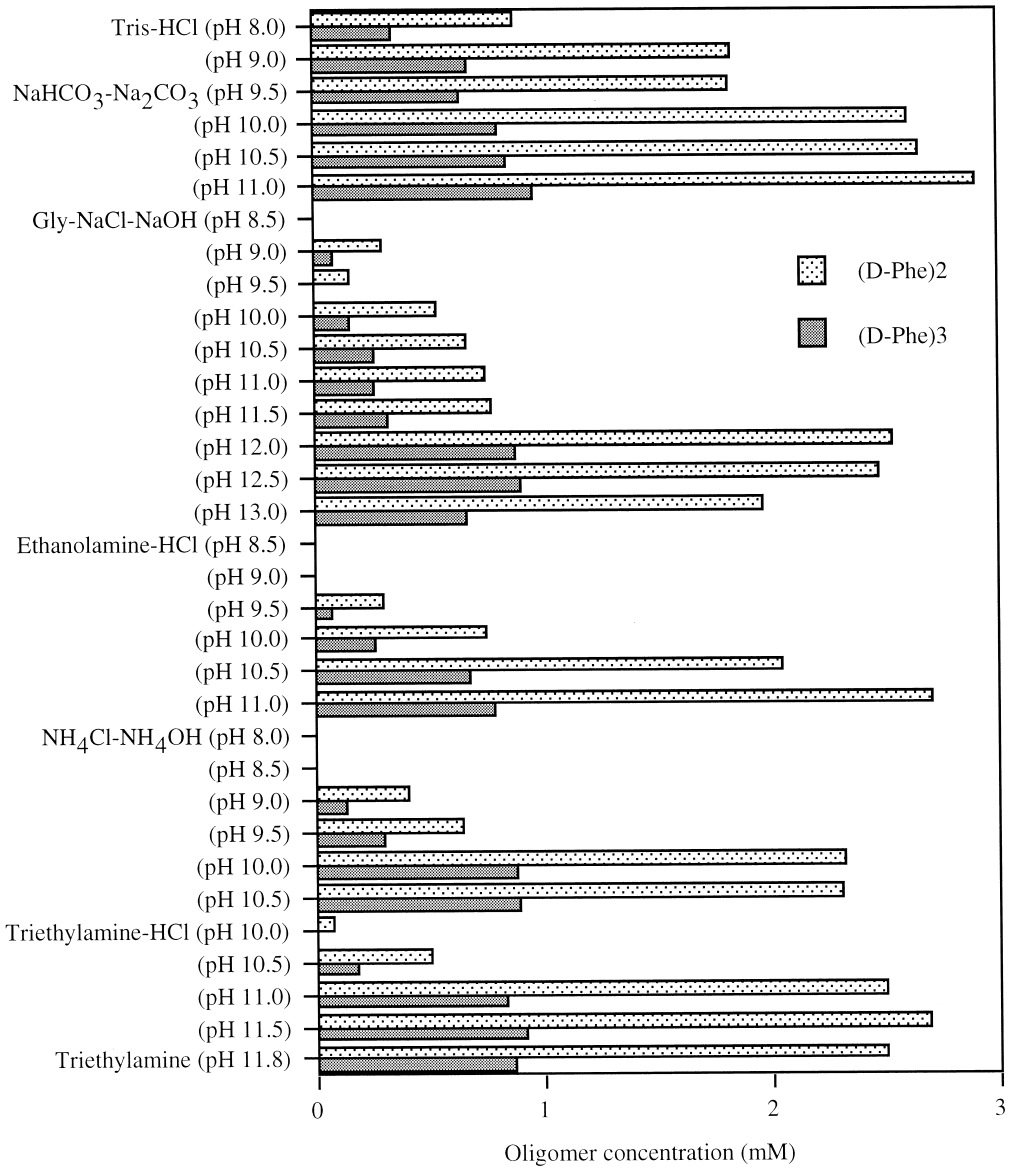

Fig. 2. Effect of buffer and pH on the enzymatic oligomerization reaction. Reaction mixtures (1 ml) containing 50 mM of D-phenylalanine methylester hydrochloride, 100 mM of each buffer, 2 mM of $MgSO_4$, and ADP (0.6 U) were incubated for 1 h at 30°C.

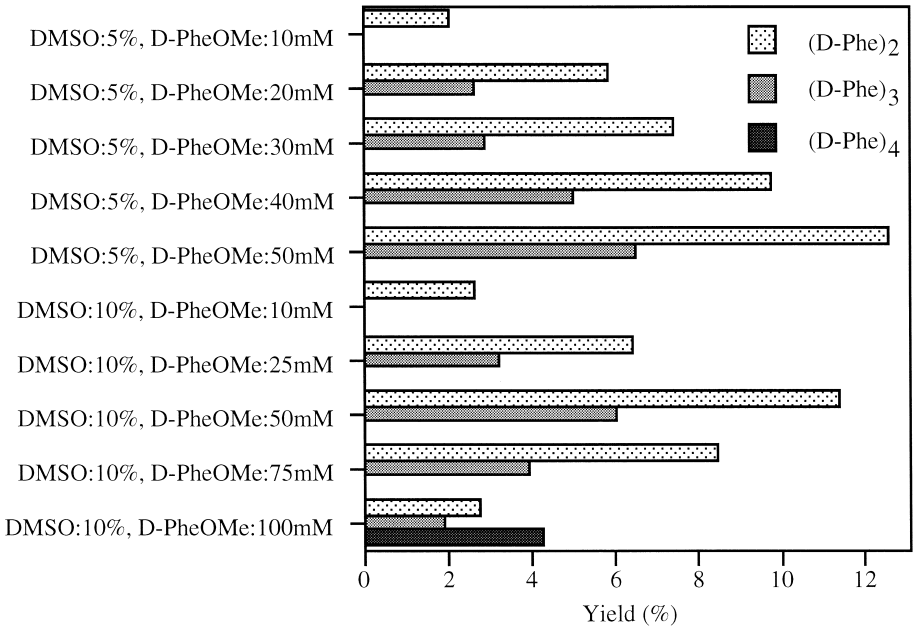

Fig. 3. Effect of substrate concentration on the enzymatic oligomerization reaction. Reaction mixtures (1 ml) containing various concentration of D-phenylalanine methylester hydrochloride, 100 mM of triethylamine–HCl (pH 11.5), 2 mM of $MgSO_4$, and ADP (0.6 U) were incubated for 1 h at 30°C. Concentration of the dimethyl sulfoxide (DMSO) used to solubilize the substrate was also investigated.

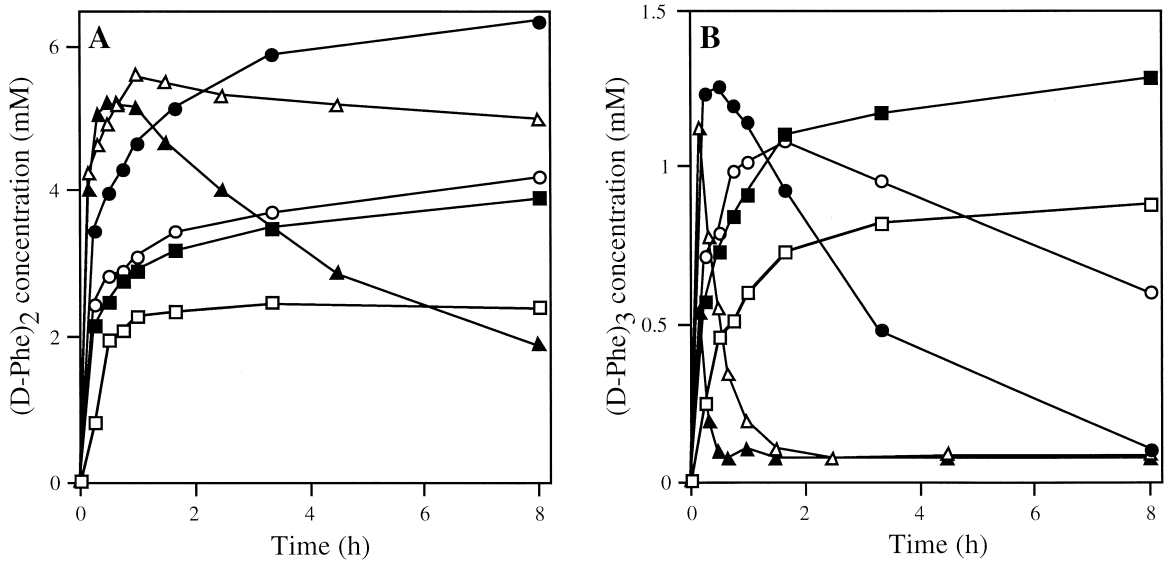

Fig. 4. Time course of the enzymatic oligomerization reaction. (A) Formation of $(D\text{-Phe})_2$. (B) Formation of $(D\text{-Phe})_3$. Reaction mixtures as described in Materials and methods containing various amounts of enzymes were incubated at 30°C. The amount of enzymes is as follows; (□) 0.2 U, (■) 0.4 U, (○) 0.8 U, (●) 2 U, (△) 10 U, (▲) 30 U.

alanine to synthesize the D-Phe oligomers. Incubation of the ADP with a free D-phenylalanine as a substrate in the reaction mixture containing 0.1 M Tris–HCl (pH 9.0) or 0.1 M triethylamine–HCl (pH 11.5) gave no oligomerization products, suggesting that the oligomerization by the ADP proceeded not through a reversal of hydrolysis but a kinetically controlled synthesis.

3.2. Effect of the substrate concentration

Fig. 3 shows the effect of substrate concentration on the oligomerization of D-phenylalanine. D-phenylalanine methylester oligomerized well when the concentration was 50 mM, with a maximum yield of $(D\text{-Phe})_2$ and $(D\text{-Phe})_3$ of 12.5% and 6.5%, respectively. The higher the substrate concentration above 50 mM in the

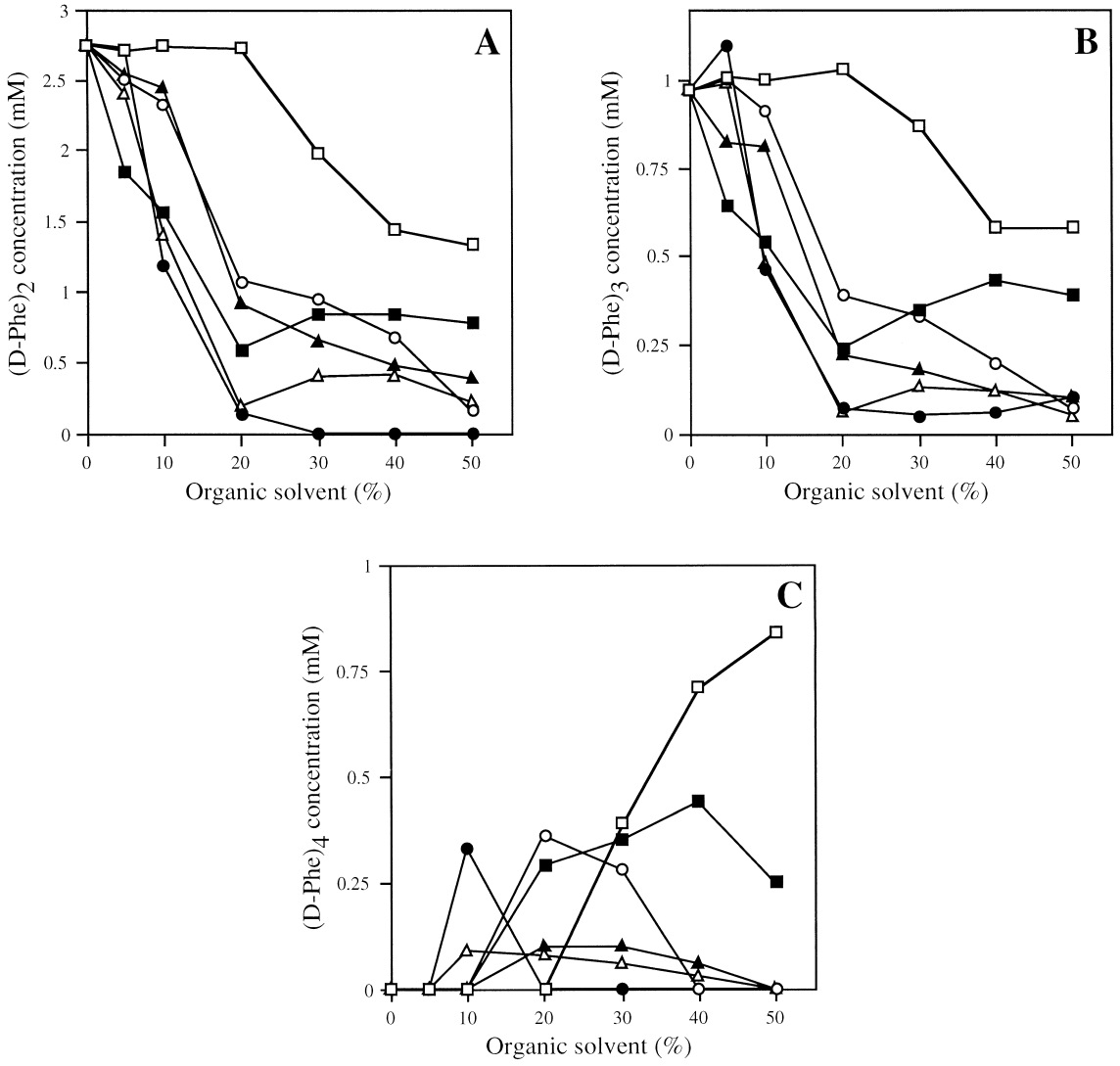

Fig. 5. Effects of organic solvents on the synthesis of $(D\text{-Phe})_2$ (A), $(D\text{-Phe})_3$ (B), and $(D\text{-Phe})_4$ (C). Reaction mixtures (1 ml) containing 50 mM of D-phenylalanine methylester hydrochloride, 100 mM of triethylamine–HCL (pH 11.5), 2 mM of $MgSO_4$, ADP (0.6 U), and various water-miscible organic solvents were incubated for 1 h at 30°C. The organic solvents used were: dimethyl sulfoxide, (□), dimethyl formamide (■), dioxane (○), tetrahydrofuran (●), acetonitrile (△), acetone (▲).

reaction mixture containing 10% dimethyl sulfoxide, the smaller were the yields of the oligomerization products, probably because of substrate inhibition of the enzyme. Lower yields were also observed when the substrate concentration was below 50 mM. Synthesis of $(D\text{-Phe})_4$ was observed only when 100 mM of substrate was used at the presence of 10% dimethyl sulfoxide.

3.3. Time course of oligomerization

The time course of $(D\text{-Phe})_2$ and $(D\text{-Phe})_3$ syntheses by the various amounts of ADP in the presence of 50 mM of the substrate, D-phenylalanine methylester in 100 mM triethylamine–HCl (pH 11.5) was investigated. As shown in Fig. 4, oligomerization progressed with time and reached a plateau in 4 h. The maximum yields of $(D\text{-Phe})_2$ and $(D\text{-Phe})_3$ were 25.4% and 8.6%, respectively, when the substrate was incubated for 8 h with the ADP (2.0 U/ml and 0.4 U/ml, respectively). The oligomerization did not yield products with higher molecular weights than $(D\text{-Phe})_3$. On the other hand, a relatively large amount of enzyme added to the reaction mixture resulted in the reduction of the oligomer synthesis. The yield of $(D\text{-Phe})_2$ decreased when the amount of the enzyme was above 10 U/ml, whereas the yield of $(D\text{-Phe})_3$ decreased when the amount of the enzyme was even above 0.8 U/ml, demonstrating that the oligomerized products might be successively hydrolyzed by the ADP. These results are consistent with the fact that the ADP had poor hydrolyzing activity toward $(D\text{-Phe})_2$; relative rate for hydrolysis of $(D\text{-Phe})_2$ was about 0.2% to those for $(D\text{-Phe})_3$ and $(D\text{-Phe})_4$ [16]. Thus the present procedure appears to be applicable to the synthesis of $(D\text{-Phe})_2$, in which an appropriate amount of the enzyme should be used.

3.4. Effect of organic solvents

The results in Fig. 5 show that most water-miscible solvents added to the reaction mixture caused reductions in the yields of $(D\text{-Phe})_2$ and $(D\text{-Phe})_3$, presumably due to an inactivation of the enzyme. However, $(D\text{-Phe})_4$ was produced in all cases tested. Especially, the addition of dimethyl sulfoxide (final conc., 50%) caused the significant increase of $(D\text{-Phe})_4$ yield (6.7%), probably due to the inhibition of the successive hydrolysis of the product $(D\text{-Phe})_4$ in the higher concentration of dimethyl sulfoxide, which relatively could not affect the enzyme stability. The yield may be much improved by a stabilization of the enzyme in organic solvents, such as immobilization to resins [14,15] or chemical modification with polyethylene glycol [20].

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