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RESEARCH PAPERS

Solid-Phase Syntheses of Two Deacetyl-Thymosin β_4 Analogues with Substitution at Position 12 and Their Effects on Impaired Blastogenic Response of T Lymphocytes of Uremic Patients

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ABSTRACT

Two deacetyl-thymosin β_4 analogues containing Phe(4Br) or D-Phe(4Br) as position 12 were synthesized by the manual solid-phase method, and their immunological effects on the impaired blastogenic response of phytohemagglutinin-stimulated T lymphocytes of uremic patients with infectious diseases were studied. Bromination of the p-position of Phe[12] resulted in a marked restorative effect on the impaired blastogenic response of T lympocytes compared with that of our synthetic deacetyl-thymosin β_4. The synthetic [Phe(4Br)[12]]deacetyl-thymosin β_4 was approximately equal in potency to our synthetic [Phe(4F)[12]]deacetyl-thymosin β_4 in uremic patients, but the other analogue, [D-Phe(4Br)[12]]deacetyl-thymosin β_4, had no effect.

INTRODUCTION

The uremic state causes T-lymphocyte immune impairment in uremic patients (1,2) reflected by a decreased response to the T-lymphocyte mitogen phytohemagglutinin. We and others have reported evidence of impaired immune function in patients with uremia (3–7). This impairment is reflected in both in vitro and in vivo depressed cell-mediated immune function.

The thymus plays an essential role in the development and maintenance of cellular immune competence, and recent evidence suggests that the thymus produces

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biologically active peptides which are responsible for the differentiation and functional maturation of precursor T lymphocytes.

The thymus shows a marked reduction in lymphoid elements and extensive replacement infiltration with fat in uremic patients. Cystic degeneration is also seen in these patients. These observations and findings suggested to us that the cell-mediated immune abnormalities seen in chronic uremia might attributable to thymus hormone deficiency.

Low et al. have reported (8) the isolation and complete amino acid sequence of thymosin β_4 purified from thymosin fraction 5. (See Fig. 1.) Thymosin β_4 is composed of 43 amino acid residues with acetyl-serine at the *N*-terminus and has an isoelectric point of 5.1. Thymosin β_4 is one of several components present in thymosin fraction 5 that participate in the regulation, differentiation, and function of thymus-dependent thymocytes. The amino acid sequences of thymosins β_8 and β_9, which were also isolated from calf thymus by Hannappel et al. (9), were found to be homologous to thymosin β_4.

In previous papers (3,10,11) we reported syntheses of deacetyl-thymosin β_4, thymosin β_8, and thymosin β_9, and showed that these synthetic thymus peptides could have restoring effects on the impaired cell-mediated immunological functions. We also noticed that the acetyl group at the *N*-terminal serine residue of thymosin β_4 is not required for the restorative effect on the impaired cell-mediated immunological functions (3).

In a preceding paper (12) we reported synthesis of [Phe(4F)^{12}]deacetyl-thymosin β_4 and showed that our synthetic [Phe(4F)^{12}]deacetyl-thymosin β_4 exhibited stronger restoring activity on the impaired blastogenic response of T lymphocytes isolated from uremic patients than that of our synthetic deacetyl-thymosin β_4.

This result prompted us to synthesize two thymosin β_4 analogues containing brominated aromatic rings instead of a fluorinated aromatic ring. This paper deals with the solid-phase syntheses of Phe(4Br)^{12}]deacetyl-thymosin β_4 and [D-Phe(4Br)^{12}]deacetyl-thymosin β_4, and an examination of the immunological effects of these analogues and our synthetic deacetyl-thymosin β_4 (3) on the impaired blastogenic response of T lymphocytes of uremic patients.

MATERIALS AND METHODS

Boc-Ser(Bzl)-Merrifield resin (0.5 mmol/g) and Boc-amino acids, Boc-Ser(Bzl)-OH, Boc-Asp(OcHex)-OH, Boc-Glu(OcHex)-OH, Boc-Lys(Z)-OH, Boc-Pro-OH, Boc-Met(O)-OH, Boc-Ala-OH, Boc-Ile-OH, Boc-Thr(Bzl)-OH, Boc-Gln-OH, Boc-Leu-OH, Boc-Gly-OH, and Boc-Asn-OH—except for Boc-Phe(4Br)-OH and Boc-D-Phe(4Br)-OH—were purchased from Protein Research Inc. (Mino, Osaka), Sigma (USA), and Kokusan Chemical Works Ltd. (Kyoto).* Boc-Phe(4Br)-OH and Boc-D-Phe(4Br)-OH were purchased from Neosystem Laboratoire (France). Other reagents and solvents were also purchased from Protein Research Inc. (Mino, Osaka) and Kokusan Chemical Works Ltd. (Kyoto). The purified synthetic [Phe(4Br)^{12}]deacetyl-thymosin β_4 and [D-Phe(4Br)^{12}]deacetyl-thymosin β_4 were chromatographed on cellulose plates (Merck). Rf^1 values refer to BuOH-AcOH-H_2O (5:1:1) and Rf^2 values refer to BuOH-pyridine-AcOH-H_2O (30:20:6:24). Kits for the fluorometric blast formation test were purchased from Japan Immunoresearch Laboratories Co. Ltd. (Japan). The fluorescence excitation spectrum was measured with an Oyo-Bunko ULOG-FLOUSPEC 11A fluorometer. Amino acid analysis was performed with a Hitachi Model 835 amino acid analyzer. HPLC was conducted with a Shimadzu LC-3A apparatus equipped with an analytical Nucleosil 5C18 column (4 × 150 mm). FAB-MS spectra were obtained on a Auto Spec Q instrument (UQ Analytical Co., England) mass spectrometer equipped with OPUS data processor.

Patient Selection

Two uremic patients who were suffering from recurrent infectious diseases were selected. Examination of the cellular immunocompetence of these patients revealed a significant decrease in blast formation by PHA. SI values of these patients were 111.4 and 108.6, respectively (normal value: 283.5). Venous blood was obtained from these uremic patients for the fluorometric blast formation test. Venous blood samples from three healthy donors were used as control.

*Abbreviations used: Z, benzyloxycarbonyl; OcHex, cyclohexyl ester; Bzl, benzyl; Boc, *tert*-butoxycarbonyl; Et_3N, triethylamine; DMF, dimethylformamide; DCC, dicyclohexylcarbodi-imide; HOBT, *N*-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; PHA, phytohemagglutinin; FCS, fetal calf serum; PBS, phosphate-buffered saline; RPMI, Roswell Park Memorial Institute; SDS, sodium dodecyl sulfate; AcOH, acetic acid; TFA, trifluoroacetic acid; EtOH, ethanol; FAB-MS, fast atom bombardment—mass spectrometry; Ac, acetyl.

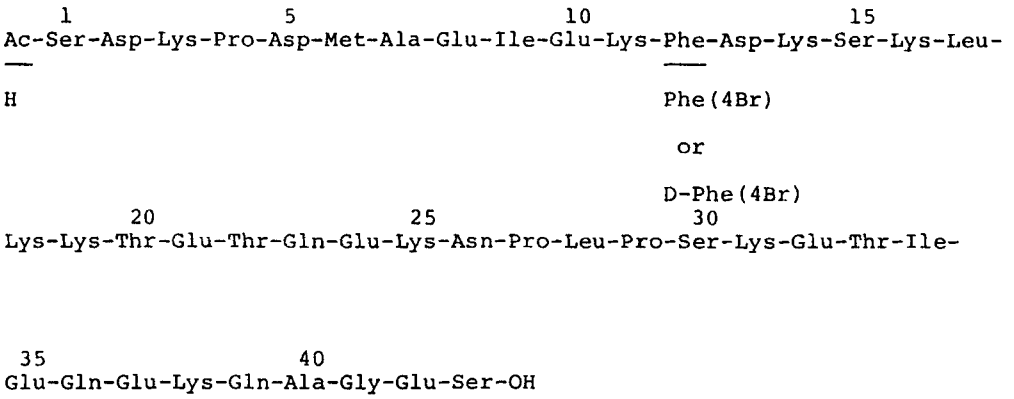

Figure 1. Amino Acid sequence of thymosin β_4.

Solid-Phase Peptide Synthesis

Solid-peptide syntheses of [Phe(Br)^{12}]deacetyl-thymosin β_4 and [D-Phe(Br)^{12}]deacetyl-thymosin β_4 were carried out manually in a glass vessel by a stepwise strategy starting with Boc-Ser(Bzl)-Merrifield resin (0.5 mmol/g, 2 g), and the synthesis was continued by sequentially incorporating amino acid residues, one at a time, into the growing peptide chains according to the general principles of the solid-phase method. In all coupling cycles (120 min) fivefold excess each of Boc-amino acid and DCC were used except in those cycles involving Asn or Gln residues, where HOBT was added in order to minimize the side reactions as usual. Double couplings were done when necessary as judged by the ninhydrin test.

The general procedure for each synthetic cycle was:

1. Three washings with CH_2Cl_2
2. Prewashing with 40% TFA in CH_2Cl_2
3. Deprotection for 30 min with 40% TFA in CH_2Cl_2
4. Three washings with CH_2Cl_2
5. Prewashing with 10% Et_3N in CH_2Cl_2
6. Neutralization for 10 min with 10% Et_3N in CH_2Cl_2
7. Three washings with CH_2Cl_2
8. Addition of 5 eq Boc-amino acid and DCC in CH_2Cl_2-DMF (1:1) or *N*-methyl-2-pyrrolidone
9. Reaction for 120 min
10. Three washings each with CH_2Cl_2, 50% EtOH in CH_2Cl_2, DMF, and then CH_2Cl_2. Double couplings were done when necessary as judged by the ninhydrin test.
11. 0.4 M acetylimidazole in DMF (×1, 30 min)
12. Two washings with DMF

The protected peptide resin thus obtained was treated with anhydrous hydrogen fluoride containing 10% anisole and Me_2Se (50 μl) as scavengers at 0°C for 60 min. After evaporation of excess hydrogen fluoride under vacuum, the crude peptide was extracted with ether and then evaporated to dryness under vacuum. The residue was dissolved in 1 N AcOH (5 ml). After being stirred with Amberlite IRA-400 (acetate form, approximately 1 g) for 30 min, the solution was adjusted to pH 6.0 with 1 N AcOH and the solution was lyophilized. The residue was dissolved in H_2O (5 ml). The solution, after addition of dithiothreitol (10 mg), was incubated at 60°C for 36 hr. The solvent was evaporated in vacuo.

Purification of Deprotected [Phe(4Br)^{12}]Deacetyl-Thymosin β_4 and [D-Phe(4Br)^{12}]-Deacetyl-Thymosin β_4

The dithiothreitol-treated product was dissolved in 1% AcOH (2 ml) and then applied to a column of Sephadex G-25 (2.5 × 94 cm), which was eluted with 1% AcOH. Individual fractions (4 ml each) were collected and the absorbance at 260 nm was determined for each fraction. The fractions corresponding to the first main peak were combined and the solvent was removed by lyophilization. The peptide thus obtained was further purified by HPLC. HPLC was conducted with a Shimadzu LC-3A apparatus. A sample (3 mg) was applied to a Nucleosil C18 column (250 × 10 mm), which was eluted with a gradient of CH_3CN (25 → 60%, 45 min)

in 0.1% aqueous TFA at a flow rate of 3.0 ml/min. The eluates corresponding to the main peaks (retention times; 21.48 min for [Phe(4Br)^{12}]deacetyl-thymosin β_4 and 21.97 min for [D-Phe(4Br)^{12}]deacetyl-thymosin β_4, detected by ultraviolet absorption measurement at 230 nm) were collected and the solvent was removed by lyophilization to give fluffy powders. The rest of the samples were similarly purified: overall yield of the two peptides were 6.3% ([Phe(4Br)^{12}]deacetyl-thymosin β_4) and 5.6% ([D-Phe(4Br)^{12}]deacetyl-thymosin β_4), respectively, based on the *C*-terminal Ser loaded on the resin. Homogeneity of the peptides was checked by TLC, analytical HPLC, FAB-MS, and amino acid analysis after 6 N HCl hydrolysis. The physicochemical data of the synthetic analogues are shown in Tables 1 and 2.

Blast Formation Test

A 3-ml aliquot of venous blood from uremic patients was drawn into a syringe containing 25 U/ml of heparin and then mixed with 3 ml of PBS. Lymphocytes were isolated in a Hypaque–Ficoll gradient as usual. Isolated lymphocytes were adjusted to 1.0×10^6/ml with PBS. The lymphocytes were cultured in 0.5 ml of RPMI 1640

Table 1

Characterization of Synthetic [Phe(4Br)^{12}]Deacetyl-Thymosin β_4 and [D-Phe(4Br)^{12}]Deacetyl-Thymosin β_4

Peptide	Yield[a] (%)	$[\alpha]_D^{21}$ (c = 0.5, 1% AcOH)	TLC[b] Rf^1	TLC[b] Rf^2	HPLC[c]	FAB-MS[d] (MH$^+$)
[Phe(4Br)^{12}]deacetyl-thymosin β_4	6.3	-82.6°	0.02	0.09	15.61	5000.1
[D-Phe(4Br)^{12}]deacetyl-thymosin β_4	5.6	-53.7	0.04	0.11	17.14	500.3

[a]Final yield after deblocking and purification starting from Boc-Ser(Bzl)-Merrifield resin.
[b]See the Materials and Methods section.
[c]HPLC was performed on an analytical Nucleosil 5C18 column (4 × 150 mm) by gradient elution with CH_3CN (20 → 45%) in 0.1% TFA at a flow rate of 1 ml/min, and eluate was monitored at 230 nm.
[d]Found values were in agreement with calculated values.

Table 2

Amino Acid Ratios in 6 N HCl Hydrolysates of [Phe(4Br)^{12}]Deacetyl-Thymosin β_4 and [D-Phe(4Br)^{12}]Deacetyl-Thymosin β_4

[Phe(4Br)^{12}]deacetyl-thymosin β_4[a]			[D-Phe(4Br)^{12}]deacetyl-thymosin β_4[a]	
Gly	1.00	(1)	1.00	(1)
Leu	2.05	(2)	2.04	(2)
Ile	1.98	(2)	2.02	(2)
Met	0.87[b]	(1)	0.89[b]	(1)
Ala	2.03	(2)	2.04	(2)
Pro	2.88	(3)	2.83	(3)
Ser	3.79	(4)	3.75	(4)
Thr	2.83	(3)	2.85	(3)
Glu	10.82	(11)	10.91	(11)
Asp	3.80	(4)	3.92	(4)
Lys	9.01	(9)	8.93	(9)
Phe(4Br)	0.90	(1)	—	
D-Phe(4Br)	—		0.87	(1)

[a]Hydrolysis was carried out in 6N HCl in an evacuated sealed tube at 120°C for 30 hr, and the results are expressed as ratios to the value for Gly, which was taken as the diagnostic amino acid in acid hydrolysates.
[b]Met + Met (O).

(Gibco) with FCS (Dainippon Pharmaceutical Co.) in microplates. Cultures of each combination were incubated at 37°C in the presence of the peptide or in the absence of the peptide in a humidified atmosphere of 5% CO_2 in the air for 12 hr, and PHA (0.125%, 0.5 ml) was added to each well. Incubation was continued under the same conditions for 60 hr. T lymphocytes in each well were transferred to a test tube and centrifuged for 10 min at 240 *g*, then the supernatant was removed. A 2-ml aliquot of 0.125% SDS was added to the residue and stirred for 20 min at room temperature; lymphocytes were completely destroyed and solubilized by this procedure. Ethidium bromide solution (2 ml) was added to the above solution and the mixture was stirred for 15 min at room temperature. The fluorescence excitation spectrum (600 nm) was measured.

RESULTS AND DISCUSSION

[Phe(4Br)^{12}]deacetyl-thymosin β_4 and [D-Phe(4Br)^{12}]-deacetyl-thymosin β_4 were synthesized using Merrifield's solid-phase peptide synthesis methodology. Boc-amino acid derivatives were coupled by the DCC coupling method and double couplings were performed if necessary judged by the results of the ninhydrin test. Unreacted peptide chains on the resin were then capped with acetylimidazole to prevent further elongation of unwanted chains. To minimize side reactions, the peptides were cleaved from the resin and freed of the side-chain protecting groups using hydrogen fluoride in the presence of 10% of anisole and Me_2Se (50 µl) as scavengers, and then incubated with dithiothreitol to reduce sulfoxide on the methionine side chain.

The crude peptides were immediately gel filtered on Sephadex G-25 to remove traces of dithiothreitol; then the crude peptide preparations were further purified by HPLC using a Nucleosil C18 column to obtain highly purified analogues. These synthetic analogues exhibited a single spot (ninhydrin and chlorine–tolidine positive) on TLC in two different solvent systems. The purity of these analogues was further confirmed by amino acid analysis after acid hydrolysis and the results of FAB-MS spectrometry. The synthetic analogues also exhibited single peaks on analytical HPLC (Fig. 2).

Immunological effect of the synethetic [Phe(4Br)^{12}]-deacetyl-thymosin β_4, [D-Phe(4Br)^{12}]deacetyl-thymosin β_4, [Phe(4F)^{12}]deacetyl-thymosin β_4^{12}, and deacetyl-thymosin β_4^3 was examined by means of the lymphocyte stimulation test by PHA. Responses of T lymphocytes to mitogenic stimulation were significantly lower in uremic patients than those of normal persons.

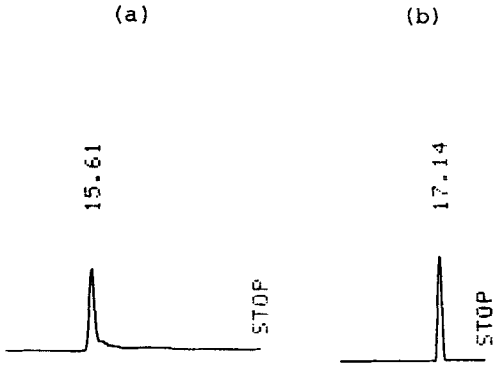

Figure 2. Analytical HPLC profiles of [Phe(4Br)^{12}]deacetyl-thymosin β_4 (a) and [D-Phe(4Br)^{12}]deacetyl-thymosin β_4 (b).

The in vitro effect of the four synthetic peptides on the impaired PHA response of T lymphocytes from uremic patients is shown in Table 3.

In our previous paper (12) we reported that our synthetic [Phe(4F)^{12}]deacetyl-thymosin β_4 showed stronger restoring activity than that of our synthetic deacetyl-thymosin β_4. In that paper (12) we concluded that the strong electron-withdrawing fluoride atom on the para position of the aromatic ring results in an analogue that posses stronger activity than that of the parent molecule. This result prompted us to synthesize two analogues which contain an bromide atom on the para position of Phe^{12} or D-Phe^{12} in deacetyl-thymosin β_4.

Interestingly, our synthetic [Phe(4Br)^{12}]deacetyl-thymosin β_4) showed stronger restoring activity than that of our synthetic deacetyl-thymosin β_4. Our synthetic [Phe(4Br)^{12}]deacetyl-thymosin β_4 was approximately equal in potency to our synthetic [Phe(4F)^{12}]deacetyl-thymosin β_4. The other analogues, [D-Phe(4Br)^{12}]deacetyl-thymosin β_4, had no restoring effect as high as 5 µg/ml.These results seem to suggest that the replacement of a hydrogen atom at the para position of the aromatic ring in the Phe^{12} residue by high electron withdrawing atoms F or Br drastically changes their immunological activity, although there is the exception of the inactive analog, [D-Phe(4Br)^{12}]deacetyl-thymosin β_4. This fact seems to give an expectation for the development of halogen atom containing thymosin β_4 analogues for patients suffering from immunodeficiency, although

Table 3

Effect on the Synthetic Deacetyl-Thymosin β_4, [Phe(4F)^{12}]Deacetyl-Thymosin β_4, [Phe(4Br)^{12}]Deacetyl-Thymosin β_4, and [D-Phe(4Br)^{12}]Deacetyl-Thymosin β_4 on the Impaired PHA Stimulation of T Lymphocytes of Uremic Patients

Peptide	Dose (μg/ml)	*SI*[a,b]
—[c]	—	284.2 ± 50.2
—[d]	—	110.3 ± 49.4[f]
Deacetyl-thymosin β_4[d,e]	0.1	108.7 ± 51.6
Deacetyl-thymosin β_4[d,e]	1.0	186.5 ± 52.1[g]
[Phe(4F)^{12}]deacetyl-thymosin β_4[d,e]	0.1	205.4 ± 50.3[g]
[Phe(4F)^{12}]deacetyl-thymosin β_4[d,e]	1.0	223.8 ± 48.7[g]
[Phe(4Br)^{12}]deacetyl-thymosin β_4[d,e]	0.1	199.7 ± 49.0[g]
[Phe(4Br)^{12}]deacetyl-thymosin β_4[d,e]	1.0	226.2 ± 51.7[g]
[D-Phe(4Br)^{12}]deacetyl-thymosin β_4[d,e]	0.1	112.4 ± 53.1
[D-Phe(4Br)^{12}]deacetyl-thymosin β_4[d,e]	1.0	109.5 ± 52.4
[D-Phe(4Br)^{12}]deacetyl-thymosin β_4[d,e]	5.0	107.6 ± 50.9

[a]Each value represents the mean ± SD of triplicate measurements.
[b]*SI* (stimulation index) was calculated according to the following formula: SI = $[(I_2 - I_0)/(I_1 - I_0)] \times 100$, where I_2 = mean fluorescence intensity of PHA-activated lymphocytes, I_1 = fluorescence intensity of non-PHA-activated lymphocytes, and I_0 = fluorescence intensity of ethidium bromide.
[c]Normal venous lymphocytes.
[d]Patient's venous lymphocytes.
[e]Incubation was carried out at 37°C in a humidified atmosphere of 5% CO_2 in air for 12 hr.
[f]$p < 0.05$, when compared to the normal persons using Student's *t* test.
[g]$p < 0.02$, when compared to the uremic patients using Student's *t* test.

the Phe residue in thymosin β_4 is considered to be L-configuration for keeping immunological activity.

In the case of normal subjects, in vitro addition of these peptides had no effect on the mitotic activity induced by PHA stimulation under the same conditions (data not shown).

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