Chem. Pharm. Bull. 38(9) 2602—2605 (1990) Vol. 38, No. 9

Immunological Effect of Two Synthetic Peptides Containing Alanine or D-Alanine Instead of Acetyl Group of Thymosin β_4 after Treatment of Human Serum[1)]

Takashi ABIKO* and Hiroshi SEKINO

Kidney Research Laboratory, Kojinkai, 1–6 Tsutsujigaoka 2-chome, Miyagino-ku, Sendai 980, Japan. *Received February 1, 1990*

Two analogs of thymosin β_4 the N-terminal acetyl groups of which were substituted by Ala or D-Ala, were synthesized by the solution method and studied for their immunological effect on the impaired blastogenic response of T-lymphocytes isolated from uremic patients after treatment of human serum. One of the synthetic analogs, D-Ala-thymosin β_4 demonstrated a restorative effect on these patients when incubated in human serum, but the other analog, Ala-thymosin β_4, showed no restorative effect under the same conditions. These results seem to suggest that D-Ala-thymosin β_4 increases resistance to proteolytic degradation by exopeptidases more than Ala-thymosin β_4.

Keywords thymosin β_4 analog synthesis; trifluoromethanesulfonic acid deprotection; dithiothreitol reduction; impaired T-lymphocyte blastogenic response; human serum treatment; serum stability

Thymosin β_4 consists of 43 amino acid residues with a molecular weight of 4963 and an isoelectric point of 5.1.[2)] The N-terminus of the peptide is blocked by an acetyl group. This peptide exhibits important activities in the regulation and differentiation of thymus-dependent lymphocytes.[3)] It induces expression of TdT activity in TdT-negative thymocytes both *in vivo* and *in vitro*.[3,4)]

The amino acid sequences of thymosins β_8 and β_9, which were also isolated from calf thymus by Hannappel *et al.*,[5)] were found to be homologous to thymosin β_4.

In previous papers,[6−8)] we reported the syntheses of deacetyl-thymosin β_4, thymosins β_8 and β_9, and showed that these synthetic thymus peptides had a restorative effect on impaired cell-mediated immunological functions. Therefor, we concluded that the acetyl group at the N-terminal Ser residue of thymosin β_4 is not required for immunological activity on impaired T-lymphocytes of uremic patients. In addition, N-terminal acetylation of thymosin β_4 increases resistance to proteolytic degradation by exopeptidases.[9)]

Following our solution syntheses of deacetyl-thymosin β_4,[6)] thymosins β_8[7)] and β_9,[8)] we wish to report the solution syntheses of two thymosin β_4 analogs in which the N-terminal acetyl group of thymosin β_4 is replaced by Ala or D-Ala, and the *in vitro* effect of these peptides on the impaired blastogenic response of T-lymphocytes of uremic patients after treatment of human serum.

For synthetic reasons, in contrast to our previous syntheses of deacetyl-thymosin β_4,[6)] thymosins β_8[7)] and β_9[8)] the thioanisole-mediated trifluoromethanesulfonic acid (TFMSA) deprotection procedure[10,11)] was applied in the final step in place of hydrogen fluoride.

Our synthetic routes to Ala-thymosin β_4 and D-Ala-thymosin β_4 are illustrated in Fig. 2.

As shown, the TFA-labile Boc group for N^{α}-protection

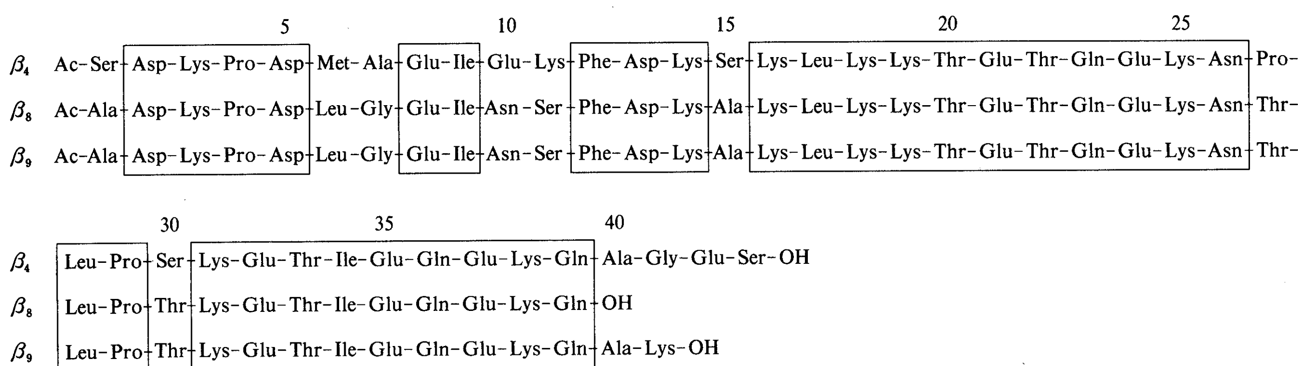
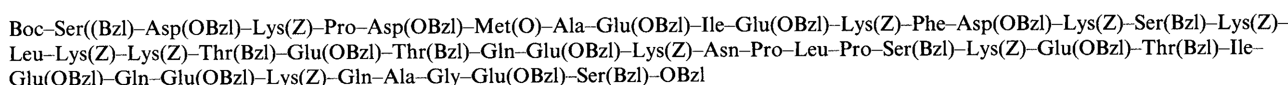
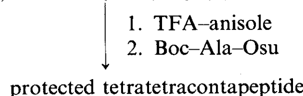
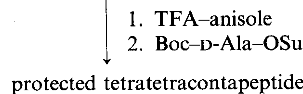


Fig. 1. Comparison of Amino Acid Sequences of Thymosins β_4, β_8 and β_9

Identical sequences are shown in boxes.







protected tetratetracontapeptide

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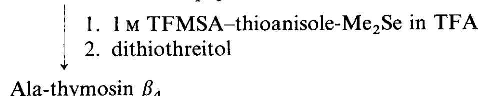

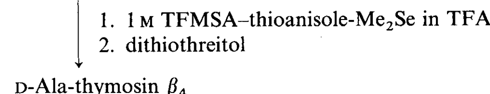

Ala-thymosin β_4

D-Ala-thymosin β_4

Fig. 2. Synthetic Routes to Ala-thymosin β_4 and D-Ala-thymosin β_4

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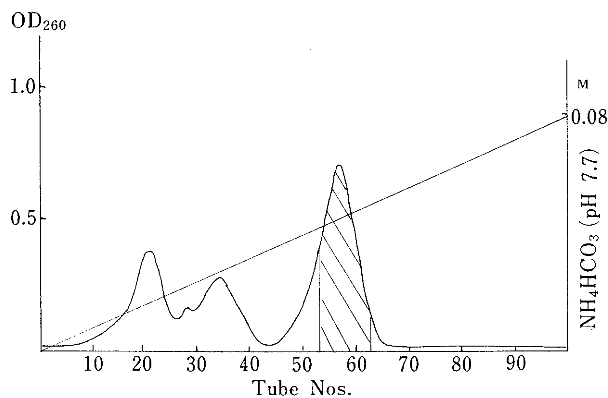

Fig. 3. Purification of Synthetic Ala-thymosin β_4 by Ion-Exchange Chromatography on a DEAE-Cellulose Column

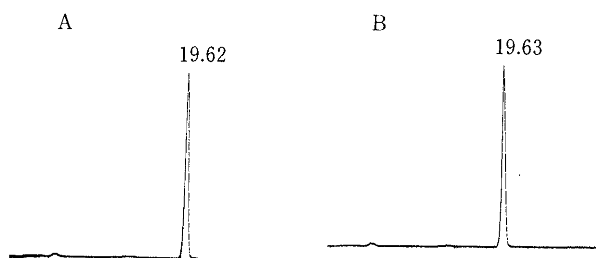

Fig. 4. HPLC of Synthetic Ala-thymosin β_4 (A) and D-Ala-thymosin β_4 (B)

and amino acid derivatives bearing protecting groups removable by 1 M TFMSA–thioanisole in TFA[10,11] were employed, *i.e.*, Ser(Bzl), Thr(Bzl), Lys(Z), Glu(OBzl), Asp(OBzl) and Ser(Bzl)–OBzl. The Met residue was reversibly protected as its sulfoxide[12] in order to prevent irreversible partial air oxidation during the synthesis. Boc(1—43)–OBzl was available from our previous synthesis of deacetyl-thymosin β_4.[6] The Boc group of Boc–(1—43)–OBzl[6] was removed by the usual TFA–anisole treatment and the corresponding free amine was condensed with Boc–Ala–OSu or Boc–D-Ala–OSu to give Boc–Ala–(1—43)–OBzl or Boc–D-Ala–(1—43)–OBzl, which purified by reprecipitation from DMF with MeOH. The homogeneity of these protected peptides was assessed by elemental analysis, TLC and amino acid analysis of the acid hydrolysates. The protected peptide esters I or III were then treated with 1 M TFMSA–thioanisole in TFA in the presence of Me_2Se. Me_2Se was employed to facilitate acidic cleavage of protecting groups.[13] Each of the deprotected peptides was next precipitated with peroxide-free ether, converted to the corresponding acetate with Amberlite IRA-400 (acetate form) and then treated with 1 N NH_4OH at pH 8.0 to reverse a possible N→O shift[14] at the Ser and Thr residues. The Met(O) residue was reduced back to Met in two steps, firstly with thioanisole and Me_2Se[13] during the above acid treatment, and secondly, with dithiothreitol during incubation of each of these deprotected peptides. Each of these reduced peptides was purified by gel-filtration on Sephadex G-25, followed by ion-exchange column chromatography on a DEAE-cellulose column by linear-gradient elution using pH 7.7 NH_4HCO_3 buffer, followed by preparative TLC. Desalting on a Sephadex G-25 column gave a fluffy powder, which exhibited a single spot (ninhydrin- and chlorine-tolidine-positive) on TLC in two different solvent systems and on a paper electrophoresis (pH 7.3 pyridinium–acetate buffer). The purity of each peptide was further confirmed by amino acid analysis after acid hydrolysis. These two peptides each exhibited a single peak on HPLC.

The immunological effect of these synthetic peptides was examined by means of the JIMRO (Japan Immunoresearch Laboratories Co., Ltd.) fluorometric blast-formation test according to Itoh and Kawai.[15] Responses of T-lymphocytes to mitogenic stimulation were lower in uremic patients than those of normal persons. The *in vitro* effect of these two synthetic peptides after treatment of human serum on the impaired PHA response of T-lymphocytes from the uremic patients is shown in Table I.

Table I. Effect of the Synthetic Ala-thymosin β_4 and D-Ala-thymosin β_4 on the Impaired PHA-Stimulation of T-Lymphocytes of Uremic Patients after Treatment of Human Serum

Peptide	Dose (μg/ml)	$SI^{a,b)}$
—[c]	—	281.4±48.2
—[d]	—	110.3±50.1[g]
Ala-thymosin β_4[d,e]	10	226.4±51.6[h]
D-Ala-thymosin β_4[d,e]	10	223.9±53.1[h]
Ala-thymosin β_4[d,f]	10	103.5±52.7[h]
D-Ala-thymosin β_4[d,f]	10	187.4±51.4[h]

a) Each value represents the mean±S.D. of triplicate measurements. *b) SI* (stimulation index) was calculated according to the following formula:

$$SI=\frac{I_2-I_0}{I_1-I_0}\times 100$$

where I_2 = mean fluorescence intensity of PHA-activated lymphocytes, I_1 = fluorescence intensity of PHA-nonactivated 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 h using each synthetic peptide without treatment of human serum. *f)* Each synthetic peptide was incubated in human serum at 37 °C for 30 min and then lymphocytes were incubated with one of the human serum treated peptides at 37 °C for 12 h. *g)* $p<0.05$, when compared to the normal persons by using Student's *t* test. *h)* $p<0.01$, when compared to the uremic patients by using Student's *t* test.

Comparison of the stimulation index (*SI*) values of the blastogenic transformation of T-lymphocytes into lymphoblasts with mitotic activity upon PHA stimulation shows that the synthetic D-Ala-thymosin β_4 still demonstrated a restorative effect in the uremic patients investigated after treatment of human serum, although the restorative effect after incubation in human serum was a little weaker than that of the same peptide without incubation. On the other hand, *in vitro* addition of the synthetic Ala-thymosin β_4 after treatment of human serum had no effect on the mitotic activity induced by PHA stimulation under the same conditions.

It appears from these results that substitution of the acetyl group by Ala did not improve stability in serum, but substitution of the acetyl group by D-Ala gave an analog which was fairly stable.

Experimental

General experimental procedures used were essentially the same as previously described.[6-8] Melting points are uncorrected. Rotations were measured with an Atago Polax machine (cell length: 10 cm). The amino acid compositions of the acid hydrolysates were determined with a Hitachi 835-50 type amino acid analyzer. Solutions were concentrated in a rotary evaporator under reduced pressure at a temperature of 30—45 °C. Boc

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groups of the protected peptides were removed by TFA–anisole treatment. The resulting amino components were chromatographed on silica gel plates (Kieselgel G, Merck) and Rf^1 values refer to the following solvent system: $CHCl_3$–MeOH–H_2O (8 : 3 : 1). The final products corresponding to the two peptides, Ala-thymosin β_4 and D-Ala-thymosin β_4, were chromatographed on cellulose plates (Merck). Rf^2 values refer to BuOH–AcOH–H_2O (4 : 1 : 5) and Rf^3 values refer to BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24).[16] Kits for the fluorometric blast-formation test were purchased from Japan Immunoresearch Laboratories Co., Ltd., Japan. 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 112.3 and 107.4, respectively (normal value: 281.4). 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 and for stability studies for the synthetic peptides. The fluorescence excitation spectrum was measured with an Oyo-Bunko ULOG-FLOUSPEC 11 A fluorometer. HPLC was conducted with a Shimadzu LC-3A apparatus equipped with a Nucleosil 5C18 column.

Boc–Ala–Ser(Bzl)–Asp(OBzl)–Lys(Z)–Pro–Asp(OBzl)–Met(O)–Ala–Glu(OBzl)–Ile–Glu(OBzl)–Lys(Z)–Phe–Asp(OBzl)–Lys(Z)–Ser(Bzl)–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–Glu(OBzl)–Thr(Bzl)–Gln–Glu(OBzl)–Lys(Z)–Asn–Pro–Leu–Pro–Ser(Bzl)–Lys(Z)–Glu(OBzl)–Thr(Bzl)–Ile–Glu(OBzl)–Gln–Glu(OBzl)–Lys(Z)–Gln–Ala–Gly–Glu(OBzl)–Ser(Bzl)–OBzl [I] The protected tritetracontapeptide ester, Boc–(1—43)–OBzl corresponding to the protected deacetyl-thymosin β_4[6] (82 mg), was treated with TFA–anisole (2—0.4 ml) in an ice-bath for 40 min, and TFA was then removed by evaporation. The residue was washed with ether, dried over KOH pellets *in vacuo* for 2 h, and then dissolved in DMF–HMPA (1 : 1, 2 ml) containing NMM (0.001 ml). To this solution, Boc–Ala–OSu (5.7 mg) was added, and the mixture was stirred at room temperature for 24 h. The reaction mixture was evaporated and then triturated with 5% citric acid. The powder obtained was washed successively with 5% citric acid, H_2O, 5% $NaHCO_3$, H_2O and MeOH. The powder was further purified by reprecipitation twice from DMF with MeOH: Yield 61 mg (73%), mp 172—186 °C (dec.), $[\alpha]_D^{21}$ −24.6° (c=0.5, DMSO), Rf^1 0.83, single ninhydrin-positive spot. *Anal.* Calcd for $C_{423}H_{529}N_{57}O_{99}S \cdot 16H_2O$: C, 61.09; H, 6.80; N, 9.60. Found: C, 59.87; H, 7.04; N, 9.32. Amino acid ratios in a 6 N HCl hydrolysate: Gly 1.00, Leu 2.04, Ile 2.06, Met(O) + Met 0.87, Ala 3.02, Pro 2.89, Phe 1.01, Ser 3.84, Thr 2.87, Glu 10.97, Asp 3.88, Lys 9.05 (recovery of Gly 83%).

H–Ala–Ser–Asp–Lys–Pro–Asp–Met–Ala–Glu–Ile–Glu–Lys–Phe–Asp–Lys–Ser–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–Pro–Leu–Pro–Ser–Lys–Glu–Thr–Ile–Glu–Gln–Glu–Lys–Gln–Ala–Gly–Glu–Ser–OH (Corresponding to Ala-thymosin β_4) [II] The protected tetratetracontapeptide [I] (50 mg) was treated with 1 M TFMSA–thioanisole in TFA (2 ml) in the presence of Me_2Se (60 μl) in an ice-bath for 110 min, then peroxide-free dry ether was added. The resulting powder was collected by centrifugation, dried over KOH pellets for 2 h and dissolved in 1 N AcOH (5 ml). The solution, after being stirred with Amberlite IRA-400 (acetate form, approximately 1 g) for 30 min, was filtered. The pH of the filtrate was adjusted to pH 8.0 with 1 N NH_4OH and after 30 min to pH 6.0 with 1 N AcOH. The solution was incubated with dithiothreitol (30 mg) at 40 °C for 12 h and then lyophilized. The product was purified by gel-filtration on a Sephadex G-25 column (3.2 × 90 cm) using 2% AcOH as an eluant. The fractions (5 ml each) corresponding to the front main peak (tube Nos. 51—62, determined by UV absorption measurement at 260 nm) were combind and the solvent was removed by lyophilization to give a fluffy powder. The product was dissolved in H_2O (3 ml) and the solution was applied to a column of DEAE-cellulose (Brown, 2.3 × 12.4 cm), which was eluted with a linear gradient of 300 ml each of H_2O–0.08 M NH_4HCO_3 buffer at pH 7.7. Individual fractions (5 ml each) were collected and the absorbancy at 260 nm was determined. Main peak fractions of the gradient eluates (tube Nos. 54—63) were combined and the solvent was evaporated *in vacuo*. Analysis by TLC revealed the presence of three ninhydrinpositive spots with Rf^2 0.03 (main), 0.21 (minor) and 0.44 (minor). The crude product was dissolved in a small amount of water and subjected to preparative TLC (cellulose plate, 20 × 40 cm) using BuOH–AcOH–H_2O (4 : 1 : 5, upper layer) as a developing solvent. The zone corresponding to Rf^2 0.03 was separated and extracted with 2% AcOH. The extracts were concentrated to a small volume, applied to a Sephadex G-25 column (3.2 × 90 cm) and eluted with 2% AcOH as described above and the solvent was removed by lyophilization: Yield 6.3 mg (21%), $[\alpha]_D^{21}$ −74.3° (c=0.3, 2% AcOH), Rf^2 0.03, Rf^3 0.10, single ninhydrin- and chlorine–tolidine-positive spot. The synthetic peptide exhibited a single spot on paper electrophoresis: Toyo Roshi No. 51 (2 × 40 cm), pyridinium–acetate buffer at pH 7.3, mobility 1.7 cm from the origin toward the anode after running at 2 mA, 600 V for 80 min. Amino acid ratios in a 6 N HCl hydrolysate: Gly 1.00, Leu 2.02, Ile 1.98, Met 0.89, Ala 2.99, Pro 2.91, Phe 1.04, Ser 3.87, Thr 2.90, Glu 10.94, Asp 3.91, Lys 9.05 (recovery of Gly 84%). The synthetic peptide exhibited a single peak on HPLC using an analytical Nucleosil 5C18 column (4 × 150 mm) at a retention time of 19.62 min, when eluted with a gradient of acetonitrile 20 to 40% in 0.1% TFA at a flow rate of 1 ml per min (Fig. 4).

Boc–D-Ala–Ser(Bzl)–Asp(OBzl)–Lys(Z)–Pro–Asp(OBzl)–Met(O)–Ala–Glu(OBzl)–Ile–Glu(OBzl)–Lys(Z)–Phe–Asp(OBzl)–Lys(Z)–Ser(Bzl)–Lys(Z)–Leu–Lys(Z)–Lys(Z)–Thr(Bzl)–Glu(OBzl)–Thr(Bzl)–Gln–Glu(OBzl)–Lys(Z)–Asn–Pro–Leu–Pro–Ser(Bzl)–Lys(Z)–Glu(OBzl)–Thr(Bzl)–Ile–Glu(OBzl)–Gln–Glu(OBzl)–Lys(Z)–Gln–Ala–Gly–Glu(OBzl)–Ser(Bzl)–OBzl [III] This compound was prepared from the protected tritetracontapeptide ester corresponding to the protected deacetyl-thymosin β_4[6] (82 mg) and Boc–D-Ala–OSu (5.7 mg) essentially as described for the preparation of I: Yield 55.2 mg (67%), mp 181—195 °C (dec.), $[\alpha]_D^{21}$ −17.3° (c=0.5, DMSO), Rf^1 0.84, single ninhydrin-positive spot. *Anal.* Calcd for $C_{423}H_{529}N_{57}O_{99}S \cdot 13H_2O$: C, 61.49; H, 6.77; N, 9.66. Found: C, 61.71; H, 7.02; N, 9.83. Amino acid ratios in a 6 N HCl hydrolysate: Gly 1.00, Leu 2.01, Ile 2.09, Met(O) + Met 0.89, Ala 3.05, Pro 2.90, Phe 0.95, Ser 3.88, Thr 2.89, Glu 11.01, Asp 3.91, Lys 8.95 (recovery of Gly 84%).

H–D-Ala–Ser–Asp–Lys–Pro–Asp–Met–Ala–Glu–Ile–Glu–Lys–Phe–Asp–Lys–Ser–Lys–Leu–Lys–Lys–Thr–Glu–Thr–Gln–Glu–Lys–Asn–Pro–Leu–Pro–Ser–Lys–Glu–Thr–Ile–Glu–Gln–Glu–Lys–Gln–Ala–Gly–Glu–Ser–OH (Corresponding to D-Ala-thymosin β_4) [IV] The protected tetratetracontapeptide ester [III] (45 mg) was treated with 1 M TFMSA–thioanisole in TFA (2 ml) in the presence of Me_2Se (60 μl) as described above and the resulting powder was dissolved in 1 N AcOH (5 ml). The solution, after being stirred with Amberlite IRA-400 (acetate form, approximately 1 g) for 30 min, was filtered. The pH of the filtrate was adjusted to 8.0 with 1 N NH_4OH and after 30 min to pH 6.0 with 1 N AcOH and lyophilized. The product was purified by gel-filtration on a Sephadex G-25 column (3.2 × 90 cm) using 2% AcOH as an eluant. The fractions (5 ml each) corresponding to the front main peak (tube Nos. 56—65; determined by UV absorption measurement at 260 nm) were combined. The solvent was removed by lyophilization to give a fluffy powder. The product was dissolved in H_2O (3 ml) and the solution was applied to a column of DEAE-cellulose (Brown, 2.3 × 11.6 cm), which was eluted with a linear gradient of 300 ml each of H_2O–0.08 M NH_4HCO_3 buffer at pH 7.6. Individual fractions (5 ml each) were collected and the absorbancy at 260 nm was determined. Main peak fractions of the gradient eluates (tube Nos. 56—66) were combined and the solvent was evaporated *in vacuo*. Analysis by TLC revealed the presence of two ninhydrin-positive spots with Rf^2 0.03 (main) and 0.29 (minor). The crude product was dissolved in a small amount of water and subjected to preparative TLC (cellulose plate, 20 × 40 cm) using BuOH–AcOH–H_2O (4 : 1 : 5, upper layer) as a developing solvent. The zone corresponding to Rf^2 0.03 was separated and extracted with 2% AcOH. The extracts were concentrated to a small volume, applied to a Sephadex G-25 column (3.2 × 90 cm) and eluted with 2% AcOH as described above and the solvent was removed by lyophilization: Yield 5.4 mg (20%), $[\alpha]_D^{21}$ −60.7° (c=0.3, 2% AcOH), Rf^2 0.03, Rf^3 0.19, single ninhydrin- and chlorine-tolidine-positive spot. The synthetic peptide exhibited a single spot on paper electrophoresis: Toyo Roshi No. 51 (2 × 40 cm), pyridinium-acetate buffer at pH 7.3, mobility 1.7 cm from the origin toward the anode after runing at 2 mA, 600 V for 80 min. Amino acid ratios in a 6 N HCl hydrolysate: Gly 1.00, Leu 1.96, Ile 2.09, Met 0.87, Ala 3.02, Pro 2.93, Phe 0.98, Ser 3.88, Thr 2.94, Glu 10.96, Asp 3.95, Lys 9.03 (recovery of Gly 85%). The synthetic peptide exhibited a single peak on HPLC using an analytical Nucleosil 5C18 column (4 × 150 mm) at a retention time of 19.63 min, when eluted with a gradient of acetonitrile 20 to 40% in 0.1% TFA at a flow rate of 1 ml per min (Fig. 4).

Stability Studies of the Two Synthetic Peptides 1. Treatment with Human Serum The synthetic peptides were treated with human serum to evaluate the preservation of their immunological activity. Blood was collected from normal healthy volunteers in serum separation tubes (Beckton-Dickson SST vacutainer brand tubes No. 6572) and allowed to clot at room temperature. The tubes were then centrifuged at 2000 × *g* for 15 min and the serum immediately transferred to sterile conical centrifuge tubes (Corning No. 25310) and placed on ice. Peptide solutions (1.0 mg/ml) in 0.01 M sodium phosphate buffer pH 7.4 were prepared. To 900 μl of serum in 1.5 ml polypropylene centrifuge tubes equili-

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brated at 37 °C was added 50 μg of peptide solution. The mixture was incubated at 37 °C for 30 min and then the pH of the solution was adjusted to acidic area with dil. AcOH and lyophilized.

2. Fluorometric 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.[17] Isolated lymphocytes were adjusted to 1.0×10^6/ml with PBS. The lymphocytes were cultured in 0.5 ml of PRMI 1640 (Gibco) with FCS (Dainippon Pharmaceutical Co.) in microplates. Cultures of each combination were incubated at 37 °C in the presence of the peptide in a humidified atmosphere of 5% CO_2 in air for 12 h and PHA (0.125%, 0.5 ml) was added to each well. Incubation was continued under the same conditions for 60 h. 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 was added to the above solution and the mixture was stirred for 15 min at room temperature. The fluorescence excitation spectrum was measured according to Itoh and Kawai.[15]

References and Notes

1) Abbreviations used: TdT, terminal deoxynucleotidyltransferase; TFA, trifluoroacetic acid; DMF, dimethylformamide; DMSO, dimethylsulfoxide; HMPA, hexamethylphosphamide; Boc, *tert*-butoxycarbonyl; Z, benzyloxycarbonyl; OBzl, benzyl ester; Bzl, benzyl; OSu, *N*-hydroxysuccinimide ester; NMM, *N*-methylmorpholine; AcOH, acetic acid; MeOH, methanol; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; DEAE, diethylaminoethyl; PBS, phosphate-buffered saline; RPMI, Rosewell Park Memorial Institute; SDS, sodium dodecyl sulfate; UV, ultraviolet; FCS, fetal calf serum; FTMSA, trifluoromethenesulfonic acid; Me_2Se, dimethyl selenium; PHA, phytohemagglutinin.
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