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Solid-Phase Synthesis of Thymosin β_4: Chemical and Biological Characterization of the Synthetic Peptide†

Teresa L. K. Low,* Su-Sun Wang,‡ and Allan L. Goldstein

ABSTRACT: The chemical synthesis of thymosin β_4 using a solid-phase procedure has been accomplished. The synthetic product was found to be homogeneous on paper electrophoresis at pH 6.5, high-performance liquid chromatography on a reversed-phase column, and isoelectric focusing using polyacrylamide gels. The synthetic material was also shown to be identical with the natural thymosin β_4 by tryptic peptide mapping, amino acid compositional analyses, and polyacrylamide gel isoelectric focusing. Biologically, synthetic thymosin β_4 was found to be as active as the natural compound in a terminal deoxynucleotidyltransferase induction assay and in a macrophage migration inhibition assay. The proposed structure of the peptide hormone was thus confirmed by a chemical synthesis.

It is now well established that the endocrine thymus produces a family of hormonal-like peptides which controls development of the thymic-dependent lymphoid system and participates in the process of immune regulation (Low & Goldstein, 1978; Low et al., 1979a). Our previous studies demonstrated that a partially purified thymic preparation termed thymosin fraction 5 is effective in partially or fully reconstituting immune functions in thymic-deprived or immunodeprived animals (Thurman et al., 1975; Bach et al., 1971; Dauphinee et al., 1974), as well as in humans with immunodeficiency diseases (Wara et al., 1975; Wara & Ammann, 1976) and cancer (Cohen et al., 1979; Chretien et al., 1978). We have previously reported the isolation and biological properties of several components of thymosin fraction 5, including thymosin α_1 (Low & Goldstein, 1979; Low et al., 1979b), α_7 (Goldstein et al., 1981; Ahmed et al., 1979), β_3 (Pazmino et al., 1978; Hu et al., 1981), β_4 (Hu et al., 1981; Low et al., 1981; Low & Goldstein, 1982), and polypeptide β_1 (Low & Goldstein, 1979; Low et al., 1979b). The biological studies of these purified peptides indicate that they act on various pre-T and T-cell[1] subpopulations to maintain normal immunological reactivity.

The amino acid sequence has been determined for thymosin α_1 (Low & Goldstein, 1979), β_4 (Low & Goldstein, 1982), and polypeptide β_1 (Low & Goldstein, 1979). Thymosin α_1 is a polypeptide consisting of 28 amino acid residues. It is highly active in amplifying T-cell immunity (Low et al., 1979b; Ahmed et al., 1979) and is capable of modulating the expression of terminal deoxynucleotidyltransferase (TdT) (Hu et al., 1981). Thymosin β_4 consists of 43 amino acid residues with a molecular weight of 4963 and an isoelectric point of 5.1 (Low & Goldstein, 1982). The amino acid sequence of this peptide is shown in Figure 1. This peptide exhibits important activities in the regulation and differentiation of thymus-dependent lymphocytes (Pazmino et al., 1978; Low et al., 1981; Thurman et al., 1981). It induces expression of TdT activity in TdT-negative thymocytes both in vivo (Low et al., 1981) and in vitro (Pazmino et al., 1978). It also inhibits the migration of macrophages (Thurman et al., 1981). Most recently, it has been found that β_4 exerts biological effects on the hypothalamus and pituitary (Rebar et al., 1981; Hall et al., 1982).

† From the Department of Biochemistry, The George Washington University School of Medicine and Health Sciences, Washington, DC 20037 (T.L.K.L. and A.L.G.), and Peninsula Laboratories, Inc., Belmont, California 94002 (S.S.W.). *Received August 4, 1982.* This research is supported in part by grants from the National Institutes of Health (CA 24974 and AI 17710) and Hoffmann-La Roche Inc. This paper is the fourth article in a series entitled The Chemistry and Biology of Thymosin.
‡ Present address: Alpha 1 Biomedicals, Inc., San Carlos, CA 94070.

[1] Abbreviations: T-cell, thymus-dependent lymphocytes; TdT, terminal deoxynucleotidyltransferase; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; PAM-resin, hydroxymethylphenylacetamidomethyl resin; HF, hydrogen fluoride; HPLC, high-performance liquid chromatography; MMI, macrophage migration inhibition; LRF, luteinizing hormone-releasing factor; LH, luteinizing hormone; 2-ClZ, 2-chlorobenzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; DEAE, diethylaminoethyl; TCA, trichloroacetic acid; PPD, preservative-free tuberculin-purified protein derivative; PBL, peripheral blood lymphocyte, PEC, peritoneal exudate cell; MIF, macrophage migration inhibitory factor.

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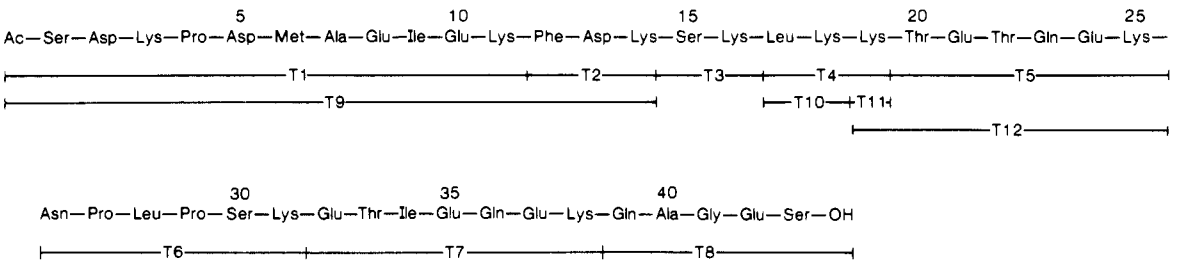

FIGURE 1: Complete amino acid sequence of thymosin β_4. Line segments (T1–T12) denote the peptides isolated after digestion of thymosin β_4 with trypsin.

In order to confirm the proposed primary structure of thymosin β_4 (Low & Goldstein, 1982) and to provide a better source of large amounts of pure material for studying the functional role of this peptide hormone in biological systems and for future clinical trials, it became necessary to chemically synthesize this peptide.

In this paper, an automated solid-phase synthesis (Merrifield, 1963; Barany & Merrifield, 1980) of thymosin β_4 according to the sequence reported previously (Low & Goldstein, 1982) is described. For the automated synthesis, Boc-Ser(Bzl)-OH was esterified onto hydroxymethylphenylacetamidomethyl (PAM) resin (Mitchell et al., 1976). The Boc-Ser(Bzl)-O-PAM-resin was then placed in the reaction vessel of a Beckman Model 990B automatic solid-phase synthesizer and the synthesis continued by sequentially incorporating amino acid residues into the growing peptide chains. The synthesized peptide was then cleaved from the resin with anhydrous fluoride (HF) (Sakakibara, 1971).

The crude peptide was purified by ion-exchange chromatography on DEAE-cellulose. The purified product was found to be homogeneous on paper electrophoresis at pH 6.5, high-performance liquid chromatography (HPLC) on a reversed-phase column, and isoelectric focusing on polyacrylamide gels. The synthetic thymosin β_4 was shown to be identical with the natural material by amino acid compositional analysis, tryptic peptide mapping, and migration pattern on isoelectric focusing polyacrylamide gels.

Furthermore, the synthetic thymosin β_4 exhibits similar biological activities as the natural compound in an in vivo assay measuring the induction of TdT (Hu et al., 1981; Low et al., 1981), in a macrophage migration inhibitory (MMI) assay (Thurman et al., 1981), and in vitro (Rebar et al., 1981) and in vivo (Hall et al., 1982) in an assay that measures the production and release of luteinizing-hormone releasing factor (LRF) and luteinizing hormone (LH). A preliminary report on the synthesis of thymosin β_4 has been presented (Wang et al., 1981).

Experimental Procedures

Materials

Chemicals. Analytical reagents obtained from either Aldrich or Eastman Kodak were used for the solid-phase synthesis. Chloromethyl resin was poly(styrene–1% divinylbenzene) beads, 200–400 mesh, from Bio-Rad. All protected amino acids were either prepared as described (Carpino, 1964) or obtained from Protein Research Foundation, Osaka, Japan. Polyacrylamide isoelectric focusing gels were purchased from LKB products (PAG_{plate}, pH 3.5–9.5). Trypsin treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TPCK) was purchased from Worthington. Fluorescamine (fluram) was obtained from Hoffmann-La Roche. Trifluoroacetic acid (TFA) was sequenal grade, obtained from Pierce. Acetonitrile was obtained from Burdick and Jackson Laboratories. Water for HPLC was obtained from Fisher (HPLC grade). Bio-Gel P-6 was procured from Bio-Rad and DEAE-cellulose was from Whatman.

Methods

Solid-Phase Synthesis of Thymosin β_4. The side chain functional groups of aspartic acid and glutamic acid were protected as benzyl esters, Boc-Glu(OBzl) and Boc-Asp(OBzl), threonine and serine as benzyl ethers, Boc-Thr(Bzl) and Boc-Ser(Bzl), and lysine as the N^{ϵ}-2-chlorobenzyloxycarbonyl derivative, Boc-Lys(2-ClZ).

The hydroxymethylphenylacetamidomethyl (PAM) resin (1% cross-linking) was prepared from chloromethyl resin [chloromethylated poly(styrene–1% divinyl benzene), 2.6% Cl], as described (Mitchell et al., 1978; Wang, 1975).

The hydroxymethyl resin was then esterified with Boc-Ser(Bzl)-OH by the dicyclohexylcarbodiimide (DCC) method (Sheehan & Hess, 1955) utilizing 4-(dimethylamino)pyridine (Steglich & Hofle, 1969) as catalysts.

Boc-Ser(Bzl)-PAM-resin (1.99 g, 0.11 mmol/g) was then placed in the reaction vessel of the Beckman 990B peptide synthesizer. The synthesis was performed under automatic mode operation by sequential incorporating one amino acid residue at a time with 10 equiv each of Boc amino acid and DCC according to the general principles of solid-phase peptide synthesis (Merrifield, 1963; Barany & Merrifield, 1980). The instrument was programmed to perform for each synthetic cycle the following 10 sequential steps: (1) three washings with CH_2Cl_2; (2) prewashing with 40% trifluoroacetic acid (TFA) in CH_2Cl_2; (3) deprotection for 28 min with 40% TFA; (4) three washings with CH_2Cl_2; (5) prewashing with 10% triethylamine in CH_2Cl_2; (6) neutralization 10 min with 10% triethylamine; (7) three washings with CH_2Cl_2; (8) addition of 2.25 mmol each of Boc-Glu(OBzl)-OH and DDC; (9) reaction for 120 min; (10) three washings each with CH_2Cl_2, 50% ethanol in CH_2Cl_2, and then CH_2Cl_2. The protected amino acid (2.25 mmol) was introduced at the eighth step in each cycle, according to the structure shown in Figure 1, beginning from the carboxyl-terminal end. In the synthetic cycles that involve glutamine or asparagine residue, 4.5 mmol each of 1-hydroxybenzotriazole was programmed to be added in step 8 also in order to minimize the side reactions (Barany & Merrifield, 1980). Double couplings were done when necessary as judged by the ninhydrin tests (Keiser et al., 1970). Acetyl group on the amino terminal was incorporated as acetic acid by using the same program in the last cycle. The Boc group was removed by 30-min treatment with 40% TFA containing 0.05% indole. The acetylated tritetracontapeptide resin was then cleaved with anhydrous HF (40 mL) at 0 °C for 45 min in the presence of anisole (5 mL) and dimethyl sulfide (1 mL). Evaporation of the acid and extraction of the residue with 5% acetic acid (100 mL) gave a solution which on lyophilization yielded the crude peptide.

Purification of Synthetic Thymosin β_4. The crude peptide was first desalted on a Bio-Gel P-6 column. It was then

chromatographed on a DEAE-cellulose column (Whatman DE-52, 2.5 × 50 cm), eluted with a linear gradient of 1 L each of 0.02–0.075 M ammonium bicarbonate at pH 7.8. There were several minor components in addition to a major peak which was rechromatographed on the same DEAE-cellulose column with a shallower gradient (0.02–0.025 M). The major peak was desalted and lyophilized to give 40 mg of the purified product.

Chemical Characterization of Synthetic Thymosin β_4. (I) Amino Acid Analysis. As described previously (Low & Goldstein, 1982), samples were hydrolyzed with twice distilled 6 N HCl at 110 °C for 24 h in sealed evacuated Pyrex glass tubes. Analyses were performed with a Beckman-Spinco amino acid analyzer, Model 119 CL, by employing single-column methodology on Beckman W-3 resin.

(II) Enzymatic Digestion. Trypsin digestion was performed in 1% ammonium bicarbonate (pH 8.3) at 37 °C. Trypsin was added to the protein solution to a final ratio of enzyme to substrate of 1:50 (w/w). The enzymatic digest was lyophilized immediately after 2.5 h.

(III) Peptide Mapping. Tryptic digests were separated by paper chromatography and electrophoresis as described previously (Low & Golstein, 1979). Chromatography was carried out first with the solvent system 1-butanol–glacial acetic acid–water (4:1:5, v/v). This was followed by high-voltage electrophoresis at pH 1.9 (glacial acetic acid–formic acid–H_2O, 8:2:90, v/v) for 30–40 min at 60 V/cm using a Savant apparatus. Peptides were detected by staining with cadmium–ninhydrin reagent (Dreyer & Bynum, 1967). For analysis of the amino acid composition of the peptides, the unstained chromatogram was stained with fluorescamine (Mendez & Lai, 1975). The fluorescent spots were cut out, eluted with water, hydrolyzed, and analyzed.

(IV) Isoelectric Focusing in Polyacrylamide Gels. As described (Low et al., 1979b), samples were dissolved at a concentration of 10 mg/mL in 1% (w/v) ampholine, 3.2 M urea, and 11.8% (w/v) sucrose. Twenty microliters of each sample was loaded onto Whatman No. 3 filter papers (5 mm × 10 mm) which were placed on the gel at appropriate positions. The electrolyte solutions used were 1 M NaOH for the cathode and 1 M H_3PO_4 for the anode. Isoelectric focusing was carried out for 90 min on an LKB multiphor unit with cooling to 4 °C. Constant power of 25 W was supplied by an LKB Model 2103 power supply set to a maximum current of 90 mA and a maximum potential of 1400 V. At the end of the run, 1-cm strips from the two extreme edges of the slab were sectioned into 5-mm increments, along the direction of focusing. The sections were placed in individual tubes and dialyzed against 0.5 mL of water overnight at room temperature. The pH of the resulting solutions was determined at 22 °C by using a combination microelectrode attached to a Corning 125 pH meter. The gels were then fixed in 20% trichloroacetic acid (TCA) for 1 h. The protein bands appearing on gels during fixing were photographed. The fixing solution was removed, the gels were washed for 10 min in a destaining solution containing 25% ethanol and 8% glacial acetic acid. The slab gels were stained with 0.1% Coomassie Brilliant Blue G 250 (Sigma) in destaining solution for 30 min. The gels were washed with destaining solution several times. The stained gels were photographed again and preserved by drying onto cellophane sheets.

(V) High-Performance Liquid Chromatography. Separation of peptides was performed on a Hewlett-Packard 1084B HPLC apparatus with a μBondapak C_{18} column (10 μm, 0.39 × 30 cm, Waters Associates) at 35 °C. The solvents used were 0.05% trifluoroacetic acid (TFA) (pH 2.3) in reservoir A and 0.05% TFA in acetonitrile in reservoir B. Solvents were filtered before use by using a Millipore filter apparatus (Waters Associates). Solvent A was prepared by adding 5 mL of a 10% TFA stock solution to 1 L of HPLC water. The solution was filtered through a Millipore Type HA membrane (0.45 μm, Waters Associates). Solvent B was made by adding 5 mL of filtered 10% TFA stock solution to 1 L of filtered acetonitrile (filtered through a Millipore type FH membrane). Detection of the peptides was accomplished with a variable-wavelength detector (Hewlett-Packard) set at 210 nm. Flow rate was set at 1.5 mL/min with a chart speed of 0.3 cm/min. Peptides were eluted from the column with 10% B for 10 min, followed by a linear gradient of 10–20% B in 10 min and a second gradient of 20–45% B in 45 min.

Biological Assay Systems. (I) In Vivo TdT Assay. The assay was performed as described (Hu et al., 1981). Groups of 6-week-old male C57Bl/6J mice with six to eight animals in each group were injected intraperitoneally with 1.25 mg of hydrocortisone acetate/animal. Twenty-four hours later, they received daily injections of thymosin fraction 5, spleen fraction 5, natural β_4, synthetic β_4, or saline for 11 days. Spleen fraction 5 was used as a control preparation in our assay systems and was prepared from calf spleen by using identical procedures as for the preparation of thymosin fraction 5. Mice were sacrificed 24 h after the last injection. Thymus glands were removed and a single cell suspension was prepared for each group. Cells (no less than 5×10^7) were resuspended in CAK buffer (20 mM potassium cacodylate–0.5 M KCl–1 mM 2-mercaptoethanol, pH 7.5) at a final concentration of 10^8 cells/mL. Cells were then pulsed disrupted in an ice bath by a sonicator (Heat System, Ultrasonics W-225 R, Plainview, NJ). The cell homogenate was ultracentrifuged (Beckman L3-50 Ultracentrifuge) at 100000*g* for 1 h. The supernatnat was used as crude TdT extract. The TdT activity in the crude extract was determined as reported by Barton et al. (1976). One unit of enzyme activity was defined as the amount catalyzing the incorporation of 1 nmol of dGTP into trichloroacetic acid insoluble material per h. The specific activity was calculated on the basis of the total enzyme activity recovered per 10^8 nucleated viable cells determined by trypan blue exclusion.

(II) In Vitro Macrophage Migration Inhibition (MMI) Assay. Both specific and nonspecific MMI assays were performed as described previously (Thurman et al., 1981).

(A) Preparation of Cells. Hartley guinea pigs were immunized with preservative-free tuberculin-purified protein derivatives (PPD-CT68, Connaught Laboratories, Toronto, Canada). Ten days later, the animals were thymectomized. Animals were bled by cardiac puncture 48 or 72 h postsurgery, and peripheral blood lymphocytes (PBL) were obtained by centrifuging the heparinized blood through a Ficoll gradient (Lymphoprep, Nyegaard and Co.). The cells recovered from the interface were washed and resuspended in HRPMI medium containing 100 μg/mL streptomycin and 100 units/mL penicillin and adjusted to 5×10^6 cells/mL. Frozen PBL, maintained at −70 °C, were thawed, washed, and utilized as described below. Peritoneal exudate cells (PEC) were prepared in Hartley strain guinea pigs by injecting mineral oil 3–5 days prior to the assay. These cells were washed 3 times to remove residual oil and adjusted to 45×10^6 cells/mL. The PBL were mixed with the PEC at a 1:9 ratio.

(B) Specific MMI Assay. The PBL–PEC cell mixture was centrifuged at 200*g* for 10 min. Sea plaque agarose (Microbiological Associates, Bethesda, MD) in HRPMI was added

Table I: Amino Acid Composition of Synthetic and Natural Thymosin β_4

amino acid	synthetic thymosin β_4[a]	natural thymosin β_4[b]	from sequence result[c]
aspartic acid	4.00	4.23	4
threonine	2.82	2.62	3
serine	3.61	3.73	4
glutamic acid	11.10	11.58	11
proline	3.00	3.85	3
glycine	0.98	1.35	1
alanine	1.96	2.30	2
valine	0.00	0.37	0
methionine	0.92	0.52	1
isoleucine	1.62[d]	1.85	2
leucine	1.95	2.05	2
phenylalanine	0.97	0.85	1
lysine	8.97	8.71	9

[a] Hydrolysis was carried out in 6 N HCl in an evacuated sealed tube at 110 °C for 24 h. [b] From Low & Goldstein (1982). [c] Number of residues obtained from the sequence result (Low & Goldstein, 1982). [d] Hydrolysis for 96 h gave 1.82 for isoleucine.

to the cell button. Cell agarose droplets were placed in the center of wells of a microtiter plate. They were allowed to solidify at 20 °C. HRPMI was then added (containing either no additives, PPD, thymosin, or thymosin and PPD) to the microtiter plates. Following a 24-h incubation at 37 °C in a humid 5% CO_2 in air incubator, the areas of migration were measured by projecting the image of the droplet and cells on a grid with a Bausch & Lomb microprojector. The percent specific inhibition was calculated as

$$\mathrm{PSI} = 100 - \left[\frac{\text{area(thymosin + PPD)}}{\text{area(thymosin)}}\right]\left[\frac{\text{area(HRPMI)}}{\text{area(PPD)}}\right] \times 100$$

If the area of the thymosin and PPD replicates was not statistically less ($P < 0.05$ by the Student's t test) than the area with PPD alone, the inhibition was considered insignificant. Percent specific inhibition of less than 20% was also considered insignificant.

(*C*) *Nonspecific MMI Assay.* The nonspecific MMI assay did not involve antigen and measured the direct effect thymosin had on macrophage inhibition. The percent nonspecific inhibition (PNSI) was calculated as

$$\mathrm{PNSI} = 100 - \frac{\text{area(thymosin)}}{\text{area(HRPMI)}} \times 100$$

If the means of the replicates for various polypeptides were not significantly different from the mean of the HRPMI control by the Student's t test ($P < 0.05$), the inhibition was considered insignificant. Percent nonspecific inhibition of migration of less than 20% was also considered insignificant.

Results

Synthesis and Purification of Thymosin β_4. On completion of the synthesis, 3.28 g of protected peptide resin was obtained. It was then treated with HF to give 0.78 g of crude synthetic thymosin β_4. Purification of the crude peptide on DE-52 gave a major fraction which was lyophilized to yield 120 mg of partially purified material. The sample was rechromatographed on the same column with a shallower gradient which gave a broad peak. The front half of the peak was collected and lyophilized to give 38 mg of homogeneous product. The second half of the peak gave 45 mg of material still slightly contaminated with a small amount of impurity.

Amino Acid Analysis of Synthetic Thymosin β_4. Amino acid analysis of the acid hydrolysate (6 N HCl, 110 °C, 24 h) of the purified synthetic thymosin β_4 is shown in Table I. For comparison, the amino acid analysis of natural thymosin β_4 (Low & Goldstein, 1982) is also listed. Their amino acid compositions appear to be identical.

Tryptic Peptide Mapping. A tryptic peptide map of synthetic thymosin β_4 is shown in Figure 2a. The chromatogram was stained with cadmium–ninhydrin reagent. The peptides were identified by amino acid analysis on the fluorescamine-stained peptide spots. The analysis results are listed in Table II. Figure 2b shows the tryptic peptide map of natural thymosin β_4. The amino acid analyses of these tryptic peptides have been presented previously (Low & Goldstein, 1982). The location of these tryptic peptides in the sequence of β_4 is indicated in Figure 1. From the results obtained, the synthetic material contains identical tryptic peptides as the natural thymosin β_4.

Isoelectric Focusing on Polyacrylamide Gels. As shown in Figure 3, on an LKB PAG_{plate} at pH 3.5–9.5, the synthetic thymosin β_4 moves to the similar position as the natural material with p*I* of 5.1. These strong protein bands were formed during the fixing step by using 20% trichloroacetic acid. It was found that these bands disappeared during subsequent staining and destaining procedures. Therefore, it is important to photograph the gels right after the fixing step for records before staining the gels.

High-Performance Liquid Chromatography. The elution profile of the purified synthetic β_4 on HPLC is shown in Figure 4a. Ten micrograms of synthetic β_4 in 20 μL of 0.05% TFA was loaded on the column. This peptide was eluted at 28.6 min as a single peak with a solvent system containing 0.05% TFA and acetonitrile. The gradient used is described under

Table II: Amino Acid Composition[a] of Tryptic Peptides of Synthetic Thymosin β_4

amino acid	T1	T2	T3	T4	T5	T6	T7	T8	T10	T12
aspartic acid	2.05 (2)	1.17 (1)				1.24 (1)				
threonine					2.05 (2)		1.18 (1)			1.65 (2)
serine	0.80 (1)		0.92 (1)			0.96 (1)		0.71 (1)		
glutamic acid	2.04 (2)				3.12 (3)		4.15 (4)	2.01 (2)		3.21 (3)
proline	0.83 (1)					2.01 (2)				
glycine								1.27 (1)		
alanine	0.93 (1)							0.97 (1)		
methionine	0.67 (1)									
isoleucine	0.98 (1)						0.73 (1)			
leucine				0.95 (1)		0.94 (1)			0.88 (1)	
phenylalanine		0.79 (1)								
lysine	2.04 (2)	0.82 (1)	1.15 (1)	2.09 (2)	0.78 (1)	0.90 (1)	1.08 (1)		1.07 (1)	1.98 (2)
total	11	3	2	3	6	6	7	5	2	7

[a] Results from 6 N HCl hydrolysates at 110 °C for 24 h.

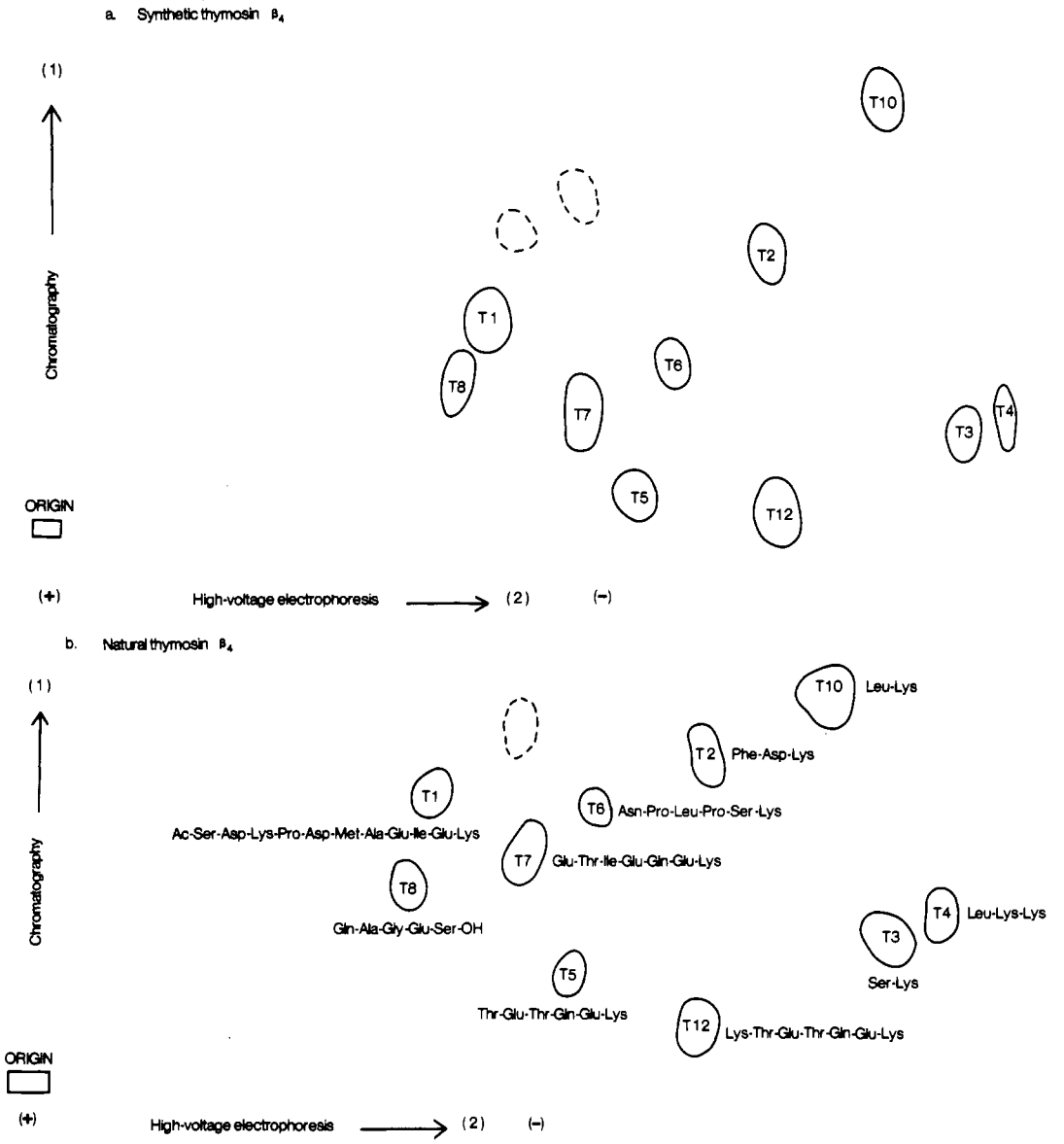

FIGURE 2: Tryptic peptide maps of synthetic and natural thymosin β_4. Chromatography was carried out in the first dimension in 1-butanol–glacial acetic acid–water (4:1:5 v/v) and high-voltage paper electrophoresis at pH 1.9 was carried out in the second dimension. (a) Tryptic map of synthetic thymosin β_4. The amino acid composition of the tryptic peptides is listed in Table II. (b) Tryptic map of natural thymosin β_4. The amino acid composition and sequence of the peptides were from Low & Goldstein (1982). These peptides are indicated in the sequence of β_4 as shown in Figure 1. All tryptic peptides which were isolated previously during sequence studies (Low & Goldstein, 1982) were found on the map with the exception of T9 and T11. These two peptides were not consistently recovered on the map.

Methods and also indicated in the profile. As shown in Figure 4b, when 10 μg of natural β_4 in 20 μL of 0.05% TFA was injected onto the column, a major peak was eluted at 28.8 min. When they were mixed and injected together onto the HPLC, they coeluted at 29.0 min.

Biological Activities. (I) Terminal Deoxynucleotidyltransferase (TdT) Assay. The enzyme TdT has been demonstrated to uniquely associate with T cells during their early stages of differentiation (Chang, 1971; Coleman et al., 1974; Greogoire et al., 1977; Barton et al., 1976). Thymosin fraction 5 and natural thymosin β_4 are potent inducers of TdT activity in vivo (Hu et al., 1981) and in vitro (Pazmino et al., 1978) in TdT-negative thymocytes. As shown in Table III, the synthetic thymosin β_4 is as active as the natural β_4 in inducing TdT activity in vivo in thymocytes of immunosuppressed mice. Thymosin β_4 is about 100–1000 times more active than its parent compound, thymosin fraction 5, in this assay system.

(II) In Vitro MMI Assay. As shown in Table IV, thymosin fraction 5 and thymosin α_1 were active in the specific MMI assay, indicating their ability to induce development of immature lymphocytes, as measured by their ability to respond to an antigenic challenge by releasing the lymphokine MIF

Table III: In Vivo Induction of TdT by Thymosin in Thymocytes from Hydrocortisone Acetate Treated C57Bl/6J Mice

preparation[a]	dose (μg)	TdT sp act.[b]	% increase
saline		38.3	
thymosin fraction 5	100	51.0	33.2
spleen fraction 5	100	42.2	10.1
natural thymosin β_4	1	52.5	37.1
	0.1	46.7	21.9
synthetic thymosin β_4	1	54.2	41.5
	0.1	47.5	24.0

[a] Mice were treated with 1.25 mg of hydrocortisone acetate, followed by daily injection of the indicated agents for 11 days. Amount used per injection is given in the dose column. [b] Specific activity is expressed as nanomoles of dGTP incorporation per 10^8 cells per hour.

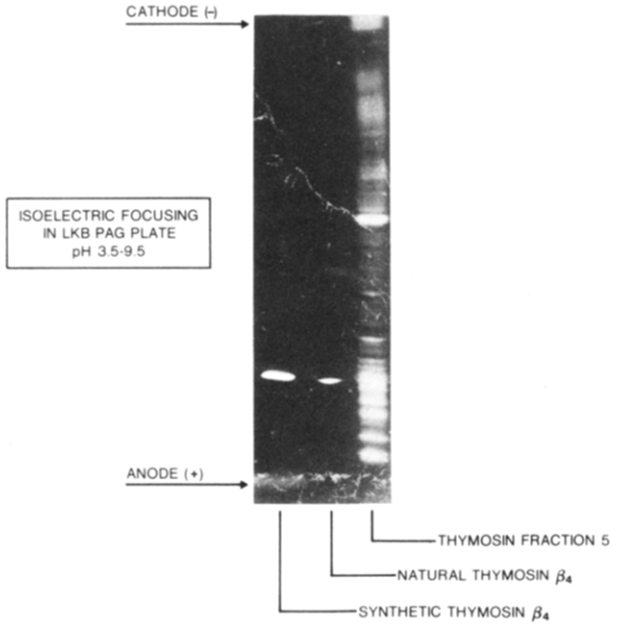

FIGURE 3: Isoelectric focusing gel of thymosin fraction 5, natural thymosin β_4, and synthetic thymosin β_4 on LKB PAG plate at pH 3.5–9.5. The amount loaded on the gel was 200 μg each of thymosin fraction 5 and synthetic thymosin β_4 and 50 μg of natural thymosin β_4. Isoelectric focusing was carried out for 90 min on an LKB multiphor unit with cooling to 4 °C. The gel was fixed in 20% trichloroacetic acid for 1 h to reveal the protein bands as shown in this picture.

(macrophage migration inhibitory factor). On the other hand, thymosin β_4, though not active in the specific MMI assay, was able to inhibit the migration of macrophages in a nonspecific manner without antigen. Spleen fraction 5 was not active in either the specific or the nonspecific MMI assay. Thymosin fraction 5, which is active in the specific MMI assay, is not active in the nonspecific assay. As indicated in Table IV, the synthetic compound appears to have better activity in the nonspecific MMI assay than the natural β_4 on a molar basis. The higher potency of synthetic β_4 might reflect the differences in purity of the two preparations.

Discussion

Thymosin β_4, synthesized by a solid-phase method using PAM-resin, was shown to be chemically and biologically indistinguishable from natural thymosin β_4. The yield of the final purified product was 5.2%. In addition, 5.8% of synthetic material was also obtained in slightly contaminated form. Amino acid analysis of the protected peptide resin indicated that over 95% of the peptide chains remained on the resin at the end of the synthesis. The high recovery yield can be accounted for by the use of a PAM-resin which enhanced the stability of the anchoring bond of the support to TFA, thus minimizing chain loss in the course of the synthesis.

The activity of thymosin β_4 to induce TdT-negative stem cells to express TdT in vivo and in vitro suggests that this peptide is acting on a less mature T cell at an early stage in the T-cell maturation process (Pazmino et al., 1978). Furthermore, thymosin β_4 was active in inducing TdT in vivo in immunosuppressed mice (Hu et al., 1981). This in vivo TdT model was used to evaluate the biological activity of synthetic β_4 in comparison with that of the natural material in the study described in this paper.

In this system, hydrocortisone acetate was used to destroy the cortisone-sensitive TdT-positive thymocytes. The involution of TdT activity in the thymus started within 24 h after injection (Hu et al., 1981). TdT activity was undetectable from day 2 to day 4. A spontaneous recovery of TdT occurred around day 6 and reached the highest level at day 12. When hydrocortisone treatment was followed by daily injections of saline, the recovery of TdT activity in the thymus was highly depressed. This might be caused by the continuous release of steroid in the animals induced by the injections. However, daily injection of thymosin fraction 5 and thymosin β_4 significantly increases TdT activity in hydrocortisone-treated thymus and overcomes the depression of TdT recovery as seen in the saline-treated group (Table III). The results indicate that thymosin fraction 5 and β_4 are acting on TdT-negative bone marrow stem cells to accelerate the repopulation of

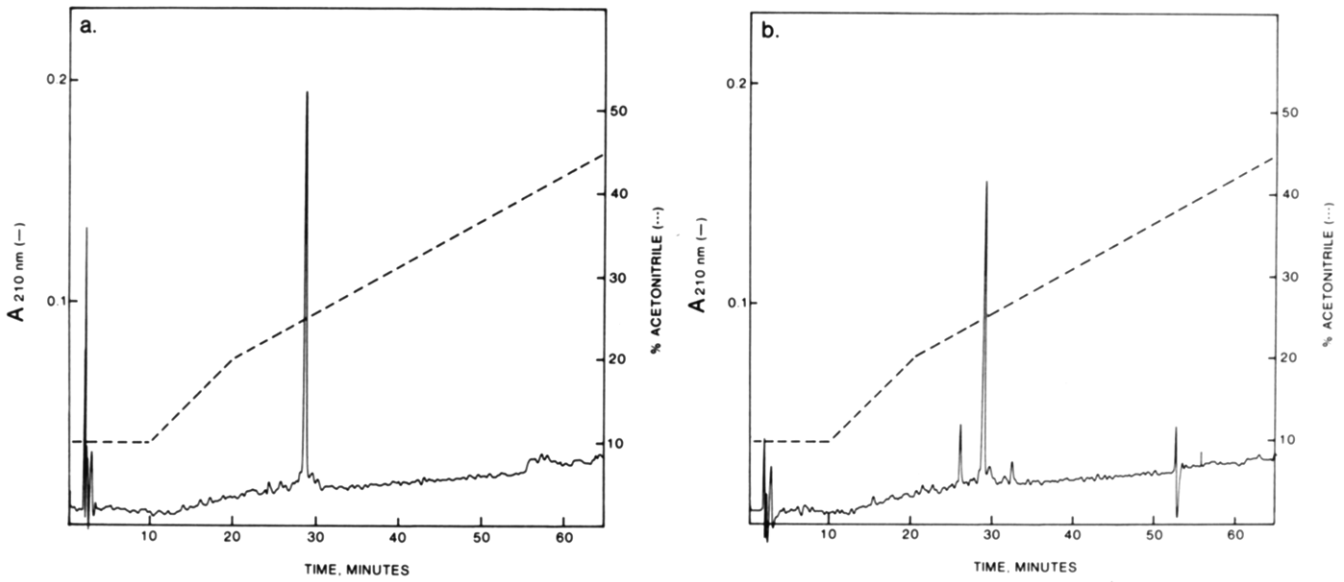

FIGURE 4: High-performance liquid chromatographic analysis of synthetic and natural thymosin β_4 on a 0.39 × 30 cm μBondapak C_{18} column (10 μm, Waters Associates) at 35 °C. The solvents used were 0.05% trifluoroacetic acid (TFA) (pH 2.3) in reservoir A and 0.05% TFA in acetonitrile in reservoir B. The peptides were eluted with 10% B for 10 min followed by a linear gradient of 10–20% B in 10 min and a second gradient of 20–45% B in 45 min. The flow rate was set at 1.5 mL/min, with a chart speed of 0.3 cm/min. (a) Ten micrograms of synthetic thymosin β_4 in 20 μL of 0.05% TFA was injected onto the column. The peptide was eluted at 28.6 min as a single peak. (b) Natural thymosin β_4 (20 μg in 20 μL) was eluted at 28.8 min as a major peak. Minor contaminating peaks were found in the natural thymosin β_4 preparation.

Table IV: Effect of Thymosin Polypeptides in the Macrophage Migration Inhibition (MMI) Assay[a]

preparation	concentration		specific MMI response to PPD (%)	nonspecific migration inhibition (%)
	μg/mL	nmol/mL		
thymosin fraction 5	200		35	none[b]
	20		none	none
spleen fraction 5	200		none	none
synthetic thymosin α_1	1.5	5×10^{-1}	51	none
	1.5×10^{-1}	5×10^{-2}	38	none
	1.5×10^{-2}	5×10^{-3}	25	none
natural thymosin β_4	2.5	5×10^{-1}	none	35
	2.5×10^{-1}	5×10^{-2}	none	32
	2.5×10^{-2}	5×10^{-3}	none	23
	2.5×10^{-3}	5×10^{-4}	none	23
	2.5×10^{-4}	5×10^{-5}	none	20
	2.5×10^{-5}	5×10^{-6}	none	18
synthetic thymosin β_4	2.5	5×10^{-1}	none	45
	2.5×10^{-1}	5×10^{-2}	none	34
	2.5×10^{-2}	5×10^{-3}	none	32
	2.5×10^{-3}	5×10^{-4}	none	25
	2.5×10^{-4}	5×10^{-5}	none	33
	2.5×10^{-5}	5×10^{-6}	none	26

[a] See Thurman et al. (1981) for details of the assay. [b] Less than 15% inhibition.

TdT-positive thymocytes in the thymus. Table III also indicates that the synthetic β_4 was as active as the natural β_4 in inducing TdT in vivo in immunosuppressed mice.

As shown in Table IV, both natural β_4 and synthetic β_4 were found to be active in inhibiting the migration of macrophages independent of antigen. Previous studies (Thurman et al., 1981) indicated that β_4 was acting directly on macrophages to inhibit the migration and not on lymphocytes to release MIF. It was shown (Thurman et al., 1981) that β_4 inhibited migration of PEC without added PBL. Peritoneal exudate cells from T-cell deficient nude mice, as well as WEHI-3 cells (known to be a macrophage cell line), were also inhibited (Thurman et al., 1981).

Recently, synthetic thymosin β_4 has been found to stimulate the secretion of luteinizing hormone-releasing factor (LRF) from superfused medial basal hypothalami of female Sprague-Dawley rats (Rebar et al., 1981). In addition, luteinizing hormone was released from pituitary glands superfused in sequence with hypothalami (Rebar et al., 1981) and when injected directly into the ventricles of male mice (Hall et al., 1982). These reports suggest a potentially important role for thymic peptide(s) on reproductive function.

Since thymosin β_4 and most of the other thymosins isolated to date are present in relatively low concentrations in tissue or serum, isolating large quantities of natural β_4 for experimental studies and clinical trials is impractical. However, the complete elucidation of the structure of thymosin β_4 (Low & Goldstein, 1982), thymosin α_1 (Low & Goldstein, 1979), and other thymic factors (Bach et al., 1977; Schlesinger & Goldstein, 1975) and the successful chemical synthesis of these molecules (Wang et al., 1978; Birr & Stolenwerk, 1979; Folkers et al., 1980; Wang et al., 1980; Wong & Merrifield, 1980; Fujino et al., 1977; Bliznakov et al., 1978) render large-scale production necessary for complete elucidation of biological activities and clinical trials of purified thymic peptides feasible.

In the present study, we have used the synthetic thymosin β_4 to establish that the biological activities observed with the natural β_4 were not derived from some minor contaminant(s) that exist(s) in the natural product.

The site of synthesis of thymosin β_4 has been studied by several investigators. Using an indirect fluorescent antibody technique, Hirokawa et al. (1982) demonstrated that thymosin β_4 was detected specifically in epithelial cells covering the cortical surface whereas thymosin α_1 was detected specifically in thymic epithelial cells of the medulla. Most recently, Xu et al. (1982) reported that thymosin β_4 was synthesized by peritoneal macrophages and adherent spleen cells. Since macrophages are known to represent a heterogeneous population of cells, the study did not exclude the possibility that β_4 is synthesized by other cells and tissues. More studies are needed to define the site of synthesis of β_4. Nevertheless, it appears that β_4 may be synthesized by more than one cell type and is probably involved in more general biological functions.

Our ongoing studies on the chemistry and biology of thymosin have documented a family of polypeptide components in thymosin fraction 5, such as thymosin α_1 (Low & Goldstein, 1979), thymosin α_7 (Goldstein et al., 1981; Ahmed et al., 1979), and thymosin β_4 (Hu et al., 1981; Low et al., 1981), that appear to be acting on different subsets of T cells or T-cell precursors to influence their maturation and function (Goldstein et al., 1981). Many questions remain regarding the precise interactions and mode of action of these peptides in lymphoid and non-lymphoid systems. However, the fact that we have now successfully synthesized a new thymic peptide, thymosin β_4, which is chemically and biologically identical with the natural compound, would facilitate further experimental and clinical studies to document the role of this molecule in the immune and reproductive systems.

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Registry No. Thymosin β_4, 77591-33-4; deoxynucleotidyltransferase, 9012-90-2.

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