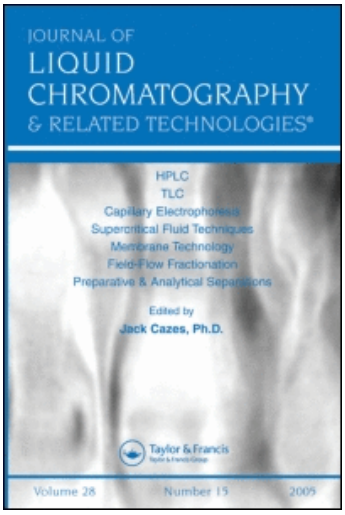

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Badamchian, Mahnaz , Strickler, M. Patricia , Stone, M. Jude and Goldstein, Allan L.(1990) 'Rapid Micro Isolation of Thymosin β4 from Human Thymus by Reversed-Phase High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 13: 17, 3439 — 3453
To link to this Article: DOI: 10.1080/01483919008049112
URL: http://dx.doi.org/10.1080/01483919008049112

JOURNAL OF LIQUID CHROMATOGRAPHY, 13(17), 3439-3453 (1990)

RAPID MICRO ISOLATION OF THYMOSIN $\beta 4$ FROM HUMAN THYMUS BY REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Thymosin β_4 ($T\beta_4$) is a peptide composed of 43 amino acid residues with a molecular weight of 4982 and a pI of 5.1. It was originally isolated from calf thymus [Low, et al. PNAS 78, (1981)]. We have developed a rapid, efficient and reproducible method for the purification of $T\beta_4$ from human thymus. A newly developed method was employed for the extraction of $T\beta_4$. This extraction is simple and minimizes the possibility of proteolytic modification. This purification is based on the use of high performance reversed phase (Delta Pak C_{18}) chromatographic columns. The HPLC retention time, RIA, IEF, and SDS-PAGE have shown that purified $T\beta_4$ from human thymus is homogeneous and is identical to synthetic $T\beta_4$.

INTRODUCTION

$T\beta_4$, a biologically active peptide was first isolated from a calf thymus preparation, thymosin fraction 5 (TF5) (1-2). $T\beta_4$ has been characterized, sequenced, and synthesized (2,4). This peptide is composed of 43 amino acids with an acetyl group at the N-terminal end. It has a molecular weight of 4982 and an isoelectric point of 5.1. $T\beta_4$ affects the early stages of T-cell differentiation (2), and may play a role in the immune mechanisms (5). $T\beta_4$ is a highly conservative polypeptide and is distributed in different tissues of vertebrates ranging from amphibia to mammals (6). This would suggest that $T\beta_4$ is probably involved in more general biological functions. Lack of signal peptides in rat and human $T\beta_4$ genes leads to speculation that the $T\beta_4$ molecule may not be a secretory peptide (7-9). However $T\beta_4$ may be a precursor molecule of a tetrapeptide that was recently extracted from fetal calf bone marrow. The tetrapeptide has the same sequence as the N-terminal sequence of $T\beta_4$. This tetrapeptide strongly inhibits entry of murine CFU-S into the cell cycle (10). In vivo the tetrapeptide increased the survival of mice given lethal doses of cytosine arabinonucleoside by keeping CFU-S out of the cycle (11,12). Comparison of tetrapeptide with $T\beta_4$, a potential precursor of tetrapeptide, indicated that $T\beta_4$ is more potent than the the tetrapeptide. $T\beta_4$ increased the survival of the arabinonucleoside treated mice by 20% compared to tetrapeptide at 0.1 μg dose/injection (Goldstein et al., unpublished results). These results support the hypothesis that the tetrapeptide is derived in vivo from proteolysis of $T\beta_4$.

$T\beta_4$ has also been isolated using a multi-step procedure, from fresh calf thymus (13). Previously, we developed and reported on a two step reversed-phase HPLC (RP-HPLC) purification of $T\beta_4$ from calf TF5 (14). In the present study we report a two step purification of $T\beta_4$ from fresh human thymus. The extraction conditions used in this procedure for the isolation of $T\beta_4$ from fresh thymus gland greatly reduces the possibility of proteolytic digestion of $T\beta_4$ and other thymic peptides.

MATERIALS AND METHODS

Human thymus (male-16 months) and synthetic $T\beta_4$ were provided as a gift from Dr. Marcelo B. Sztein and Alpha One Biomedicals (Washington, D.C. U.S.A) respectively. Delta-Pak C_{18} columns and chemicals for the Pico-Tag system were from Waters Chromatography Division of Millipore (Milford, MA, U.S.A.). Water for HPLC was purified with a Milli-Q water system (Millipore, Bedford, MA, U.S.A.). IEF gels (Pagplate) were purchased from Pharmacia/LKB Nuclear, Inc. (Gaithersburg, MD, U.S.A.). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) chemicals were of molecular-biology grade and were purchased from Sigma (St. Louis, MO, U.S.A.). The SDS low-molecular weight protein standards and silver stain kit were purchased from Bio-Rad Lab. (Richmond, CA, U.S.A.). All buffers and solvents were of HPLC grade (Fisher Scientific, Pittsburgh, PA, U.S.A.). All HPLC solutions were filtered through a 0.45-μm membrane (Millipore) prior to use.

Sample preparation for HPLC

Human thymus (male-16 months) was immediately frozen and stored at -90°C. 2.5 g of tissue was homogenized in 7.5 ml of ice-cold 0.5 N perchloric acid with a teflon pestle at 840 rpms with 100 up and down strokes. The homogenate was centrifuged at 1000 g for 10 minutes. The pH of the supernatant was adjusted to 7 with 1 N potassium hydroxide added over a period of 30 minutes. The solution was stirred for 60 minutes at 4°C and centrifuged at 20,000 g for 30 minutes. The supernatant was aliquoted and stored at -20°C.

The concentration of protein in the supernatant was estimated to be 0.8 mg/ml by the method of Lowry (15) using bovine serum albumin as the standard.

HPLC Methods

Preparative reversed-phase chromatography of 1.8 ml of human thymus extract (1.44 mg of protein, as determined by Lowry assay) was performed on a Model 600 HPLC system equipped with a Model 490 multi-wavelength detector (Waters), set a 214 nm, and a 7.8-mm x 30

cm Delta-Pak, 300 Å, 15-μm C_{18} column (Waters). Eluent A was 0.02 M ammonium acetate, (pH 6.8), and eluent B was acetonitrile. A 60 minute linear gradient from 0-80% B was run at a flow rate of 2 ml/min. One minute fractions were collected, and assayed for Tβ_4 activity by RIA.

Tβ_4 active fractions 20 and 21 (4 ml) from the preparative separation of human thymus extract were pooled and concentrated to 300 ul. Semi-preparative RP-HPLC of the pooled fractions was carried out on a 3.9-mm x 15 cm Delta Pak, 300-Å, 5-μm C_{18} column (Waters). Eluent A was 0.1% phosphoric acid in water and eluent B was acetonitrile containing 0.1% phosphoric acid. Purification of Tβ_4 (44 ug) was achieved with a 10 minute linear gradient from 0-14% B, followed by a 10 minute hold at 14% B and a 10 minute linear gradient to 18% B at a flow rate of 1 ml/min. One minute fractions were collected and assayed for Tβ_4 activity.

Analytical RP-HPLC of 100 ul aliquots of immunoreactive fractions 35 and 36 from the semi-preparative separation was performed using the conditions described for the semi-preparative separation. The remaining material (43 ug) was pooled subjected to amino acid composition analysis, SDS-PAGE and iso-electric focusing.

Data from all the HPLC procedures was collected using a Model 860 chromatography work station.

Electrophoresis

A. Analytical isoelectric focusing in thin layer polyacrylamide gels was performed on Pharmacia-LKB Pagplates. Pagplates provide a pH range of 3.5-9.5, a gel concentration of 5%, and a 3% degree of cross-linkage. The ampholine concentration is 2.4% (wt/vol). Natural and synthetic Tβ_4 samples (about 20-50 μg) were applied 2 cm from cathode strip. The electrolyte solutions used were 1 N NaOH for the cathode and 1 M H_3PO_4 for the anode. Isoelectric focusing was carried out for 90 minutes on a Pharmacia-LKB multiphor unit with cooling to 4°C. Constant power of 25 watts was supplied by a Pharmacia-LKB Model 2103 power supply set of a maximum current of 90 mA and a maximum potential of 1400 volts. At

the end of the run, gels were fixed in 20% trichloroacetic acid and 3.5% sulphosalicylic acid for one hour.

B. SDS-PAGE was performed using 12% polyacrylamide slab gels (16 cm x 18 cm x 1.5 mm), according to the method of Laemmli (16). Natural and synthetic $T\beta_4$ samples (10-20 μg) were incubated at 90°C for 5 min before gel electrophoresis. Proteins were visualized in the gels by silver staining with the Bio-rad silver-stain kit.

Amino Acid Analysis

Amino acid analysis was performed with Pico-Tag amino acid analysis system of Waters-Millipore. The method is based on the formation of a phenylthiocarbamyl (PTC) derivative of the amino acids from acid-hydrolyzed proteins. $T\beta_4$ samples (about 1-5 μg) were hydrolyzed in 200 μl of a constant boiling HCl atmosphere containing 1% (v/v) phenol, at 110°C for 48 h in the Pico-Tag work station. The hydrolysates were dried and the amino acids were derivatized with phenylisothiocyanate (PITC) for 20 minute at room temperature to yield the corresponding PTC derivative (17). These derivatives were analyzed with the Pico-Tag amino-acid analysis system, which had been previously calibrated with a standard mixture of amino acid.

Protein Determinations

Protein was estimated by the methods of Lowry et al. (15) with bovine serum albumin (BSA) as standard in the case of crude preparations and by amino acid analysis in the case of highly purified samples.

Radioimmunoassay(RIA)

The RIA for $T\beta_4$ was performed according to the method of Naylor et al. (18). Fractions collected from the HPLC separation were lyophilized and resuspended in 1 ml HPLC-grade water and aliquots of these fractions were used for the RIA. RIA buffer, 0.01 M sodium phosphate buffer (pH 7.4), containing 0.165 M sodium chloride, 0.05% sodium azide, 0.091 mM EDTA, and 0.1% BSA was added to each tube to

bring the volum to 400 μl. A 50 μl aliquot of stock antiserum and 50 μl of tracer labeled tyrosine (Tyr^1-C13-Tβ_4) were added to each tube. The tubes were mixed in an vortex mixer and incubated for 24 h at 4°C. Separation of free from bound tracer was carried out by the addition of 50 μl of goat anti-rabbit IgG in 0.5 M phosphate buffer solution. After mixing, the tubes were incubated overnight at 4°C. The immunoprecipitates were pelleted by centrifugation at 1500 g for 25 minute at 4°C. The supernatants were aspirated and discarded, and the radioactivity in the immunoprecipitates was measured in an automatic Beckman Gamma 4000 spectrometer (Columbia, MD, U.S.A.).

RESULTS AND DISCUSSION

We have developed a rapid, efficient, and reproducible HPLC method for the purification of Tα_1 and Tβ_4 from TF5 (14,19). The primary goal of our present work was to apply this HPLC method to the micro-purification of thymic peptides from fresh thymus. The preparation of TF5 from calf thymus allows for the proteolysis of thymic peptides, therefore, it is not possible to isolate peptides that may serve as precursors. It is also not possible to estimate with any certainty the quantity of any of the thymic peptides present in the fresh tissue. Micro-isolation of the thymic peptides from fresh thymus, that has been extracted to minimize proteolysis, would allow for the isolation of precursors, as well as, a means of quantifying the amount of peptide present in fresh tissue. The purification of Tβ_4 can be used as an example for the isolation of precursors and the quantitative isolation of other thymic peptides from fresh thymus in sufficient quantity for characterization.

To minimize the possibility of proteolytic modification, thymic peptides, thymus was immediately frozen and stored at -90°C, after its removal from human. Tβ_4 was extracted from a perchloric acid homogenate of frozen thymus, as described in Materials and Methods. The low pH of perchloric acid inhibits proteolysis. This protocol was carried out on three different human

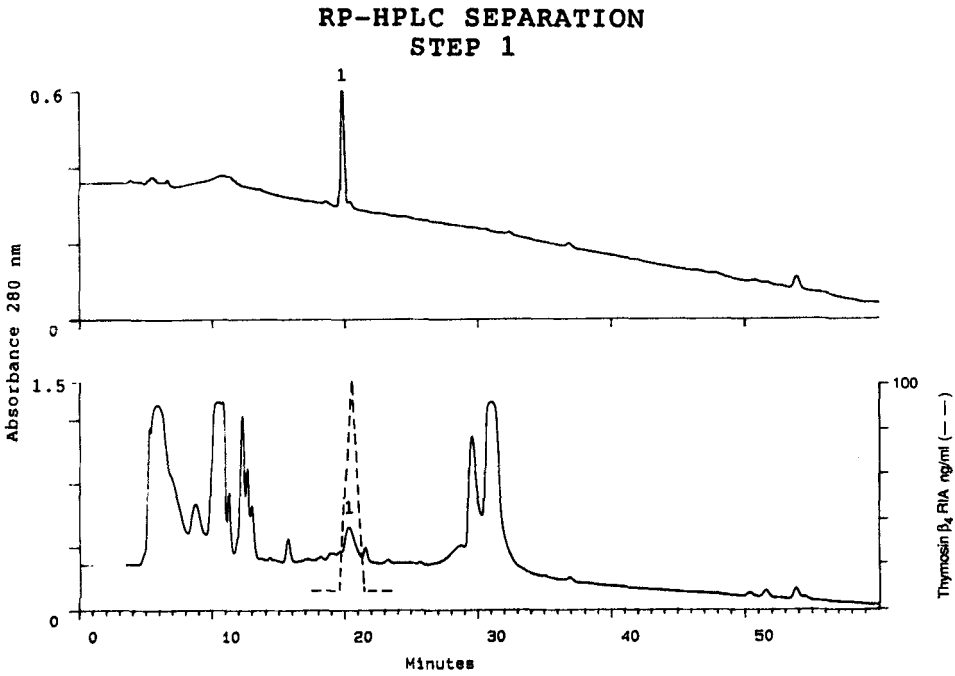

Figure 1. RP-HPLC of 20 ug of synthetic Tβ_4 (1), top panel, and 1.8 ml of human thymus extract (1.44 mg of protein as determined by a Lowry assay), bottom panel, on a 7.8-mm x 30 cm, Delta pak, 300 Å, 15-um C_{18} column. Eluent A was 0.02 M ammonium acetate (pH 6.8) and eluent B was acetonitrile. A 60 minute, linear gradient from 0-80% B was run at a flow rate of 2 ml/min. Detection, 280 nm. One minute fractions were collected and assayed for Tβ_4 using RIA. Results are overlaid on the chromatogram.

thymus glands. The recovery of Tβ_4, as determined by RIA and HPLC, was similar, suggesting that proteolysis was inhibited.

Preparative reversed-phase isolation of Tβ_4 from human thymus extract was carried out on a 7.8-mm x 30 cm Delta-pak, 300-Å, 15-μm C_{18} column. The RIA analysis of the HPLC fractions (Fig. 1, bottom panel) indicated that immunoreactive Tβ_4 elutes in fractions 20 and 21. Synthetic Tβ_4 was also chromatographed under the same condition (Fig. 1, top panel). Synthetic Tβ_4 had similar retention time to

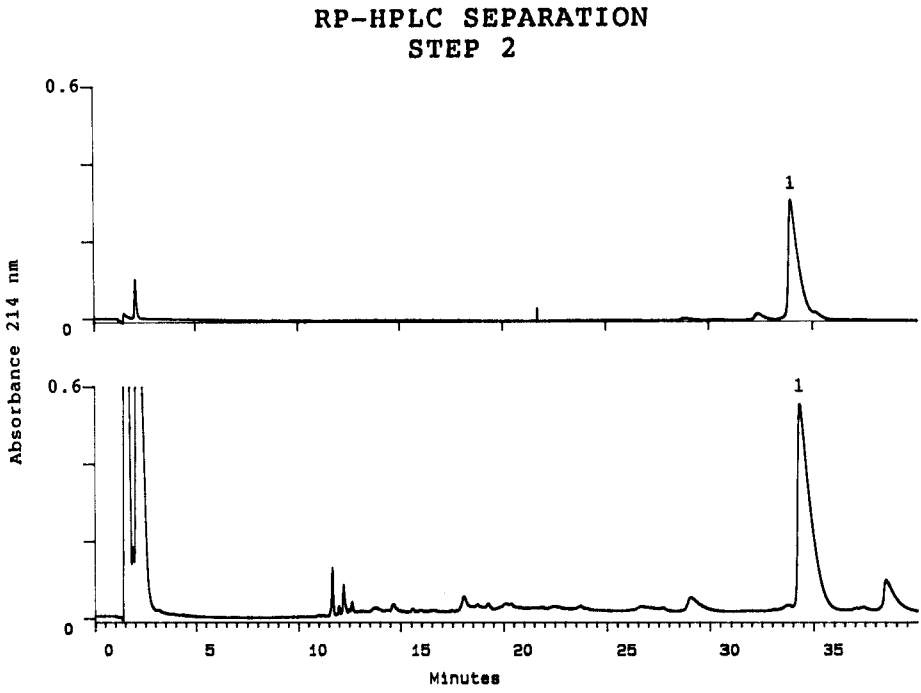

Figure 2. RP-HPLC separation of 20 ug of synthetic Tβ_4 (1), top panel and 44 ug of Tβ_4 from the pooled fractions 20 and 21 from the separation of human thymus extract, bottom panel, on a 3.9-mm x 15 cm, Delta Pak, 300 Å, 15-um, C_{18} column. Eluent A was 0.1% phosphoric acid in water and eluent B was 0.1% phosphoric acid in acetonitrile. Separation of Tβ_4 was achieved by a 10 minute, linear gradient to 14% B, followed by a 10 minute hold and a 10 minute, linear gradient to 18% B. The flow rate was 1 ml/min. Detection at 214 nm. One minute fractions were collected and assayed for Tβ_4 using RIA.

immunoreactive Tβ_4 and was eluted in Fraction 20. Tβ_4 active fractions 20 and 21 were pooled and rechromatographed on a 3.9-mm x 15 cm, Delta-pak, 300 Å, 5-μm C_{18} column (Fig. 2, bottom panel). Figure 2 (top panel) also shows the RP-HPLC separation of synthetic Tβ_4 under similar conditions. RP-HPLC analyses of immunoreactive Tβ_4 in fractions 35 and 36 from the semi-preparative separation, using

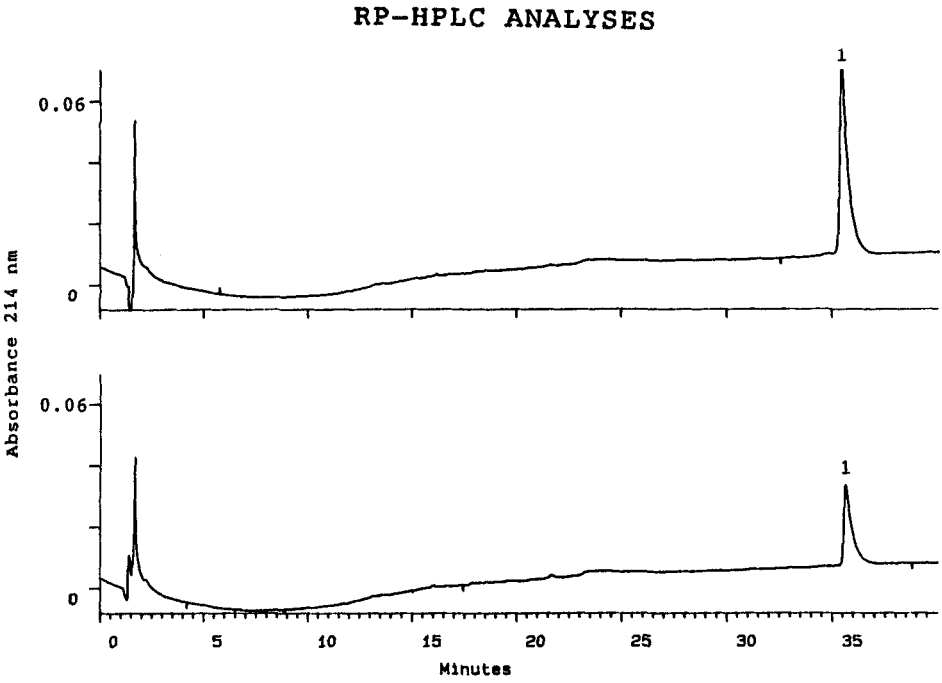

Figure 3. Analytical RP-HPLC of 100 ul aliquots of fractions 35 and 36 from the separation shown in Figure 2, bottom panel. 100 ul of water was added to each sample before analysis. The chromatographic conditions, described in Figure 2, were employed. $T\beta_4(1)$.

the same conditions as the semi-preparative separation, are shown in Fig 3. The HPLC isolated $T\beta_4$ was homogeneous and had a similar HPLC retention time as the synthetic $T\beta_4$. Fractions 35-36 from the semi-preparative separation were pooled and used as the final preparation of $T\beta_4$ for IEF, SDS-PAGE, and amino acid composition analysis.

SDS-PAGE analysis of the natural and synthetic $T\beta_4$, illustrated in Fig. 4 was carried out as described in Materials and Methods. When the protein bands on SDS gels were silver stained, a single

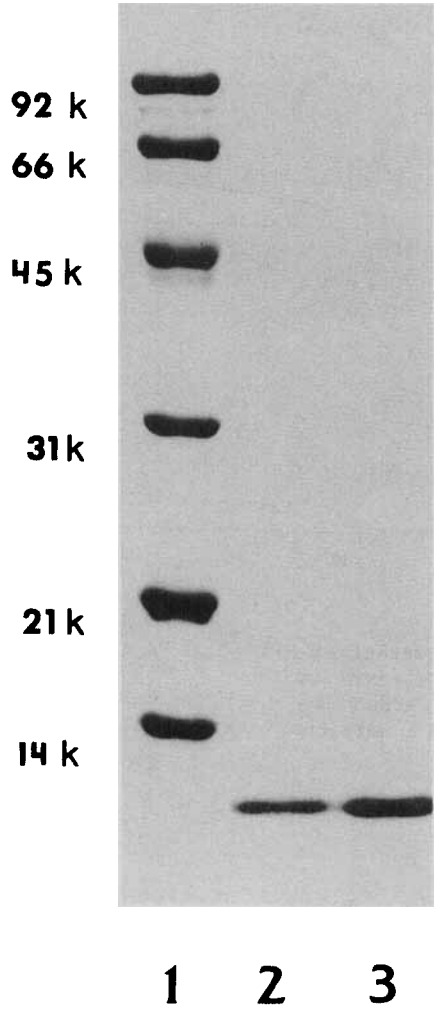

Figure 4. SDS-PAGE of natural and synthetic $T\beta_4$. About 10-20 ug of the samples were run on a 1.5 mm 12% SDS-polyacrylamide gel according to the method of laemmeli (8) and stained with silver stain. Lane 1: protein standards, Lane 2 and 3: natural and synthetic $T\beta_4$, respectively.

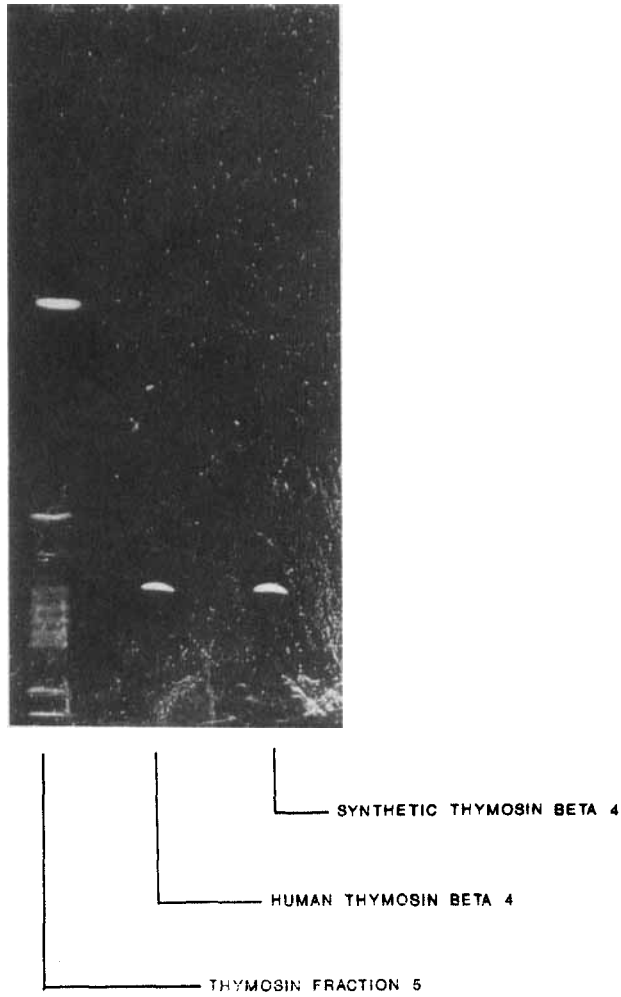

Figure 5. Isoelectric focusing gel of natural and synthetic $T\beta_4$. About 20-50 ug of the samples were run on Pharmacia/LKB Pagplates (3.5 - 9.5). The protein was precipitated with 20% trichloroacetic acid (TCA) and 3.5% sulfosalicylic acid for one hour.

TABLE 1

Amino acid composition* of natural and synthetic $T\beta_4$

Amino Acid	Synthetic $T\beta 4$	Natural $T\beta_4$	Reported Sequence**
Asp	3.9	4.2	4
Glu	10.8	11.1	11
Ser	3.5	3.9	4
Gly	1.1	1.4	1
Thr	2.8	2.9	3
Ala	2.2	2.4	2
Pro	2.9	3.1	3
Met	0.8	1.2	1
Ile	1.6	2.3	2
Leu	1.6	2.3	2
Phe	0.9	0.7	1
Lys	8.5	9.4	9

Amino acid analysis was performed with a Pico-Tag amino acid analysis system. About 1-5 μg samples of synthetic and natural $T\beta_4$ were hydrolyzed with 6N HCl, containing 1% phenol by volume at 110°C for 48 h. The hydrolysates were dried and used for amino acid analysis by the Pico-Tag amino-acid analysis system (15).

* The data are presented as assumed numbers of residues per molecule.

** Number of residues obtained from reported sequence (2).

identical protein band with a low molecular weight was evident for both natural and synthetic $T\beta_4$ samples. IEF analysis of the natural and synthetic $T\beta_4$ on an isoelectric focusing slab gel of pH range 3.5-9.5 also revealed a single identical protein band with pI of 5.1 for both natural and synthetic $T\beta_4$ samples (Fig. 5).

Amino acid analysis of the synthetic and natural $T\beta_4$ are shown in Table 1. Our results indicate that synthetic and natural $T\beta_4$ have the identical amino acid composition. These results also indicate that the amino acid composition of calf thymus $T\beta_4$ is identical to human thymus $T\beta_4$.

In summary, we were able to purify $T\beta_4$ from human thymus using a 2-step RP-HPLC procedure. The presence of $T\beta_4$ was followed by RIA and HPLC retention time. SDS-PAGE and IEF analysis of the HPLC purified $T\beta_4$, revealed single peptide bands with molecular weights below 10 Kd and pI of 5.1. The HPLC isolated $T\beta_4$ was homogeneous and had a similar HPLC retention time and amino acid composition as the synthetic $T\beta_4$. Finally, an overall 98% recovery of $T\beta_4$ from the two step RP-HPLC separation was determined by comparison of peak areas with a $T\beta_4$ standard. The amount of protein in the thymus extract was determined by Lowry assay. The amount of $T\beta_4$ isolated in this procedure was determined by comparison of peak areas with $T\beta_4$ standard. From this we estimate that human thymus contains 3% $T\beta_4$.

ACKNOWLEDGEMENTS

The authors thank Laraine Danes and Jung Lim for manuscript preparation, Mike Erdos, Christopher Rogers and Tania Damavandy for technical assistance. This research was supported in part by grant CA24974 from National Cancer Institutes to A.L.G. and a grant from Institute for Advanced Studies in Immunology and Aging to M.B.

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