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RAPID ISOLATION OF THYMOSIN β_4 FROM THYMOSIN FRACTION 5 BY PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We have developed a rapid, efficient, and reproducible two-step method for the purification of thymosin β_4 (Tβ_4) from thymosin fraction 5 (TF5). This purification is based on the use of high-performance preparative/semi-preparative and analytical reversed-phase (Delta-Pak C_{18}) chromatographic columns. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and amino acid compositional analysis have shown that natural, purified Tβ_4 is identical to synthetic Tβ_4. This procedure can be used to isolate other biologically active peptides from TF5 in sufficient quantity for characterization.

INTRODUCTION

A partially purified thymosin preparation from calf thymus termed "thymosin fraction 5" (TF5)[1] has been studied extensively for biological activity[2–8], and in clinical trials[9–13]. TF5 has been shown to modulate several *in vivo* and *in vitro* immune responses[13,14]. TF5 consists of a family of biologically active polypeptide components with hormone-like activities. Thymosin β_4 (Tβ_4) is one of several peptides that is present in TF5 and participates in the process of regulation, differentiation, and function of thymus-dependent thymocytes. Tβ_4 has been isolated from TF5 and completely characterized. Tβ_4 is composed of 43 amino acid residues and has a molecular weight of 4982 and p*I* of 5.1. The amino-terminus of the peptide is blocked by an acetyl group[14]. Tβ_4 has been purified from TF5 by conventional column techniques for large-scale purification using ion-exchange chromatography on a carboxymethyl-cellulose column, followed by gel chromatography on Sephadex G-50 in 6 *M* guanidinium chloride. The desalted Tβ_4 on Sephadex G-10 had a yield of 0.45%[15]. Tβ_4 has also been isolated by high-performance liquid chromatographic (HPLC)

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techniques for analytical purposes, using a μBondapak C_{18} column[16] with a recovery of 2–3%. Low recoveries have always been a problem in the isolation of TF5 peptides and have limited the amount of purified peptide available for further characterization and comparison of the different forms of the biologically active peptides. In the present study, we report a very fast, reproducible, and efficient large-scale isolation procedure as well as an analytical reversed-phase HPLC (RP-HPLC) procedure for the purification of $T\beta_4$ from TF5.

EXPERIMENTAL

Materials

Clinical-grade TF5 (Lot C114080-02) and synthetic $T\beta_4$ were provided as a gift from Alpha One Biomedicals (Washington, DC, U.S.A.). 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.) 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.

HPLC Methods

Optimization of reversed-phase chromatographic conditions for the fractionation of TF5 was carried out on a Model 600 HPLC system (Waters), equipped with a Model 490 multi-wavelength detector set at 280 nm and a 300 mm × 3.9 mm I.D. Delta-Pak, 300-Å, 15-μm, C_{18} column (Waters). Eluent A was 0.02 *M* ammonium acetate, (pH 6.8); eluent B was acetonitrile. A 60-min linear gradient from 0 to 80% B was run at a flow-rate of 0.5 ml/min. The sample was 900 μg of TF5, dissolved in the initial buffer.

Preparative-scale reversed-phase chromatography of TF5 (1.5 g) was performed on a Delta-Prep HPLC system (Waters), equipped with a Model 481 variable-wavelength detector with a semi-preparative flow-cell, set at 280 nm, and a 300 mm × 50 mm I.D. 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-min linear gradient from 0 to 80% B was run at a flow-rate of 80 ml/min. TF5 was dissolved in eluent A and applied to the column through a port in the solvent delivery system. Fractions of 1 min were collected, and a 1-ml aliquot was used for radioimmunoassay (RIA).

Semi-preparative reversed-phase HPLC of an aliquot of a $T\beta_4$-active fraction No. 20 (5.6 mg) from the preparative separation, was carried out on a Model 600 HPLC system 1 equipped with a Model 490 multi-wavelength detector, set at 214 nm, and a 150 mm × 3.9 mm 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. Separation of $T\beta_4$ was achieved with a 10-min linear gradient from 0 to 14% B, followed by a 10-min hold at 14% B and a 10-min linear gradient to 18% B at a flow-rate of 1 ml/min. Fractions of 1 min were collected and assayed by RIA for

Tβ_4. The peak of immunoreactive Tβ_4 (fraction 33, 100 μl) was diluted with 100 μl of water and rechromatographed under the same conditions. One half minute fractions were collected. Fractions 33 and 33.5, collected from this separation were pooled, reduced to a volume of 250 μl, diluted with 250 μl of water, and rechromatographed similarly. The peak was collected and subjected to amino acid analysis and SDS-PAGE.

A crude mixture, containing synthetic Tβ_4 (50 μg) was also chromatographed on the 5-μm Delta-Pak C_{18} column under the conditions described above. The major peak (33.5 min) was identified as Tβ_4 by RIA. Data from all the chromatographic procedures was collected with a Model 840 chromatography workstation (Waters).

Gel electrophoresis

SDS-PAGE was performed according to the method of Laemmli[17]. Tβ_4 samples were incubated at 90°C for 5 min before gel electrophoresis. Proteins were visualized by silver staining with the Bio-Rad silver-stain kit.

Amino acid analysis

This procedure was performed with the 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. The Tβ_4 samples (about 5–10 μg) were hydrolyzed in 200 μl of a constant-boiling hydrochloric acid 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 min at room temperature to yield the corresponding PTC derivatives[18]. These derivatives were analyzed with the Pico-Tag amino acid analysis system, which had been previously calibrated with a standard mixture of amino acids.

Protein determinations

Protein was estimated by the method of Lowry *et al.*[19] 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

The RIA for Tβ_4 was performed according to the method of Naylor *et al.*[20]. 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, 0.01 m*M* EDTA, and 0.1% BSA was added to each tube to bring the volume 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 a 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 min 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

The primary goal of our ongoing thymosin research program is to develop a rapid and efficient method for the isolation of the TF5 peptides. The isolated peptides will then be further characterized and their individual biological functions will be determined. In the present study, separation of thymic peptides in TF5 was carried out by RP-HPLC on the Delta-Pak columns.

Fig. 1 shows an analytical RP-HPLC separation of TF5. TF5 (900 μg) was fractionated on a 300 mm × 3.9 mm I.D. Delta-Pak, 300-Å, 15-μm C_{18} column under the conditions described in *HPLC methods*. Under these conditions many peptides were eluted as distinct peaks in the first half of the chromatogram, whereas a broad heteregeneous peak was eluted in the second half of the chromatogram. We were unable to improve the resolution of the 30–60 min region of TF5 by using different chromatographic conditions and HPLC columns, *e.g.*, ion-exchange, reversed-phase, size exclusion, chromatography, etc. (results are not shown). However, the fractionation of TF5 on the Delta-Pak, 300-Å, 15-μm C_{18} column generated the greatest number of peptide peaks compared to all other HPLC columns we have tried. Therefore, the Delta-Pak C_{18} column was selected as the initial step for the fractionation of TF5 peptides.

Preparative scale-up isolation of Tβ_4 from TF5 was achieved with a 300 mm × 50 mm I.D. preparative Delta-Pak 300-Å, 15-μm C_{18} column (Fig. 2). This was the initial step used for the fractionation of all the peptides in TF5. Isolation of Tβ_4 from this preparative chromatogram can be used as an example of the further isolation of new TF5 peptides. The RIA analysis of the fractionated TF5, as shown in Fig. 2,

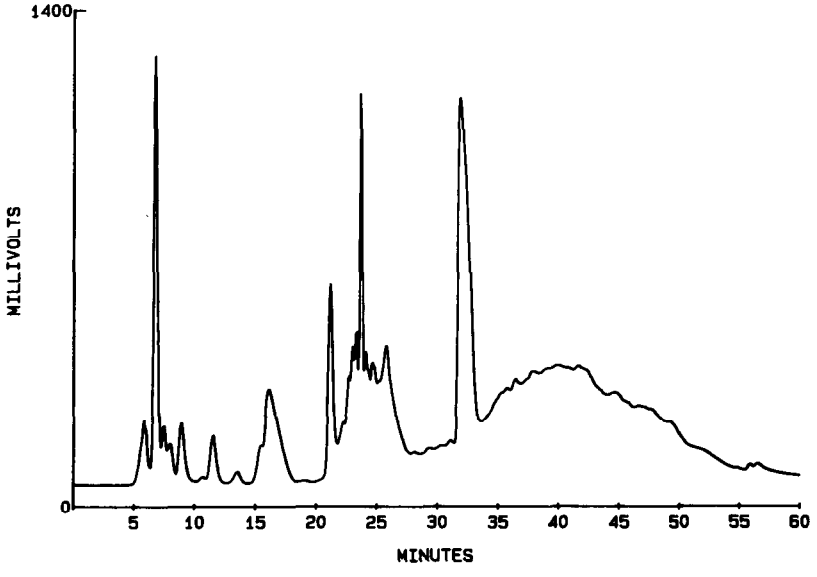

Fig. 1. RP-HPLC separation of 900 μg of TF5 on a 300 mm × 3.9 mm I.D. Delta-Pak, 300 Å. 15-μm, C_{18} column. Eluent A was 0.02 *M* ammonium acetate, (pH 6.8), and eluent B was acetonitrile. A 60-min linear gradient from 0 to 80% B was run at a flow-rate of 0.5 ml/min. Detection, 280 nm, 1.4 a.u.f.s.

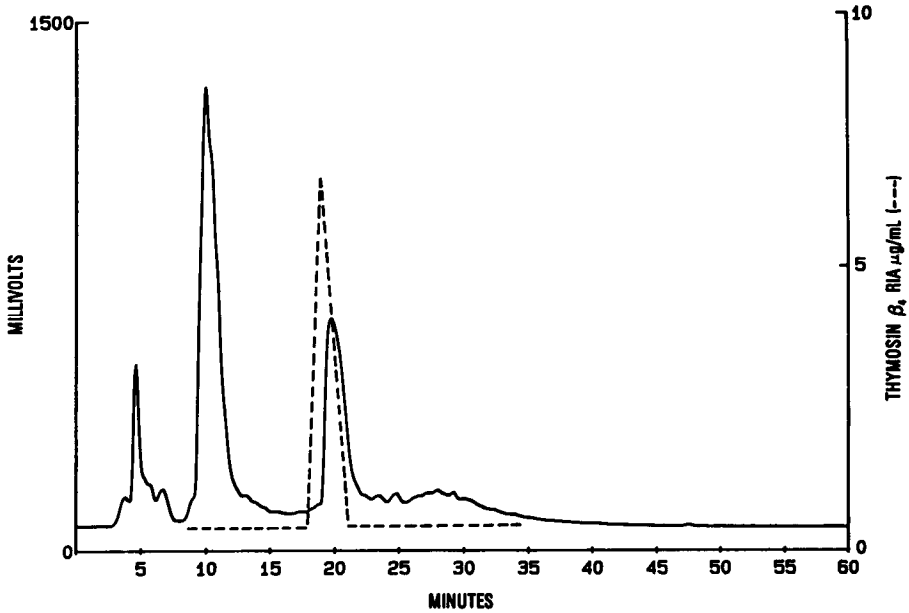

Fig. 2. RP-HPLC separation of 1.5 g of TF5 on a 300 mm × 50 mm I.D. Delta-Pak, 300-Å, 15-μm, C_{18} column. Eluent A was 0.02 *M* ammonium acetate, (pH 6.8) and eluent B was acetonitrile. A 60-min linear gradient from 0 to 80% B was run at a flow-rate of 80 ml/min. Detection, 280 nm at 1.5 a.u.f.s. Fractions were assayed for Tβ_4 by RIA. Results are superimposed on the chromatogram.

indicated that immunoreactive Tβ_4 was eluted between 18 and 21 min. Fraction 20 (F20) was used for further purification.

Optimal separation conditions for the F20 peptides were determined with a 150 mm × 3.9 mm I.D. analytical Delta-Pak, 300-Å, 5-μm, C_{18} column (Fig. 3). Our results indicated that the major peptide peak from the chromatographic separation of F20 was Tβ_4 (peak 1). When synthetic Tβ_4 was chromatographic under the same conditions, the major Tβ_4 peak had a retention time identical to that of peak 1 (results not shown). This chromatographic step can also be used for the identification and isolation of Tβ_4 for analytical purposes.

F20 (5.6 mg) from the preparative separation (Fig. 2) was further fractionated on a 150 mm × 3.9 mm I.D. Delta-Pak, 300 Å 5-μm, C_{18} column under the conditions described previously in *HPLC methods*. As shown in Fig. 4, the scale-up isolation of Tβ_4 from F20 did not lead to appreciable loss in resolution. The RIA analysis of the fractions indicated that the major peptide peak at a retention time of 33 min was Tβ_4. Rechromatography of immunoreactive fractions 32–34 under similar conditions revealed a single major peptide peak for fraction 33, (Fig. 5), and the presence of several minor contaminants. The other fractions (not shown) contained much less Tβ_4. Fraction 34 was contaminated by the shouldering peak shown on peak 1 in Figs. 3 and 4. Therefore, fractions 33 and 33.5 (from the rechromatography of fraction 33) were pooled, rechromatographed and used as the final Tβ_4 preparation for SDS-PAGE and amino acid composition analysis.

An estimate of the homogeneity of the isolated Tβ_4 was obtained by RP-HPLC. As shown in Fig. 6, the isolated Tβ_4 from TF5 (natural Tβ_4) gave a homogeneous

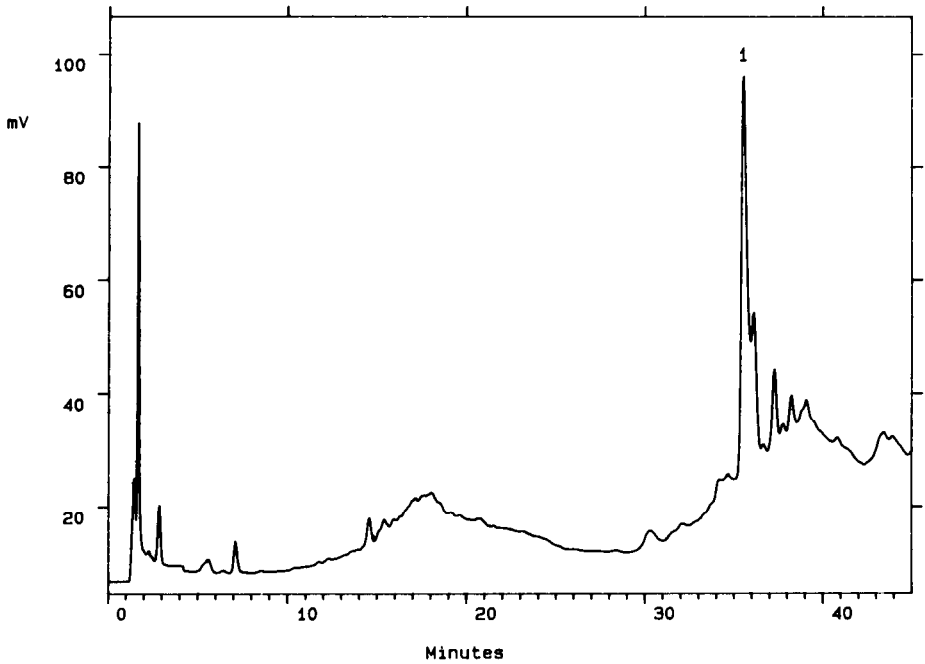

Fig. 3. RP-HPLC separation of 130 μg of fraction 20 from the 1.5 g separation of TF5 on a 150 mm × 3.9 mm I.D. Delta-Pak, 300-Å, 5-μm, C_{18} column. Eluent A was 0.1% phosphoric acid and eluent B was acetonitrile containing 0.1% phosphoric acid. Separation of Tβ_4 was achieved by a 10-min linear gradient to 14% B, followed by a 10-min hold at 14% B and a 10 min-gradient to 18% B. The flow-rate was 1 ml/min. Detection at 214 nm, 0.1 a.u.f.s.

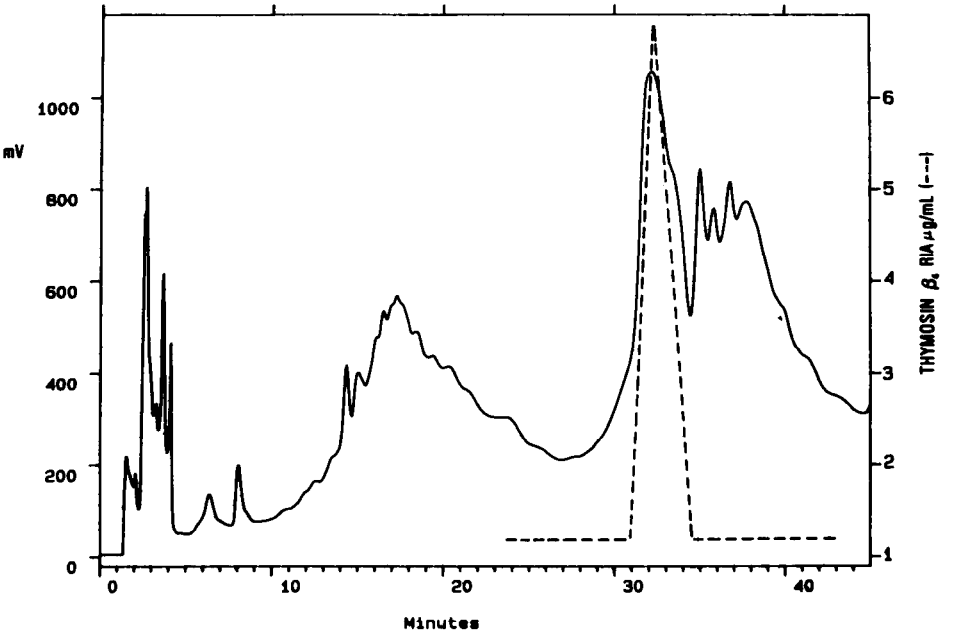

Fig. 4. RP-HPLC separation of 5.6 mg of fraction 20 from the 1.5 g separation of TF5 on a 150 mm × 3.9 mm I.D. Delta-Pak, 300-Å, 5-μm, C_{18} column, under the conditions described in Fig. 3. Detection, 214 nm, 1 a.u.f.s. Collected fractions were assayed for Tβ_4 by RIA. Results are superimposed on the chromatogram.

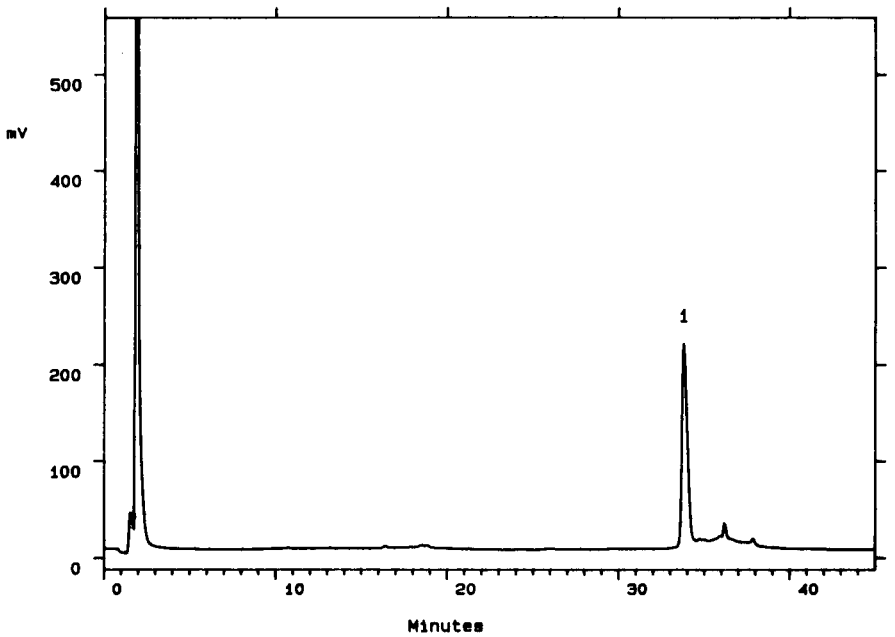

Fig. 5. Rechromatography of 100 μl of fraction 33 from the preparative separation of fraction 20. The sample was diluted with 100 μl of water and injected into the 150 mm × 3.9 mm I.D. Delta-Pak, 300-Å, 5-μm, C_{18} column, under the conditions described in Fig. 3. Detection, 214 nm, 0.5 a.u.f.s.

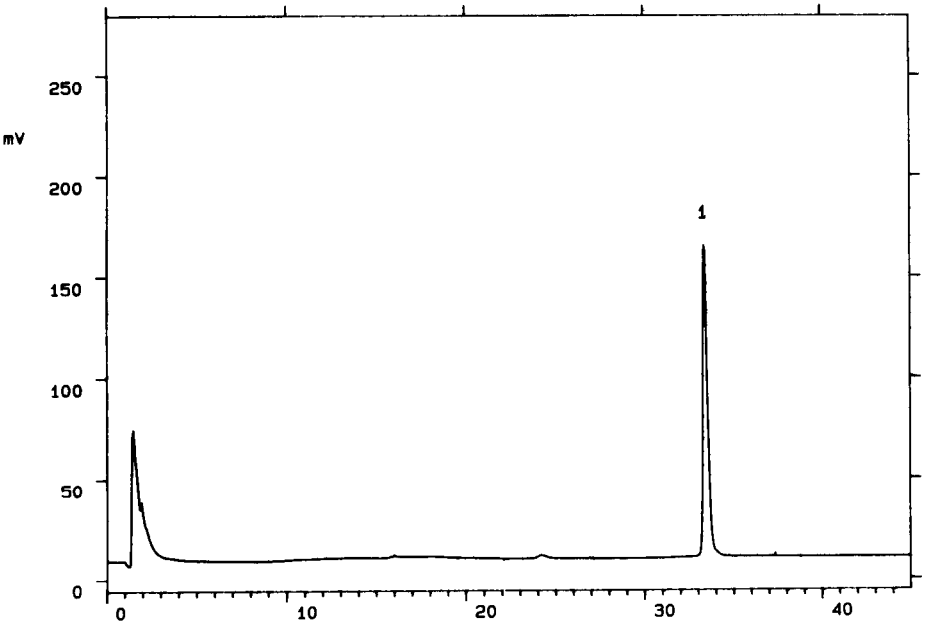

Fig. 6. Rechromatography of fractions 33 and 33.5 (from the purification of fraction 33). The sample was concentrated to 250 μl, diluted with water, and injected into the 150 mm × 3.9 mm I.D. Delta-Pak, 300-Å, 5-μm, C_{18} column, under the conditions described in Fig. 3. Detection, 214 nm, 0.5 a.u.f.s.

peak. Fig. 7 shows the comparison of the purified natural Tβ_4 by the two-step RP-HPLC procedure and the crude synthetic Tβ_4. Results indicate that natural and synthetic Tβ_4 have identical retention times.

SDS-PAGE analysis of the natural and HPLC purified synthetic Tβ_4, illustrated in Fig. 8, was carried out as described in *HPLC methods*. When the protein bands on SDS gels were initially stained with Coomassie Blue R-250, only a very faint band of Tβ_4 was evident. When the gel was then processed with the more sensitive silver stain, a single identical protein band with a low molecular weight was evident for both natural and synthetic Tβ_4 samples. Gel electrophoresis of Tβ_4 has in the past always been done by isoelectric focusing (IEF) using relatively huge amounts of Tβ_4 (200 μg). In addition, the Tβ_4 band on IEF gels was only visible during the fixing step of the gel by using 20% trichloroacetic acid, and it disappeared during subsequent staining and destaining procedures[10]. The SDS-PAGE and staining procedure used for the Tβ_4 peptide has two advantages over the previously reported IEF method: (1) it requires 10 times less protein and (2) the stained Tβ_4 band is intense and stable.

Amino acid analysis of the purified synthetic and natural Tβ_4 are shown in Tabel I. Our results indicate that the synthetic and purified natural Tβ_4 have the identical amino acid composition.

In summary, we were able to purify Tβ_4 from TF5 in a two-step RP-HPLC procedure. The presence of Tβ_4 was followed by RIA and HPLC retention time. In the first step of the purification the fractions containing Tβ_4 (18–21) were identified in the RIA. In the second step only F20 was selected for further purification. Fractions

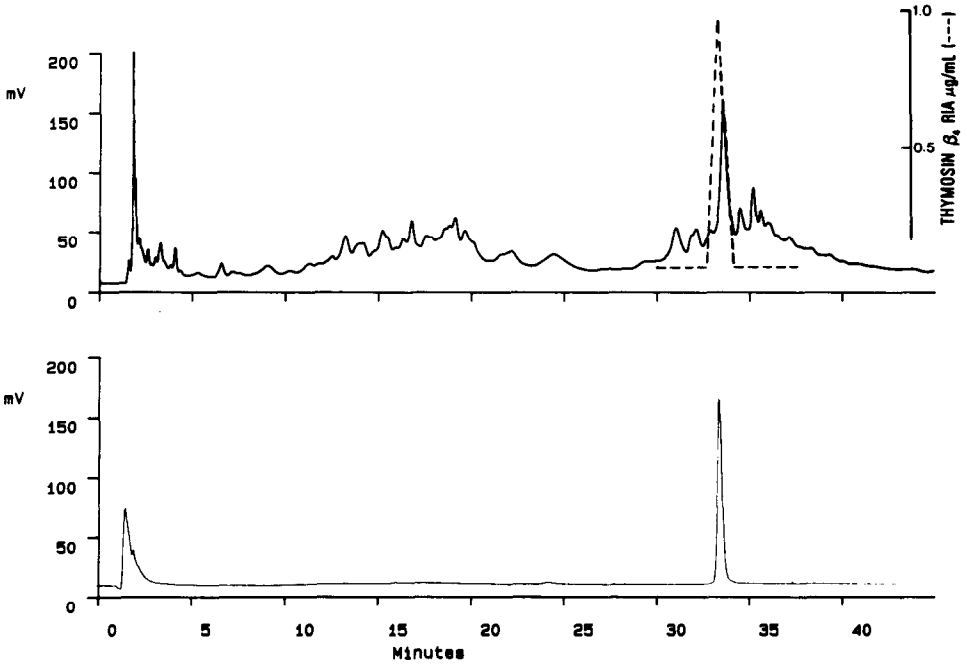

Fig. 7. Comparison of 50 μg of crude synthetic Tβ_4 and Tβ_4 isolated by the two-step RP-HPLC procedure on the 150 mm × 3.9 mm I.D. Delta-Pak, 300-Å, 5-μm column with the gradient described previously (Fig. 3). The synthetic Tβ_4 was identified by RIA, and the results are superimposed on the chromatogram.

TABLE I

AMINO ACID COMPOSITION OF NATURAL AND SYNTHETIC THYMOSIN β_4

Amino acid analysis was performed with a Pico-Tag amino acid analysis system. About 10-μg samples of synthetic and isolated Tβ_4 were hydrolyzed with 6 *M* hydrochloric acid, containing 1% (v/v) phenol at 110°C for 48 h. The hydrolysates were dried and used for amino acid analysis by the Pico-Tag standard procedure[18]. The data are presented as assumed numbers of residues per molecule.

Amino acid	*Synthetic Tβ_4*	*Natural Tβ_4*	*From reported sequence**
Asp	3.9	3.8	4
Glu	10.8	10.6	11
Ser	3.5	3.8	4
Gly	1.1	1.2	1
Thr	2.8	3.2	3
Ala	2.2	1.8	2
Pro	2.9	2.9	3
Met	0.8	0.7	1
Ile	1.6	1.9	2
Leu	1.6	2.2	2
Phe	0.9	0.8	1
Lys	8.5	8.6	9

* Number of residues obtained from the reported sequence result[14].

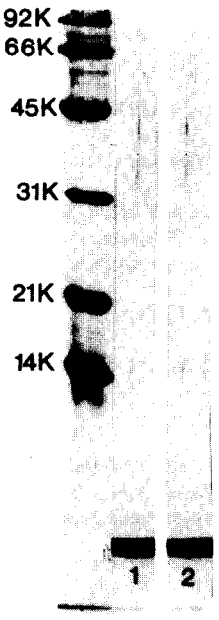

Fig. 8. SDS-PAGE of synthetic and isolated Tβ_4. About 10–20 μg of Tβ_4 samples were electrophoresed on a 1.5-mm 12% SDS-polyacrylamide gel according to the method of Laemmli[17] and stained with silver stain. Lanes 1 and 2, synthetic and natural Tβ_4, respectively. On the left are standard proteins. K = Kilodalton.

32–34 from F20 were immunoreactive. However, only the peak of immunoreactivity, (fraction 33), was used for further purification and analysis. From fraction 33 we obtained 5.1 mg of Tβ_4 which correlates well with 5.4 mg calculated by RIA. The mass recovery of Tβ_4 from F20 was greater than 80% as calculated by the peak area of Tβ_4 compared to the peak area of an HPLC purified synthetic β_4 standard. More Tβ_4 could be obtained from the other immunoreactive fractions (18, 19 and 21) from the preparative separation of TF5. Our goal, however, was to develop a simple procedure for the isolation of biologically active peptides from TF5 in sufficient quantity for characterization. The isolation of Tβ_4 is presented as an example.

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