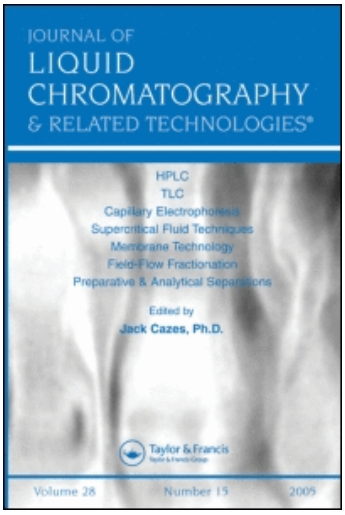

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Rapid Micro Isolation of Thymosin α₁ from Thymosin Fraction 5 by Reversed-Phase High Performance Liquid Chromatography

Manhaz Badamchian[a]; Allan L. Goldstein[a]; M. Patricia Strickler[b]; M. Jude Stone[b]
[a] Department of Biochemistry, The George Washington University School of Medicine and Health Sciences, Washington, D.C. [b] Waters Chromatography Division Millipore Corporation Life Science Application Laboratory, fair, Virginia

To cite this Article Badamchian, Manhaz , Goldstein, Allan L. , Strickler, M. Patricia and Stone, M. Jude(1989) 'Rapid Micro Isolation of Thymosin α₁ from Thymosin Fraction 5 by Reversed-Phase High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 12: 5, 845 — 860
To link to this Article: DOI: 10.1080/01483918908049211
URL: http://dx.doi.org/10.1080/01483918908049211

JOURNAL OF LIQUID CHROMATOGRAPHY, 12(5), 845-860 (1989)

RAPID MICRO ISOLATION OF THYMOSIN α_1 FROM THYMOSIN FRACTION 5 BY REVERSED-PHASED HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

MANHAZ BADAMCHIAN[1],
M. PATRICIA STRICKLER[2], M. JUDE STONE[2]
AND ALLAN L. GOLDSTEIN[1]
[1]*Department of Biochemistry*
The George Washington University
School of Medicine and Health Sciences
Washington D.C. 20037
[2]*Waters Chromatography Division*
Millipore Corporation
Life Science Application Laboratory
Fairfax, Virginia 22030

ABSTRACT

We have developed a rapid, efficient, and reproducible method for the purification of thymosin α_1 ($T\alpha_1$) from thymosin fraction 5 (TF5). This procedure can serve as a model for isolation of other biologically active peptides from TF5 in sufficient quantity for characterization. The purification procedure is based on the use of high-performance preparative/semi-preparative and analytical reversed-phase (C_{18} Delta-Pak) chromatographic columns. The HPLC retention time, pI, RIA, SDS-PAGE, and amino acid composition analysis have shown that natural, purified $T\alpha_1$ is identical to synthetic α_1.

INTRODUCTION

A thymic preparation termed thymosin fraction 5 (TF5) has been shown to be a potent immuno-potentiating agent (1). TF5 consists

of a family of biologically active polypeptide components with hormone-like activities (2). Thymosin α_1 ($T\alpha_1$) was the first biologically active polypeptide to be purified from TF5 and completely characterized. It is an acidic peptide with an isoelectric point of 4.2, and a molecular weight of 3108 (3,4). This peptide is highly active in amplifying T-cell immunity (5) and is active in modulating the expression of terminal deoxynucleotidyl transferase (TdT) (6). Intercerebral injections of $T\alpha_1$ in mice stimulates corticosterone production (7). $T\alpha_1$ has been isolated and purified from TF5 by ion-exchange chromatography on CM-cellulose and DEAE-cellulose, as well as by gel filtration chromatography on Sephadex G-75 (8). $T\alpha_1$ has also been isolated by HPLC techniques, using a μ-Bondapak C_{18} column (9) with recovery of 0.6%. 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. In this paper, we report a very fast, reproducible, and easy, large-scale isolation procedure as well as an analytical reversed-phase HPLC (RP-HPLC) procedure for the purification of the $T\alpha_1$ and other peptides from TF5.

MATERIALS AND METHODS

Delta-Pak C_{18} columns and chemicals for the Pico-Tag system were from Waters Chromatography Division of Millipore Corporation (Milford, MA). Water for HPLC was purified with a Milli-Q water system (Millipore Corporation, Bedford, MA). All buffers and solvents were of HPLC grade (Fisher Scientific, Pittsburgh, PA). All HPLC solutions were filtered through a 0.45 μm membrane (Millipore) prior to use. Clinical grade TF5 (Lot C114080-02) and synthetic α_1 were provided as a gift from Alpha One Biomedicals, Inc. (Washington, DC). SDS-PAGE chemicals were of molecular biology grade and were purchased from Sigma Chemical Co. (St. Louis, MO). The SDS Low-molecular weight protein standards and silver stain kit were purchased from Bio-Rad Laboratories (Richmond, CA).

HPLC Methods

Preparative reversed-phase chromatography of thymosin fraction 5 (1.5 g) was performed on a Delta-Prep HPLC system equipped with a Model 481 variable-wavelength detector with a semi-preparative flow-cell, set at 280 nm, and a 300 x 50-mm 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 0-80% B was run at a flow-rate of 80 ml/min. Thymosin fraction 5 was dissolved in the initial buffer and applied to the column through a port in the solvent delivery system. One-min fractions were collected, and assayed for $T\alpha_1$ by RIA.

Optimization of reversed-phase chromatographic conditions for the purification of $T\alpha_1$ from Fractions 13 and 14 from the preparative separation of TF5 was carried out on a Model 600 HPLC system (Waters), equipped with a Model 490 multi-wavelength detector set at 214 nm and 220 nm and a 150 x 3.9-mm Delta-Pak, 300-Å, 15-μm, C_{18} column (Waters). Eluent A was 0.1% phosphoric acid in water and Eluent B was acetonitrile with 0.1% phosphoric acid. 80 μg of fraction 14 was used in each separation. Panel A was a 30-min linear gradient from 0-30% B. Panel B was a 10-min linear gradient from 0-15% B followed by a 20-min linear gradient from 15-23% B and a 10-min hold at 23% B. At 40.1-min the column was washed with 50% B for 10-min. Panel C was a 10-min linear gradient from 0-15% B with a 10-min hold at 15% B followed by a 20-min gradient from 15-20% B with a 10-min hold at 20% B. At 50.1-min the column was washed with 50% B for 10-min. Panel D shows the optimal conditions of a 10-min linear gradient from 0-15% B with a 5-min hold at 15% B followed by a 20-min gradient from 15-17% B with a 10-min hold at 17% B. At 45.1-min the column was washed with 50% B for 10-min. All the separations were run at a flow-rate of 1 ml/min. Detection was at 214 and 220 nm.

The peaks in aliquots of Fraction 13(108 μg) and 14(80 μg) from the 1.5-g separation of TF5 were identified as $T\alpha_1$ by comparing their retention times with the retention time of

synthetic α_1 on the Delta-Pak C_{18} column using the 10-min linear gradient from 0-15% B with a 5-min hold at 15% B followed by a 20-min gradient from 15-17% B with a 10-min hold at 17% B. 80 μg of Fraction 14 was also spiked with 9 μg of synthetic α_1 to further confirm the identity of $T\alpha_1$.

Semi-preparative reversed-phase HPLC of an aliquot of the $T\alpha_1$ immunoreactive Fraction 13(4.3 mg) from the preparative separation was carried out on a Model 600 HPLC using the 10-min linear gradient from 0-15% B with a 5-min hold at 15% B followed by a 20-min gradient from 15-17% B with a 10-min hold at 17% B. One minute fractions were collected and assayed for $T\alpha_1$ by RIA. The peak of immunoreactive $T\alpha_1$ (Fraction 23, 300 μl) was diluted with 300 μl of water and re-chromatographed under the same conditions. One half minute fractions were collected. Fractions 30-31.5 collected from this separation were pooled, concentrated to 800 μl to remove acetonitrile and re-injected. The peak was collected and subjected to amino acid composition analysis and SDS-PAGE.

Data from all the chromatographic procedures was collected using a Model 840 chromatography workstation (Waters).

Gel Electrophoresis

SDS-PAGE was performed according to the method of Laemmli (10). $T\alpha_1$ samples were incubated at 90°C for 5-min before gel electrophoresis. Proteins were visualized in the gels by Coomassie Blue R-250 staining.

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 $T\alpha_1$ samples (about 5-10 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-min at room temperature to yield the corresponding phenylthiocarbamyl derivatives (11). 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. (12) 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 $T\alpha_1$ was performed according to the method of McClure et al. (13) with minor modifications. Fractions collected from HPLC separation were lyophilized and resuspended in 1 ml HPLC-grade water, and aliquots of these fractions were used for the RIA. The antiserum to synthetic N14-$T\alpha_1$ and the labeled N-AC(Tyr^1) $T\alpha_1$ (^{125}I-labeled synthetic $T\alpha_1$ with the N-terminal serine substituted by a tyrosine) in 0.05 M phosphate buffer saline, containing 0.01 mM EDTA, 0.05% NaN_3 and 0.5% BSA were incubated with standard or HPLC fractions for 24 h at 4°C. A second antibody (goat anti-rabbit IgG) was used to precipitate the immune complexes. The radioactivity in the immunoprecipitates was measured in an automatic Beckman Gamma 4000 spectrometer (Columbia, MD).

RESULTS AND DISCUSSION

A primary goal of our ongoing thymosin research program is to develop more efficient and rapid methods for the isolation of sufficient quantity (15-100 μg) of the biologically active peptides in TF5. This amount of peptide is generally sufficient to characterize peptides. In the present study a simpler and faster separation of thymic peptides in TF5 has been achieved by RP-HPLC on Delta-Pak columns.

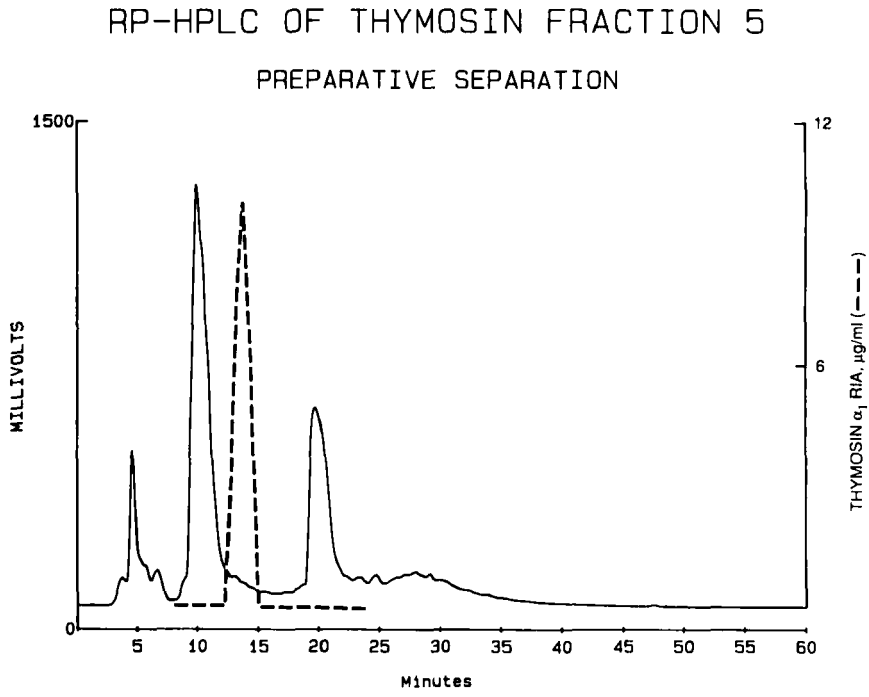

FIGURE 1. RP-HPLC separation of 1.5-g of TF5 on a 300 x 50-mm Delta-Pak, 300-Å, 15-μm, C_{18} column. Eluent A was 0.02 M ammonium acetate (pH 6.8) and Eluent B was acetonitrile. A 60-min linear gradient from 0-80% B was run at a flow rate of 80 ml/min. Detection 280 nm, 1.5 AUFS. Collected fractions were assayed for $T\alpha_1$ by RIA. Results are superimposed on the Chromatogram.

Preparative scale-up isolation of $T\alpha_1$ from TF5 was carried out on a 50-mm x 30 cm preparative Delta-Pak 300-Å, 15-μm C_{18} column. This was our general, initial step for the fractionation of TF5 described previously (14). The RIA analysis of the fractionated TF5 (Fig. 1) indicated that immunoreactive $T\alpha_1$ elutes in Fraction 13 (F13), the peak of activity, and Fraction 14 (F14). The volume of each fraction collected from each separation was 80 ml and 10 ml of each fraction was used for the rest of the study.

Optimization of chromatographic conditions was carried out on a 150 x 3.9 mm analytical Delta-Pak, 300-Å, 5-μm C_{18} column using

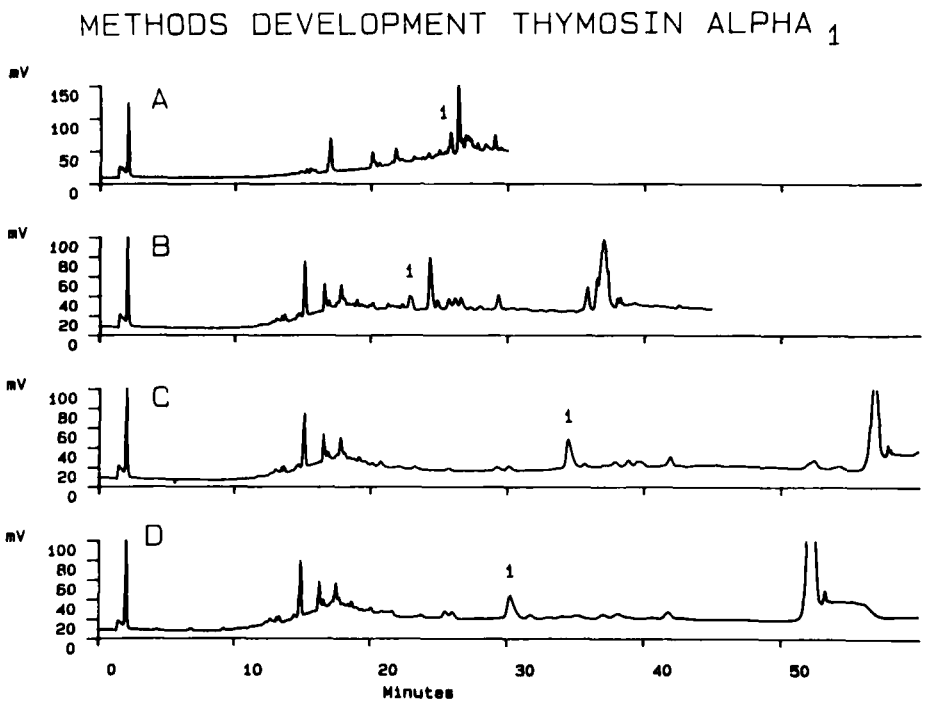

FIGURE 2. Optimization of chromatographic conditions for the isolation of $T\alpha_1$ from Fraction 14 of the 1.5-g separation of TF5 on a 150 x 3.9-mm Delta-Pak C_{18}, 300-Å, 5-μm column. Eluent A was 0.1% phosphoric acid in water and eluent B was acetonitrile with 0.1% phosphoric acid. The gradient conditions for each separation were the following: Panel A, 0-30% B, 30-min, Panel B, 0-15% B, 10-min, 15-23% B 20-min, hold 10-min, 50% B at 40.1-min, Panel C, 0-15% B, 10-min, hold 10-min, 15-20% B, 20-min, hold 10-min, 50% B at 50.1-min, Panel D, 0-15% B, 10-min, hold 5-min, 15-17% B, 20-min, hold 10-min, 50% B at 45.1-min. All the separations were run at a flow rate of 1 ml/min. $T\alpha_1(1)$ was detected at 214 nm. Panel A at 0.15 AUFS and Panels B, C, D at 0.1 AUFS.

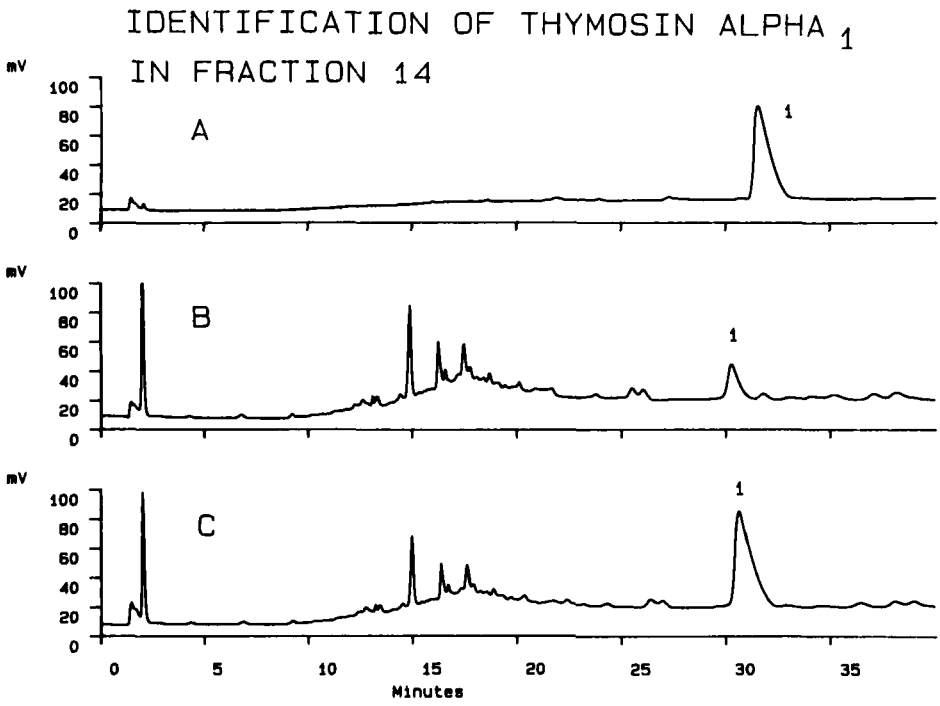

FIGURE 3. Identification of $T\alpha_1$ (1) in Fraction 14 from the 1.5-g separation of TF5 by comparison with synthetic α_1 (1) by reversed-phase HPLC. Chromatographic conditions are described in Fig. 2, Panel D. Panel A shows the separation of 11-μg of synthetic α_1. Panel B shows 80 μg of Fraction 14 and Panel C 80-μg of Fraction 14 spiked with 9 μg of synthetic α_1. Detection at 214 nm, 0.1 AUFS.

aliquots of F14, containing 80 μg of protein. Eluents of 0.1% phosphoric acid in water and acetonitrile resolved the most peaks. Maximum separation of $T\alpha_1$ (peak 1) was achieved by applying a series of gradients with decreasing amounts of acetonitrile until the minimum concentration of acetonitrile (17%) was found that would elute $T\alpha_1$ (Fig. 2). With these conditions (Fig. 2, Panel D) the hydrophilic impurities are eluted in 10-min with a gradient to 15% acetonitrile, the $T\alpha_1$ is then baseline resolved with a 20-min gradient to 17% acetonitrile. The hydrophobic impurities are

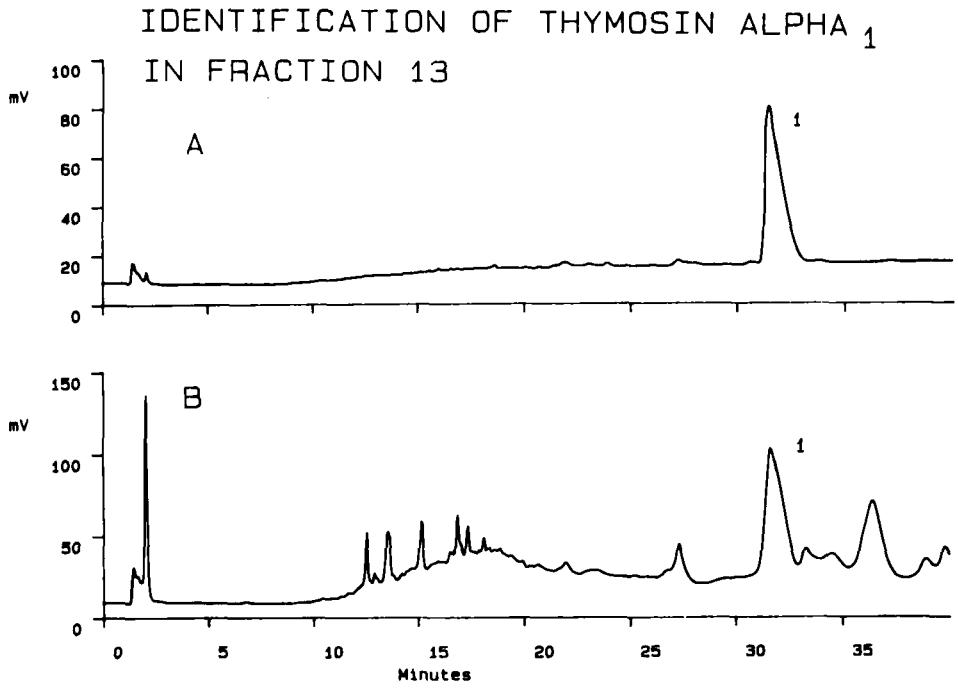

FIGURE 4. Identification of $T\alpha_1$ (1) in Fraction 13 from the 1.5-g separation of thymosin fraction 5 by comparison with synthetic α_1 by reversed-phase HPLC. Chromatographic conditions are described in Fig. 2, Panel D. Panel A shows 11-μg of synthetic α_1 and Panel B 108-μg of Fraction 13. Detection 214 nm, 0.1 AUFS (Panel A) and 0.15 AUFS (Panel B).

washed from the column with 50% acetonitrile. The $T\alpha_1$ peak was identified by running synthetic α_1 under the same conditions (Fig. 3, Panel A). Since the retention time of a peak in a complex sample such as this can shift, synthetic α_1 was added to F14 and chromatographed similarly (Fig. 3, Panel C). The area of the α_1 peak increased proportionally to the amount added. F13 was treated similarly as shown in Fig. 4. By comparing the peak areas of $T\alpha_1$ in F13 and F14 to the peak area of known amounts of synthetic α_1 the amount of $T\alpha_1$ was determined to be 594 μg in F13 and 111 μg in F14. This is in agreement with the amounts calculated by RIA.

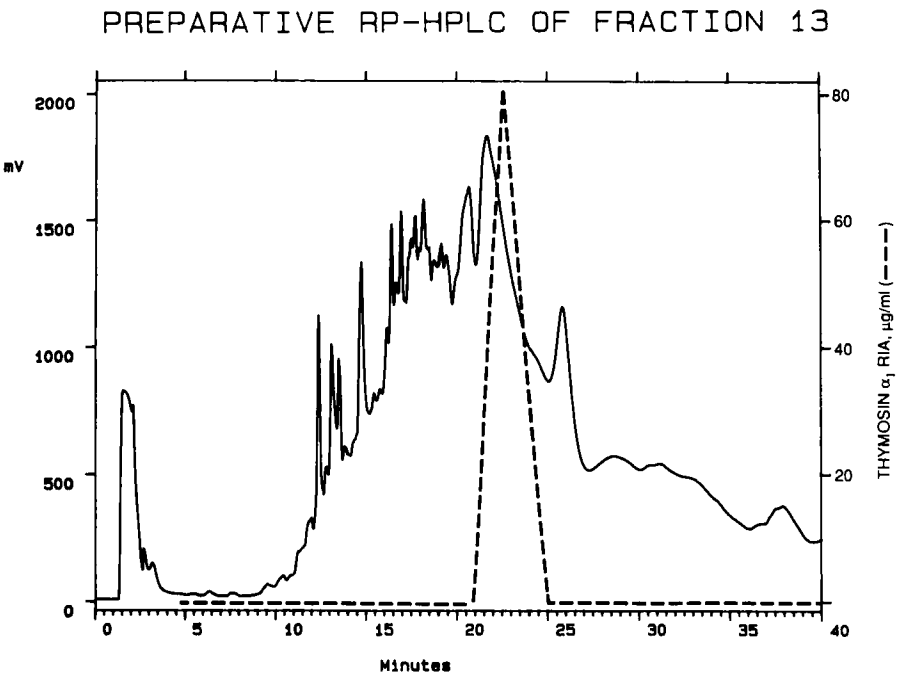

FIGURE 5. Reversed-phase HPLC separation of 4.3-mg of Fraction 13 from the 1.5-g separation of TF5. Chromatographic conditions were described in Fig. 2, Panel D. Detection 214 nm, 2.0 AUFS. Collected fractions were assayed for $T\alpha_1$ using RIA. Results are superimposed on the chromatogram.

F13 (4.3 mg) from the preparative separation (Fig. 1) was further fractionated using the conditions described previously (Fig. 2, Panel D). The RIA analysis of the Fractions (Fig. 5) indicated that the peak of $T\alpha_1$ activity was in Fraction 23 (F23). All the Fractions 22-26 containing immunoreactive $T\alpha_1$ (only F23 is shown in Fig. 6) were rechromatographed and the amount of $T\alpha_1$ was determined by comparison of peak areas with synthetic α_1 as shown in Table 1. 540 μg of $T\alpha_1$ was recovered from the 593 μg injected, a 91% recovery.

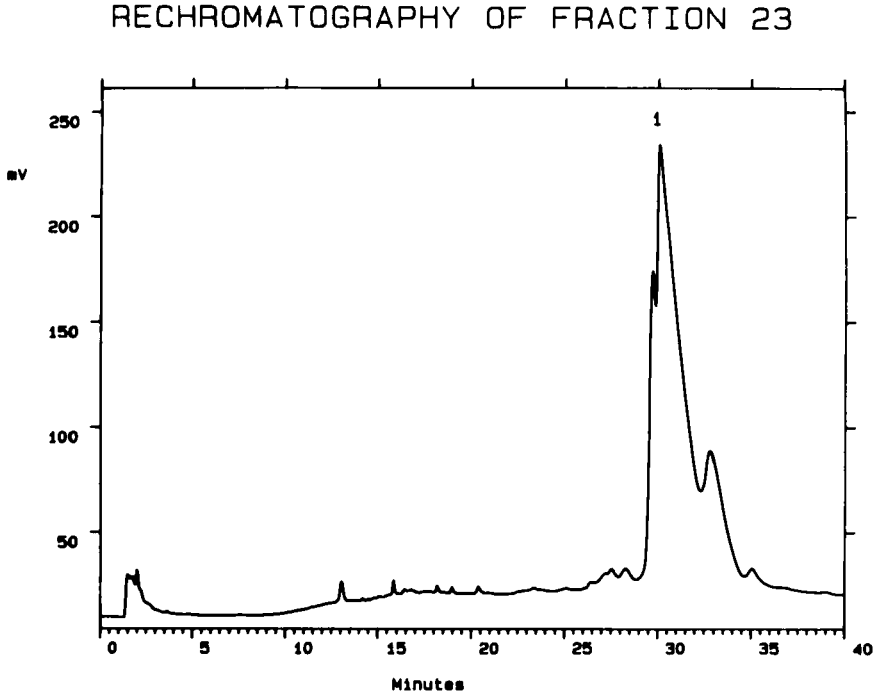

FIGURE 6. Rechromatrography of 300 μl of Fraction 23 from the preparative separation of Fraction 13. The sample was diluted with 300 μl of water and injected on to the 150 x 3.9-mm Delta-Pak C_{18} column. The chromatographic conditions are described in Fig. 2, Panel D. One half min fractions were collected across the major peak of $T\alpha_1$ (1). Detection 214 nm, 0.25 AUFS.

TABLE 1
Recovery of $T\alpha_1$ from F13

Fraction	Amount recovered in μg
22	96
23	200
24	125
25	78
26	41
Total	540

Recovery of $T\alpha_1$ from F13. F13 4.3-mg from the preparative separation (Fig. 1) was further fractionated using the conditions described in Fig. 2, Panel D. All the Fractions 22-26 containing immunoreactive $T\alpha_1$ were rechromatographed, and the amount of $T\alpha_1$ was determined by comparison of peak area with synthetic α_1.

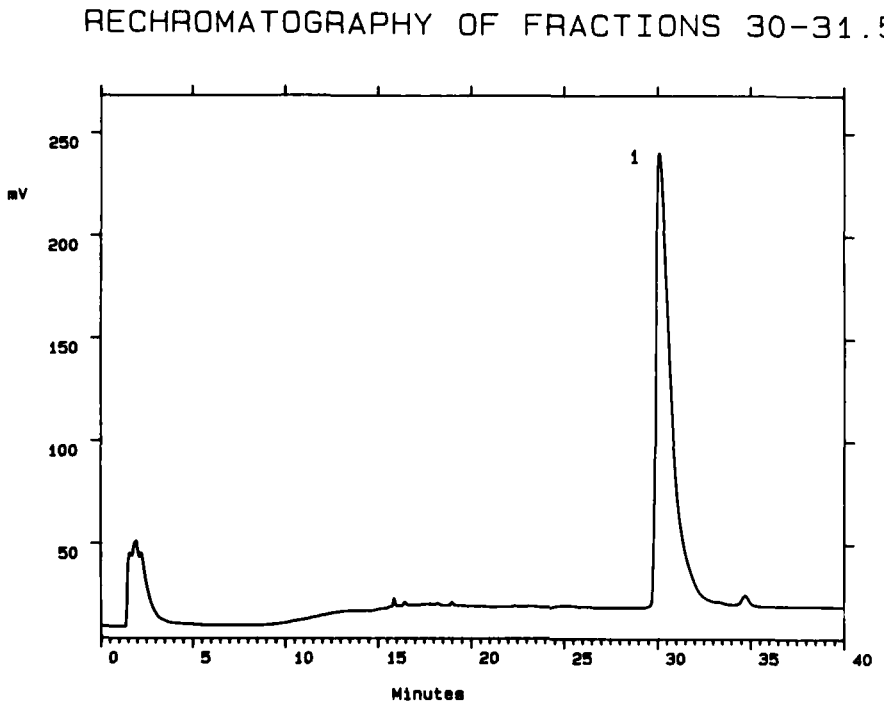

FIGURE 7. Rechromatography of Fraction 30-31.5 from the separation of Fraction 23. The pooled samples were concentrated to 800 μl to remove the acetonitrile. Chromatographic conditions were as described in Fig. 2, Panel D. The $T\alpha_1$ (1) was detected at 214 nm, 0.25 AUFS.

Fractions 30-31.5, containing 60 μg of $T\alpha_1$, from the rechromatography of F23 were pooled and used as the final preparation of $T\alpha_1$ for SDS-PAGE and amino acid composition analysis.

An estimate of the homogeneity of the isolated $T\alpha_1$ (Fig. 7) was obtained by comparing the peak area ratio at 214/220 nm to that of the synthetic α_1. Both gave a 214/220 ratio of 1.76.

SDS-PAGE analysis of the natural and HPLC purified synthetic α_1, illustrated in Fig. 8, was carried out as described in Methods. When the protein bands on SDS gels were stained with Comassie Blue

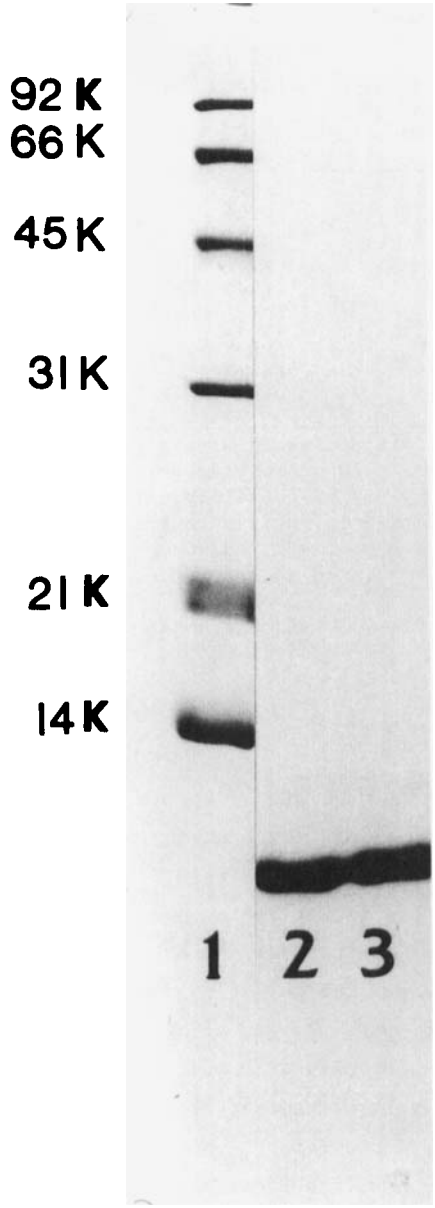

FIGURE 8. SDS-PAGE of synthetic α_1, and Tα_1. About 10-20 μg of Tα_1 samples were electrophoresed on a 1.5-mm 12% SDS-Polyacrylamide gel according to the method of Laemmli (10) and stained with Coomassie Blue R-250. Lane 1: Standard protein. Lanes 2 and 3: synthetic and natural α_1, respectively.

TABLE 2
Amino Acid Composition[a] of Natural and Synthetic Thymosin α_1

Amino Acid	Synthetic α_1	Natural Tα_1	From Reported Sequence[b]
Asp	3.6	4.15	4
Glu	5.9	6.4	6
Ser	2.7	3.1	3
Thr	2.6	2.5	3
Ala	2.9	3.4	3
Val	2.6	3.0	3
Ile	1.0	1.0	1
Leu	1.3	1.1	1
Lys	4.0	4.4	4

Amino acid analysis was performed with a Pico-Tag amino acid analysis system. About 10-μg samples of synthetic and natural α_1 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 standard procedure (11).

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

b. Number of residues obtained from the reported sequence result[4].

R-250, a single identical protein band with a low molecular weight was evident for both natural and synthetic α_1 samples. We were not able to visualize the α_1 protein bands on the SDS-gels with silver stain. As we reported previously (14), Tβ_4 behaves differently i.e. it stains with the silver stain, but not with Comassie Blue R-250. IEF analysis of the natural and HPLC purified synthetic α_1 on an isoelectric focusing slab gel of pH range 3.5-10 revealed a single identical protein band with pI of 4.2 for both natural and synthetic αl samples (results are not shown).

Amino acid analysis of the purified synthetic and natural Tα_1 are shown in Table 2. Our results indicate that synthetic and purified natural Tα_1 have the identical amino acid composition.

In summary, we were able to easily purify enough Tα_1 for characterization from TF5 using this procedure. The presence of Tα_1 was followed by RIA and comparison of the HPLC retention time to that of the synthetic α_1 . The final preparation of Tα_1 is homogenous, and had a similar HPLC retention time, pI and amino acid composition as the synthetic α_1.

ACKNOWLEDGEMENTS

This research was supported in part by a grant CA24974 from National Cancer Institute and by a gift from Alpha 1 Biomedical Inc.

REFERENCES

1. Hooper, J.A., McDaniel, M.C., Thurman, G.B., Cohen, G.H., Schulof, R.S. and Goldstein, A.L., Purification and properties of bovine thymosin, Ann. N.Y. Acad. Sci., 249, 125, 1975.

2. Low, T.L.K., Thurman, G.B., Chincarini, C., McClure, J.E., Marshall, G.D., Hu, S.K. and Goldstein A.L., Current status of thymosin research: evidence for the existence of a family of thymic factors that control T-cell maturation, Annals New York Academy of Science, 33, 33, 1979.

3. Low, T.L.K., Thurman, G.B., McAddo, M., McClure, J.E., Rossio, J.L., Naylor, P.H. and Goldstein, A.L., The chemistry and biology of thymosin. I. Isolation, characterization, and biological activities of thymosin alpha 1 and polypeptide beta 4 from calf thymus, J. Biol. Chem., 254, 981, 1979.

4. Low, T.L.K. and Goldstein, A.L., The chemistry and biology of thymosin. II. Amino acid sequence analysis of thymosin alpha 1 and polypeptide beta 4, J. Biol. Chem., 254, 987, 1979.

5. Ahmed, A., Wong, D.M., Thurman, G.B., Low, T.L.K., Goldstein, A.L., Sharkis, S.J. and Goldschneider, I., T-lymphocyte maturation: cell surface markers and immune function induced by T-lymphocyte cell-free products and thymosin polypeptides, Ann. N.Y. Acad. Sci., 332, 81, 1979.

6. Hu, S.K., Low, T.L.K. and Goldstein, A.L., Modulation of terminal deoxynucleotidyl transferase (TdT) in vitro by thymosin fraction 5 and purified thymosin α_1 in normal thymocytes, Fed Prc. Fed. Am. Soc. Exp. Biol., 9, 1133, 1980a.

7. Vahouny, G.V., Kyeyne-Nyombi, E., McGillis, J.P., Tare, N.S., Huang, K.Y., Tombers, R., Goldstein, A.L. and Hall, N.R., Thymosin peptides and lymphokines do not directly stimulate adrenal corticosteroid production in vitro, J. Immunol., 130, 791, 1983.

8. Goldstein, A.L., Low, T.L.K., McAdoo, M., McClure, J., Thurman, G.B., Rossio, J., Lai, C.-Y., Chang, D., Wang, S.-

S., Harvey, C., Ramel, A.H. and Meienhofer, J., Thymosin alpha 1: Isolation and sequence analysis of an immunologically active thymic polypeptide, Proc. Natl. Acad. Sci. USA., 74, 725, 1977.

9. Low, T.L.K., McClure, J.E., Naylor, P.H., Spangelo, L. and Goldstein, A.L., Isolation of thymosin alpha 1 from thymosin fraction 5 of different species by high-performance liquid chromatography, J. Chromatogr., 266, 533, 1983.

10. Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature, 227, 680, 1970.

11. Bidlingmeyer, B.A., Cohen, S.A. and Tarvin, S.L., Rapid analysis of amino acids using pre-column derivization, J. Chromatogr., 336, 93, 1984.

12. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with the Folin Phenol Reagent, J. Neurochem., 12, 253, 1985.

13. McClure, J.E., Lameris, N., Wara, D.W. and Goldstein, A.L., Immunochemical studies on thymosin: radioimmunoassay of thymosin α_1, J. Immunol., 128, 368, 1982.

14. Badamchian, M., Strickler, M.P., Stone, M.J. and Goldstein, A.L., Rapid isolation of thymosin β_4 from thymosin fraction 5 by preparative high performance liquid chromatography. J.Chromatogr. (In Press).