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ISOLATION AND STRUCTURAL STUDIES OF PORCINE, OVINE AND MURINE THYMOSIN β_4 BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

Rapid high-performance liquid chromatographic (HPLC) procedures have been used to isolate and characterize thymosin β_4 from different species. Crude extracts termed thymosin fraction 5A were prepared from porcine and ovine thymus glands as well as murine spleen. Each fraction 5A preparation was then fractionated by HPLC on a μBondapak C_{18} reversed-phase column. Porcine and ovine thymus fraction 5A, and murine spleen 5A, each yields a predominant peak at a retention time similar to that of bovine thymosin β_4. Amino acid analysis as well as HPLC tryptic peptide mapping of these peaks indicate that they have homologous sequences to bovine thymosin β_4. Chromatographic analysis of fresh murine thymus and spleen tissues also revealed protein peaks at the position of bovine β_4, suggesting that thymosin β_4 is the native protein present in the animal tissues.

INTRODUCTION

The central role of the thymus gland in the development and regulation of cell-mediated immunity is well documented[1]. Restoration of immune functions in neonatally thymectomized mice by thymic tissues enclosed in cell-impermeable Millipore chamber suggested a hormonal mechanism of thymic function[2]. Later, significant influences on the developing lymphoid cells in both animals and humans using crude thymus extracts and progressively well defined molecules from these extracts were reported[3,4]. We have previously reported the isolation and amino acid sequence of two immunologically active polypeptides termed thymosins α_1 and thymosin β_4[5–7]. They were isolated from crude thymic extracts termed thymosin fraction

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5 (f5) and fraction 5A (f5A) by conventional column techniques. The chemical synthesis of both α_1 and β_4 has been accomplished[8,9].

In order to study the species variations of thymic peptides, thymosins f5 and f5A of several species were prepared. The isolation of thymosin α_1 from thymosin f5 of different species by high-performance liquid chromatography (HPLC) has been reported[10]. In this paper, we present the isolation of thymosin β_4 from porcine and ovine thymosin f5A, and murine spleen f5A. The structure of β_4 from different sources was also analyzed by tryptic peptide mapping in HPLC. Porcine and ovine thymosin β_4 as well as murine spleen β_4 exhibit very similar tryptic peptide elution profiles to those of bovine thymosin β_4 indicating that they have homologous structure. HPLC analysis of mouse thymus and spleen tissue extracts also revealed protein peaks at the β_4 position. Our results demonstrate that reversed-phase HPLC greatly facilitates the isolation and identification of homologous proteins or polypeptides.

Recently, thymosin β_4 was found to be present in a number of rat and mouse tissues[11], in tissues of many vertebrate classes[12] and in cultured mammalian cell lines[13]. Our studies described in this paper support those observations. It appears that β_4 is a highly conservative protein and is distributed in different tissues. These results suggest that thymosin β_4 is probably involved in more general biological functions.

EXPERIMENTAL

Materials

Freshly frozen porcine and ovine thymus tissues from young animals (less than 1 year old) were supplied by Max Insel Cohen (Livingston, NJ, U.S.A.). Mouse thymus and spleen tissues for preparation of thymosin f5A were pooled from mice of various ages and strains. Thymus and spleen for murine tissue extractions were from 4–6 weeks-old male C57B1/B6 mice. Trypsin treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK) was purchased from Worthington. Sep-Pak C_{18} cartridges and reversed-phase columns (μBondapak C_{18}, 10 μm, 30 × 0.39 cm) were purchased from Waters Assoc. (Milford, MA, U.S.A.). Trifluoroacetic acid (TFA) was Sequenal grade, obtained from Pierce (Rockford, IL, U.S.A.). Acetonitrile was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Water for HPLC was purified using a Hydro ultrapure water system (Hydro, Rockville, MD, U.S.A.), Model DC2-18 with an HPLC-14 column, Guanidinium chloride (ultra-pure-reagent grade) was from Bethesda Research Labs. (Bethesda, MD, U.S.A.).

Methods

Preparation of porcine and ovine thymosin f5A and murine spleen f5A

Fraction 5A preparations were prepared from frozen pig and sheep thymus glands and fresh mouse spleen tissues according to the purification procedures described previously[5]. As outlined in Fig. 1, tissues were homogenized in a Waring blender. The homogenate was centrifuged at 14,000 *g* for 20 min and the supernatant heated to 80°C. The precipitate of heat-denatured protein was removed by filtration. The filtrate was cooled to 6°C and was added to acetone at −10°C. The precipitate was collected on a Buchner funnel and dried under vacuum (fraction 3). Fraction 3 was then subjected to precipitation with ammonium sulfate. The 50–95% ammonium

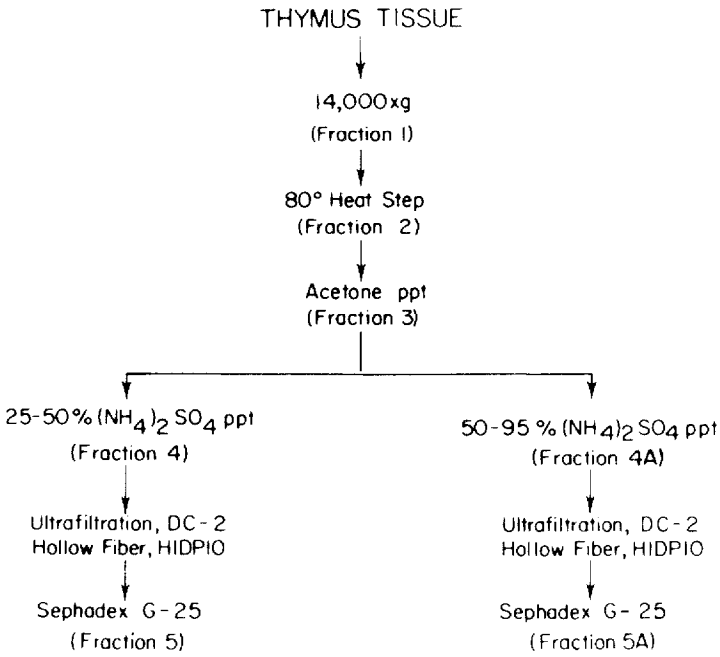

Fig. 1. Purification of bovine thymosin fractions 5 and 5A. One kg of thymus tissue homogenized in 3 l of saline solution provides the sample to initiate the procedure.

sulfate cut was collected and further purified by ultrafiltration with an Amicon DC-2 hollow-fiber system having a molecular weight cut-off at 15,000. The filtrate was desalted on Sephadex G-25. The lyophilized fraction was designated thymosin f5A (when thymus was used) or spleen f5A (when spleen was used).

Preparation of thymosin f5A samples and fresh tissue extracts for HPLC analysis

(1) *For f5A samples.* Fraction 5A preparations (5–7 mg) were suspended in 0.4–0.6 ml of 0.05% TFA, vortexed and centrifuged for 5 min at 5000 *g* on an Adams Analytical Centrifuge (Clay Adams, Parsippany, NJ, U.S.A.) to remove the insoluble materials. The supernatant was used for HPLC analysis.

(2) *For tissue extracts.* Batches of fresh mouse thymus or spleen tissues (0.5–1 g) from 15–30 animals were homogenized in 15 ml of 6 *M* guanidinium chloride using a Polytron homogenizer (Brinkman Instruments; setting 6, 20–30 sec). The homogenates were diluted in 15 ml of 0.1% TFA, vortexed and centrifuged (Sorvall RC3) at 18,000 *g* for 1 h. The floating lipid layer was removed and the supernatant forced through a primed Sep-Pak cartridge with a 20-ml Luer-Lok syringe. The cartridge was washed with 5 ml of 0.05% TFA and the peptides eluted with 2–3 ml of 50% acetonitrile containing 0.05% TFA. The eluates were lyophilized, then suspended in 0.4 ml of 0.05% TFA, vortexed and centrifuged at 5000 *g* to remove the insoluble materials. The supernatant was used for HPLC analysis.

Oxidation of f5A samples

Thymosin or spleen f5A (5–6 mg) was suspended in 5–6 ml of 0.05% TFA and centrifuged to remove the insoluble material. Two milliliters of 30% H_2O_2 were

added to the supernatant, and incubated for 1 h at room temperature. The reaction mixture was then lyophilized. The oxidized products were fractionated by HPLC.

Amino acid analysis

Samples were hydrolyzed with twice-distilled 6 *M* HCl at 110°C for 24 h in evacuated sealed Pyrex glass tubes. The analyses were performed with a Beckman-Spinco amino acid analyzer Model 119 CL, employing single-column methodology on Beckman W-3 resin.

Tryptic digestion

Tryptic digestion was performed in 1% ammonium bicarbonate at pH 8.3 for 2.5 h at 37°C. Trypsin was added to the protein solution to give a final ratio of enzyme to substrate of 1:50 (w/w). The enzymatic digests were lyophilized immediately at the termination of reaction.

Peptide mapping on paper

The tryptic digest of synthetic β_4 was separated by paper chromatography and high-voltage electrophoresis, as described previously[6]. Peptides were detected by staining with cadmium–ninhydrin reagent. To analyze the amino acid composition of the peptides, the unstained chromatogram was stained with fluorescamine. The fluorescent spots were cut out, eluted with water, hydrolyzed and analyzed.

High-performance liquid chromatography

A Hewlett-Packard 1084B liquid chromatograph equipped with a variable wavelength detector (Hewlett-Packard Model 79875A), a fraction collector (Hewlett-Packard Model 79825A) and a reversed-phase column (μBondapak C_{18}) was used. Reservoir A contained 0.05% TFA (pH 2.3) and reservoir B contained acetonitrile with 0.05% TFA. The solvents were filtered through a Millipore filter apparatus (Waters Assoc.) before use. Peak detection was made at 210 nm. The flow-rate was set at 1.5 ml/min for the f5A samples and tissue extracts, and 1 ml/min for eluting the tryptic peptides.

(1) *Fractionation of f5A samples.* An amount of 20–50 μl (0.6–1 mg) of the supernatants prepared from f5A samples as described above was directly applied for HPLC. Peptides were eluted from the column with 15% solvent B for 5 min followed by a linear gradient of 15 to 20% B in 15 min, an isocratic elution at 20% B for 10 min and a second linear gradient of 20 to 50% B in 40 min. The major peak eluting in the region where bovine thymosin β_4 elutes (25.5–27.5 min) was collected, lyophilized and analyzed for amino acid composition. Oxidized f5A samples were fractionated by HPLC using the same procedures.

(2) *Fractionation of fresh tissue extracts.* Supernatants (100 μl) from the fresh tissue extracts were eluted from the column with 10% solvent B for 10 min followed by a linear gradient of 10 to 20% B in 10 min and a second gradient of 20 to 45% B in 45 min. The major peak eluting at the retention time of bovine β_4 (30.04 min) was collected, lyophilized and analyzed for amino acid composition.

(3) *Tryptic peptide mapping.* Tryptic digests of β_4 preparations from different sources were fractionated by HPLC with 1.5% solvent B for 10 min followed by a linear gradient of 1.5 to 35% B in 120 min. Peaks were collected, lyophilized and analyzed for amino acid composition.

RESULTS

Isolation of thymosin β_4 from thymosin or spleen f5A

The separation of thymic peptides in thymosin or spleen f5A was carried out by reversed-phase HPLC on a μBondapak C_{18} column with 15–50% acetonitrile in 0.05% TFA as eluent. As shown in Fig. 2, bovine thymosins α_1 and β_4 were eluted from the column at 21.85 and 27.62 min respectively using the programmed gradient indicated in the figure. The HPLC elution profiles of porcine and ovine f5A as well as murine spleen f5A are shown in Figs. 3–5. The most predominant peak from each chromatographic separation was taken as β_4 and was collected and analyzed by tryptic peptide mapping. The retention times of these β_4 peaks varied from 25.88 min (Fig. 5, murine spleen f5A) to 27.03 min (Fig. 3, porcine f5A). The retention time for bovine thymosin β_4 (synthetic material) also varied from 25.5 to 27.5 min when this peptide was used as a standard and analyzed repeatedly by HPLC. The variation is most likely attributed to the isocratic condition in this region (from 20 to 30 min).

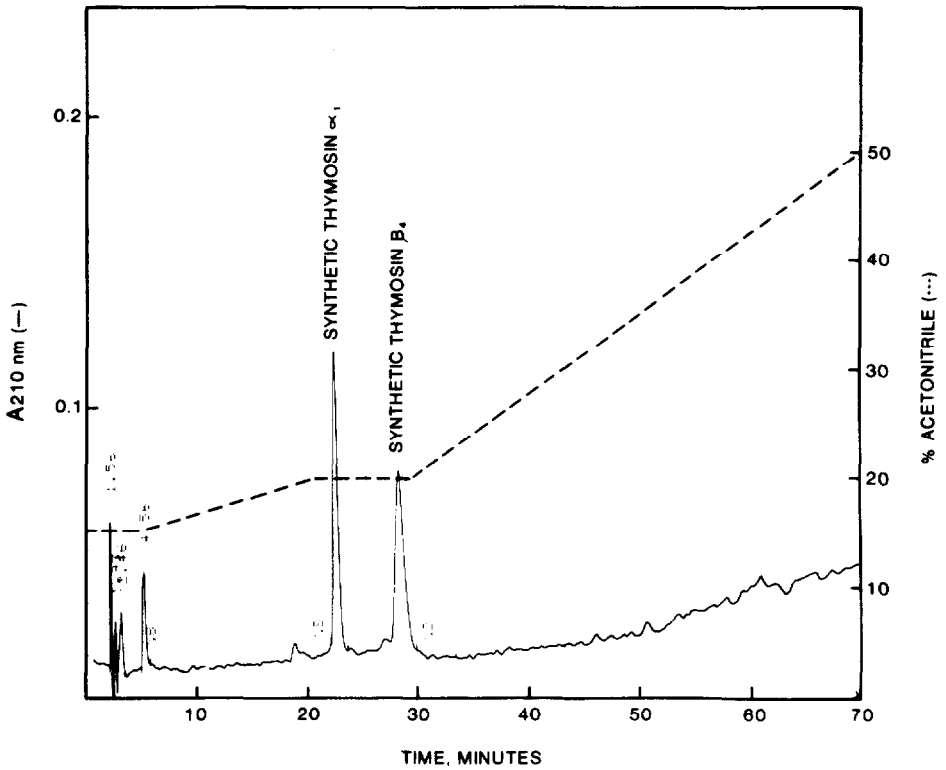

Fig. 2. HPLC analysis of synthetic thymosin α_1 (bovine) and β_4 (bovine) on a 30 × 0.39 cm μBondapak C_{18} column (10 μm, Waters Assoc.) at 35°C. The solvents used were: 0.05% TFA in reservoir A and acetonitrile containing 0.05% TFA in reservoir B. The peptides were eluted with 15% solvent B for 5 min followed by a linear gradient of 15 to 20% B in 15 min, an isocratic elution at 20% B for 10 min and a second linear gradient of 20 to 50% B in 40 min. The flow-rate was set at 1.5 ml/min, with a chart speed of 0.3 cm/min. The elution was monitored by UV absorbance at 210 nm (———). The solvent gradient is indicated by a dashed line (-------).

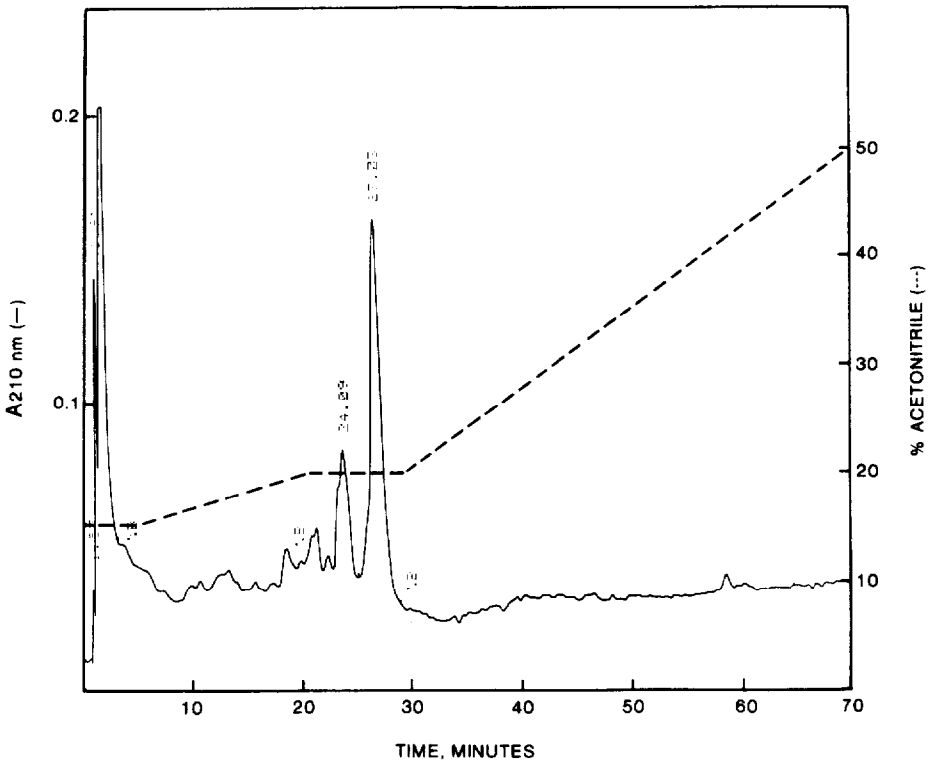

Fig. 3. HPLC analysis of porcine thymosin f5A. Conditions as in Fig. 2.

The amino acid analysis of each β_4 preparation is shown in Table I. The results indicate that porcine and ovine thymosin β_4, and murine spleen β_4, have very similar amino acid compositions to that of bovine thymosin β_4. The amount of β_4 present in f5A samples estimated from the results of amino acid analysis is shown in Table II.

Tryptic peptide mapping by HPLC

The tryptic digests of the β_4 preparations were fractionated by reversed-phase HPLC. Fig. 6 shows the tryptic peptide elution profile of bovine thymosin β_4 (chemically synthesized material). The peaks were collected, hydrolyzed and the amino acid composition determined. The results are listed in Table III. The identification of the peaks in the chromatogram was made by comparing the peptide compositions with the established bovine β_4 sequence[7] as shown in Fig. 7. Peptides T1, T2, T3, T6, T7 and T9 are easily identified. However, peak 2 appears to be a mixture of T8, T10 and T12 and peptides Ta and Tb are probably partial cleavage products and were not identified.

A tryptic peptide map of bovine thymosin β_4 obtained by two-dimensional paper chromatography and high-voltage electrophoresis is shown in Fig. 8. While peptides T8, T10 and T12 are well resolved by the peptide mapping on paper, they were poorly resolved on a μBondapak C_{18} reversed-phase column. These small or

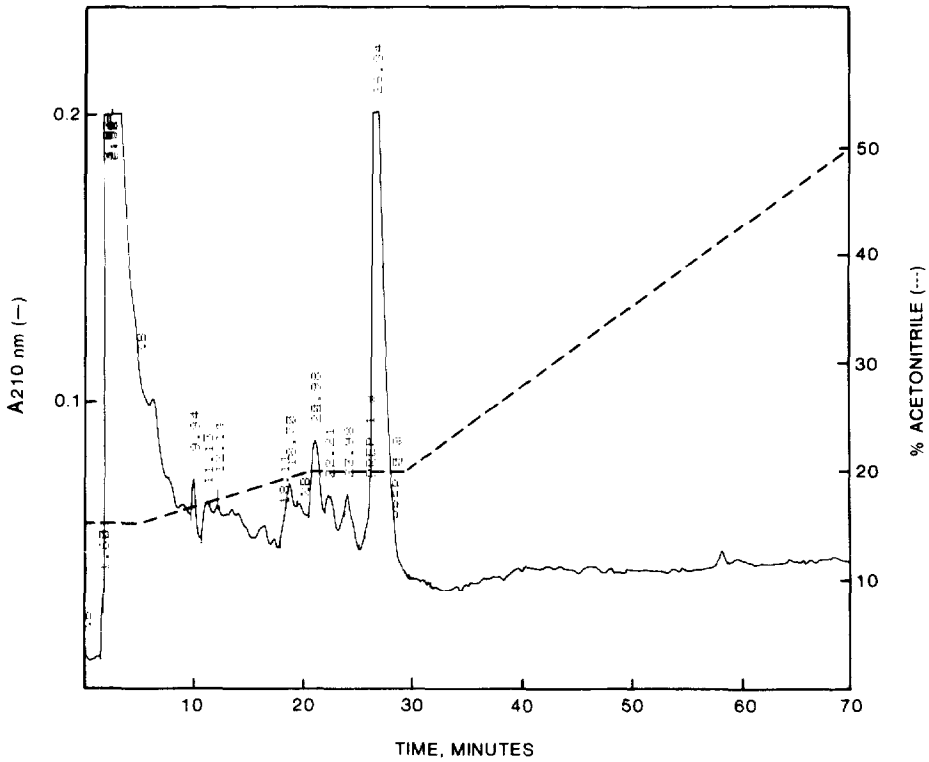

Fig. 4. HPLC analysis of ovine thymosin f5A. Conditions as in Fig. 2.

more hydrophilic tryptic peptides are probably better resolved on columns with smaller particle size, such as Altex Ultrasphere ODS.

The tryptic peptide maps of porcine and ovine thymosin β_4 as well as murine spleen β_4 are shown in Figs. 9–11. The amino acid compositions of these peptides are listed in Tables IV, V and VI. Peptides T1, T2, T6, T7 and T9 are clearly identifiable in each case. However, peak 1 in each of Figs. 9–11 appears to be a mixture of T_3 and T_8, and peak 2, a mixture of T10 and T12. The elution profiles of these tryptic digests are very similar, and the retention times for the major peaks are highly reproducible.

Isolation of β_4 from murine thymus and spleen tissue

As shown in Figs. 12 and 13, when murine thymus and spleen tissue extracts were applied directly to HPLC, predominant peaks at the β_4 position were observed. The program used for separating the tissue extracts was different from the one used for separating f5A samples. The retention time for β_4 was found to be highly reproducible. When synthetic β_4 was injected repeatedly in HPLC it was eluted at 29.6–30.1 min. HPLC tryptic peptide mapping of these peaks (data not shown) reveals that they contained all the major β_4 tryptic peptides in addition to other peaks. The results suggest that β_4 is present in murine thymus and spleen tissues. However, the

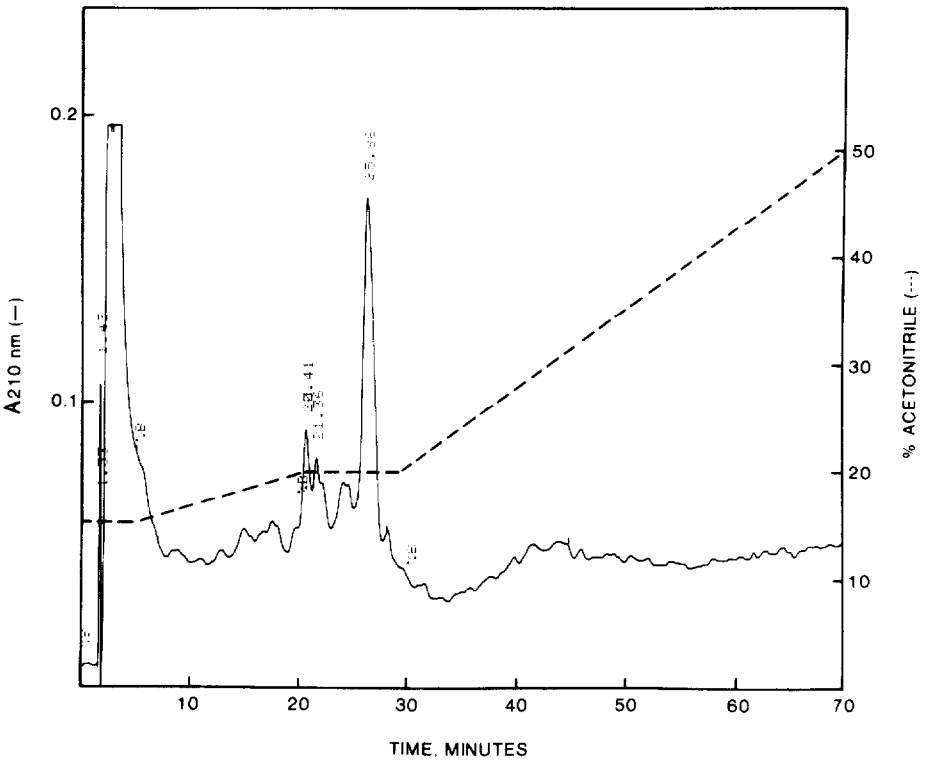

Fig. 5. HPLC analysis of murine spleen f5A. Conditions as in Fig. 2.

β_4 preparations obtained from the tissue extracts are not as pure as the β_4 obtained from f5A which has been partially purified by several conventional techniques.

Oxidation of f5A samples

Hannappel *et al.*[14] reported the isolation and sequencing of two peptides, thymosin β_8 and β_9, homologous to thymosin β_4. However, no biological study was reported for either molecule. As shown in Fig. 14, of the 38 amino acid residues in thymosin β_8, 31 are identical to the corresponding residues in thymosin β_4. Thymosin β_9 is identical to thymosin β_8 except for the presence of an additional dipeptide, -Ala-Lys-OH at the carboxyl terminus. Since thymosin β_8 and β_9 do not contain methionine as β_4 does, oxidation would modify β_4 but not β_8 or β_9. In order to be certain that the β_4 peaks from the f5A preparations were not contaminated with β_8 or β_9, f5A samples were oxidized with H_2O_2 and rechromatographed by HPLC. As shown in Figs. 15–17, the oxidized β_4 peaks were eluted 3–4 min earlier than the unoxidized peptides. No or only a trace amount of material was detected at the original β_4 positions indicating that there was no (or traces of) β_8 or β_9 coeluted with β_4 in the initial f5A elution profiles (Figs. 3–5). This was further supported by the absence of tryptic peptides from β_8 or β_9 identifiable in Figs. 9–11.

TABLE I

THE AMINO ACID COMPOSITION OF PORCINE AND OVINE THYMOSIN β_4, AND MURINE SPLEEN β_4

Results from 6 *M* HCl hydrolysates at 110°C for 24 h.

Amino acid	*Thymosin β_4 obtained from*			
	*Bovine thymus**	*Porcine thymus*	*Ovine thymus*	*Murine spleen*
Asp	4.23 (4)**	4.62	4.46	4.38
Thr	2.62 (3)	2.60	2.58	2.41
Ser	3.73 (4)	3.25	3.16	2.22
Glu	11.58 (11)	10.95	11.06	9.69
Pro	3.85 (3)	3.93	4.46	3.88
Gly	1.35 (1)	1.32	1.14	1.15
Ala	2.30 (2)	2.38	2.01	2.03
Val	0.37 (0)	0.48	0.32	0.59
1/2 Cys	0 (0)	0	0	0
Met	0.52 (1)	0.88	0.85	0.86
Ile	1.85 (2)	1.70	1.64	1.63
Leu	2.05 (2)	1.81	1.89	2.17
Tyr	0 (0)	0	0	0
Phe	0.85 (1)	0.86	0.94	0.83
Lys	8.71 (9)	8.23	8.25	7.88
His	0 (0)	0	0	0
Arg	0 (0)	0.23	0.24	0.37

* From ref. 7.
** From the sequence result[7].

DISCUSSION

This paper illustrates the use of reversed-phase HPLC for the isolation and identification of thymosin β_4 from different species using bovine thymosin β_4 as a model peptide. Thymosin β_4 preparations were identified by the HPLC retention time, amino acid analysis and HPLC tryptic peptide mapping. Our results indicate that porcine and ovine thymosin f5A, and murine spleen f5A, each contains a peptide homologous to bovine β_4. Furthermore, murine thymus and spleen tissue extracts also yield peaks at the β_4 position suggesting that β_4 is the native protein in animal

TABLE II

RECOVERY OF THYMOSIN β_4 FROM PORCINE AND OVINE THYMOSIN f5A AND MURINE SPLEEN f5A

Preparation	*Retention time of β_4 peak (min)**	*Recovery***
Porcine thymosin f5A	27.03	76.5
Ovine thymosin f5A	26.84	127.4
Murine spleen f5A	25.88	30.5

* From Figs. 3–5.
** Values are expressed as μg per mg of f5A samples.

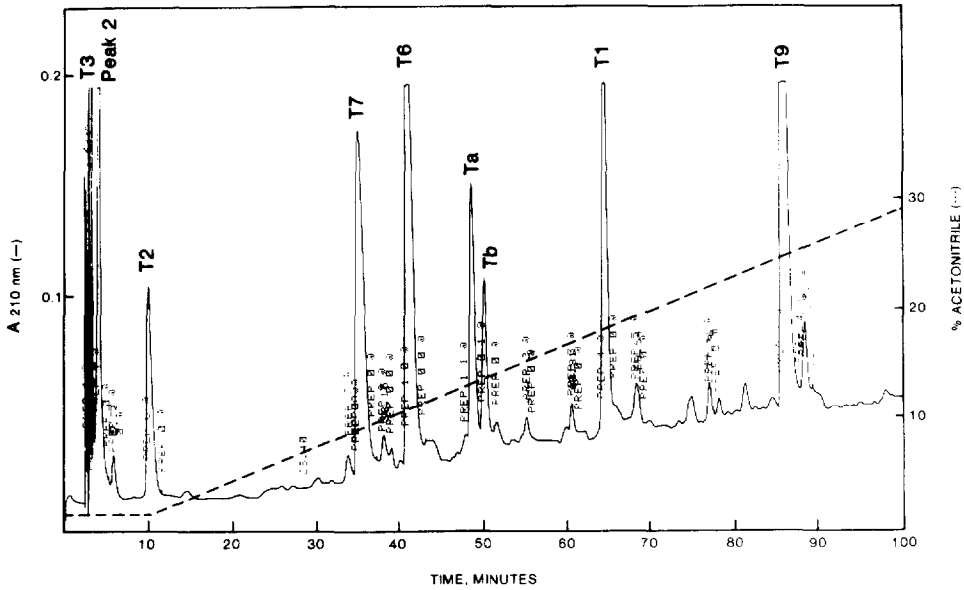

Fig. 6. HPLC tryptic peptide map of bovine thymosin β_4 (synthetic). Digestion with trypsin was carried out in 1 ml of 1% ammonium bicarbonate containing 550 μg β_4 and 11 μg trypsin for 2.5 h at 37°C. The digest was lyophilized, resuspended in 200 μl of 0.05% TFA. Forty microliters were injected onto the μBondapak C_{18} column and peptides were eluted with 1.5% solvent B for 10 min followed by a linear gradient of 1.5 to 35% B in 120 min. Peaks were collected, lyophilized and analyzed for amino acid composition (Table III). The major peaks were identified by comparison with the established bovine β_4 sequence (see Fig. 7).

TABLE III

AMINO ACID COMPOSITION OF THE TRYPTIC PEPTIDES OF BOVINE THYMOSIN β_4 (SYNTHETIC MATERIAL)

Results from 6 *M* HCl hydrolysates at 110°C for 24 h.

Amino acid	*T1*	*T2*	*T3*	*T6*	*T7*	*T9*	*Peak 2*
Asp	2.13	1.18	0.06	1.16		2.31	0.04
Thr	0.35				1.13		1.08
Ser	1.04		1.00	0.86		0.70	1.03
Glu	2.93		0.08		4.39	1.74	3.98
Pro	1.08			2.09		0.96	
Gly	0.38		0.10				0.96
Ala	1.13					1.00	1.06
Val							
Met	0.79						
Ile	0.59				1.08	1.04	
Leu				0.96			0.97
Tyr							
Phe		0.97				0.69	
Lys	2.04	0.93	1.01	0.92	0.85	2.23	3.06

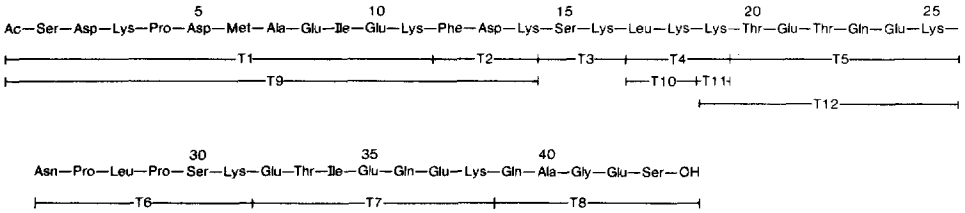

Fig. 7. Complete amino acid sequence of bovine thymosin β_4. Line segments (T1–T12) denote the peptides isolated after digestion of thymosin β_4 with trypsin.

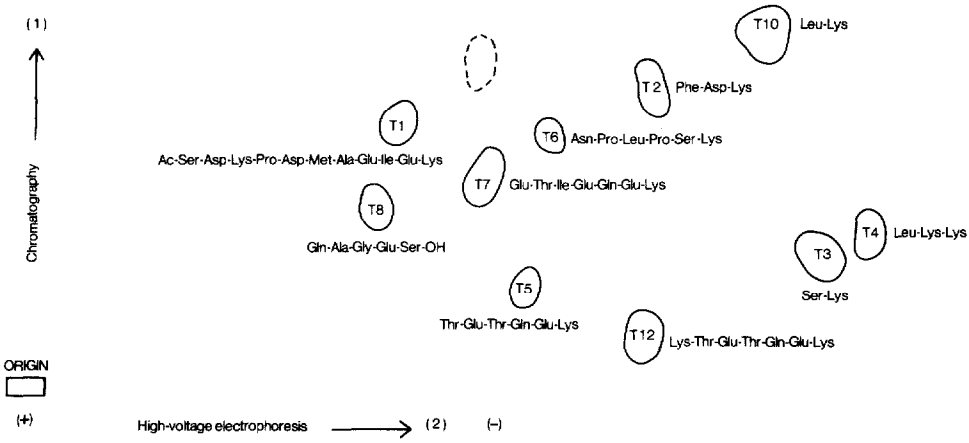

Fig. 8. Paper tryptic peptide map of bovine thymosin β_4. Chromatography was carried out in the first dimension in 1-butanol–glacial acetic acid–water (4:1:5, v/v) and high-voltage paper electrophoresis at pH 1.9 was carried out in the second dimension.

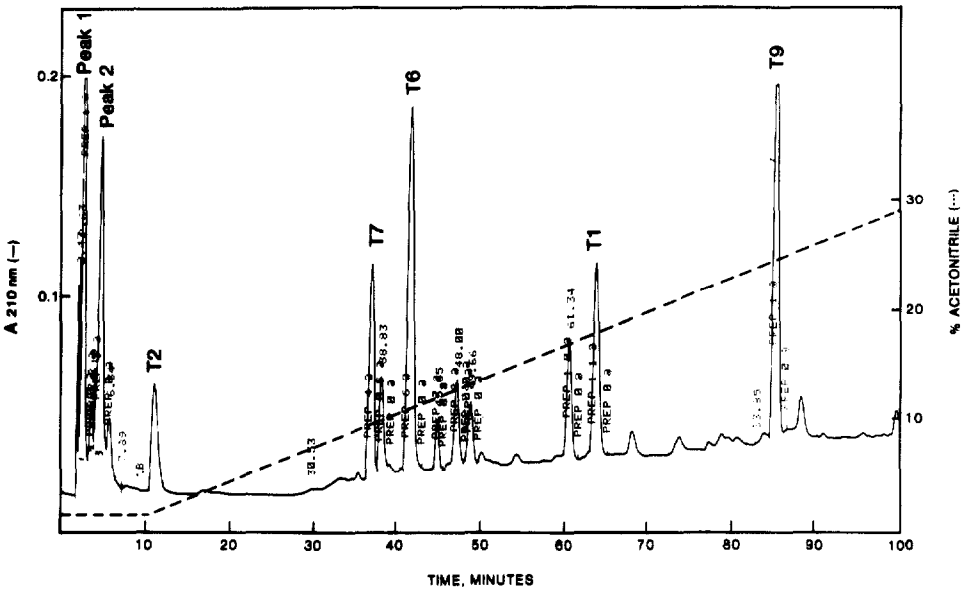

Fig. 9. HPLC tryptic peptide map of porcine thymosin β_4. An amount of 460 μg of collected β_4 peaks was digested with trypsin, lyophilized and resuspended in 400 μl of 0.05% TFA. One hundred microliters were injected for HPLC. Conditions as in Fig. 6. The aminon acid composition of the major peaks is listed in Table IV.

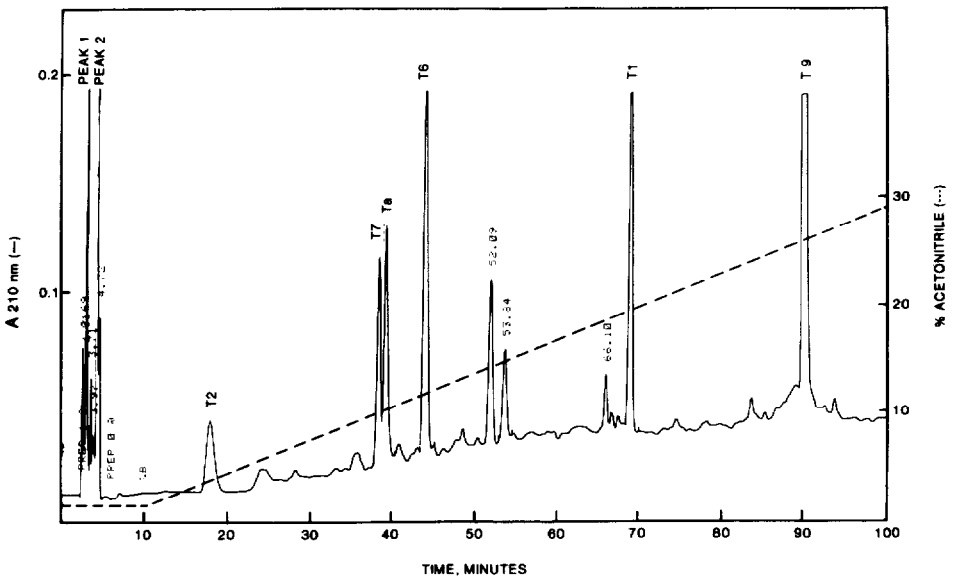

Fig. 10. HPLC tryptic peptide map of ovine thymosin β_4. An amount of 1.34 mg of collected β_4 peaks was treated with trypsin, lyophilized and resuspended in 200 μl of 0.05% TFA. Fifty microliters were injected for HPLC and analyzed as described in Fig. 6. The amino acid composition of the major peaks is listed in Table IV.

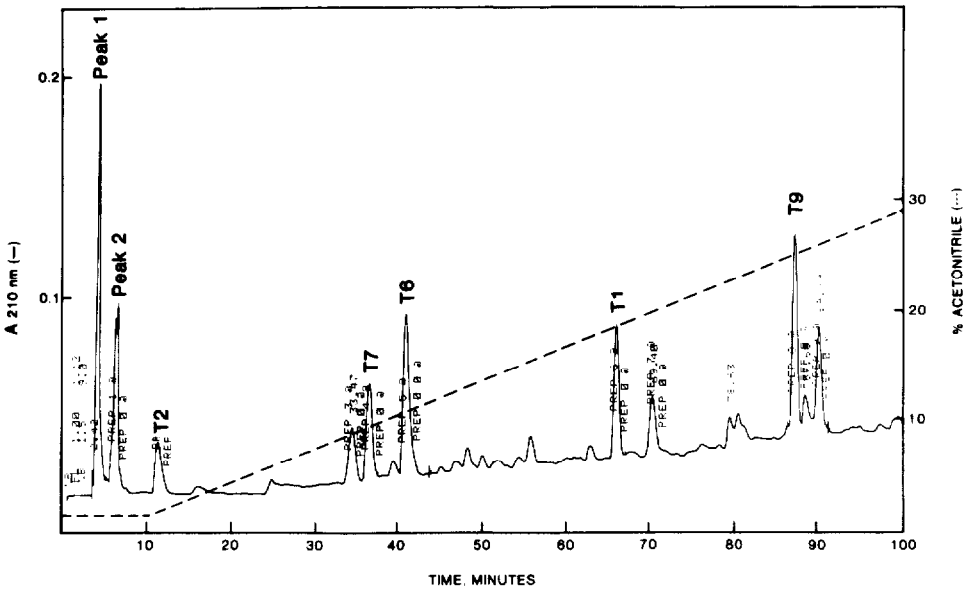

Fig. 11. HPLC tryptic peptide map of murine spleen β_4. An amount of 117 μg of collected β_4 peaks was digested with trypsin, lyophilized and resuspended in 400 μl of 0.05% TFA. Two hundred microliters were injected for HPLC and analyzed as described in Fig. 6. The amino acid composition of the major peaks is listed in Table VI.

TABLE IV

AMINO ACID COMPOSITION OF THE TRYPTIC PEPTIDES OF PORCINE THYMOSIN β_4

Results from 6 *M* HCl hydrolysates at 110°C for 24 h.

Amino acid	*T1*	*T2*	*T6*	*T7*	*T9*	*Peak 1*	*Peak 2*
Asp	1.24	1.06	0.95		2.63		
Thr				0.73			0.41
Ser	0.62		0.68		0.68	2.55	
Glu	1.40			4.11	2.25	2.25	2.79
Pro			2.40				
Gly						0.88	
Ala	0.68				0.80	0.79	
Val							
Met	0.63				0.95		
Ile	0.70			0.96	0.95		
Leu			0.95				1.06
Tyr							
Phe		0.87			0.92		
Lys	1.35	0.83	0.88	0.99	2.56	6.16	2.13

tissues. Oxidation experiments and tryptic peptide mapping indicate that no (or traces of) β_8 or β_9 was present in f5A samples.

Synthetic thymosin β_4 was chromatographed on the reversed-phase column using two different elution programs. As shown in Figs. 3–5, β_4 was eluted at 25.88–27.03 min. It was found that the retention time of synthetic β_4 varied from 25.5 to 27.5 min upon repeated injections, which may be attributed to the isocratic conditions between 20 and 30 min in this program. On the other hand, when β_4 was eluted in HPLC using a gradient program (Figs. 12, 13), the retention time upon repeated injection was highly reproducible.

TABLE V

AMINO ACID COMPOSITION OF THE TRYPTIC PEPTIDES OF OVINE THYMOSIN β_4

Results from 6 *M* HCl hydrolysates at 110°C for 24 h.

Amino acid	*T1*	*T2*	*T6*	*T7*	*T9*	*Peak 1*	*Peak 2*
Asp	2.04	0.89	0.96		2.84		
Thr				1.05		–	2.02
Ser	0.99		0.80		1.39	2.08	
Glu	1.85			4.25	2.08	1.11	3.34
Pro	0.96		2.09		0.88		
Gly						0.94	
Ala	0.87				1.20	0.78	
Val							
Met	0.45						
Ile	0.92			0.98	1.11		
Leu			1.07				0.87
Tyr							
Phe		1.07					
Lys	1.78	1.07	1.03	0.81	2.58	1.94	2.28

TABLE VI

AMINO ACID COMPOSITION OF THE TRYPTIC PEPTIDES OF MURINE SPLEEN β_4

Results from 6 *M* HCl hydrolysates at 110°C for 24 h.

Amino acid	*T1*	*T2*	*T6*	*T7*	*T9*	*Peak 1*	*Peak 2*
Asp	2.23	1.08	1.06		3.07		
Thr				1.04			2.09
Ser	0.65		0.61		0.65	2.35	
Glu	2.31			4.31	2.37	2.65	3.19
Pro	0.89		2.54		0.90		
Gly						1.33	
Ala	1.07				0.92	0.91	
Val							
Met	0.73				0.67		
Ile	0.67			0.77	0.63		
Leu			0.90				0.91
Tyr							
Phe		0.95					
Lys	1.63	0.87	0.94	0.79	2.45	5.54	1.98

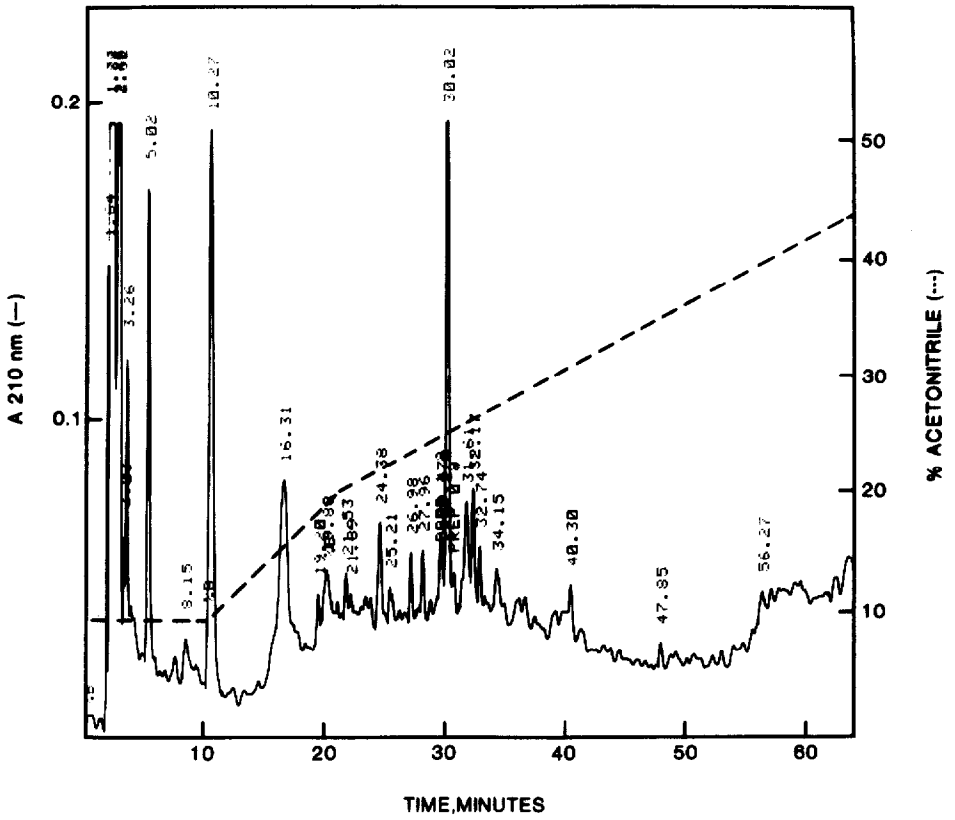

Fig. 12. HPLC analysis of murine thymus tissue extract on a 30 × 0.39 cm μBondapak C_{18} column (10 μm, Waters Assoc.) at 35°C. The peptides were eluted with 10% solvent B for 10 min followed by a linear gradient of 10 to 20% B in 10 min and a second gradient of 20 to 45% B in 45 min. Other conditions as in Fig. 2.

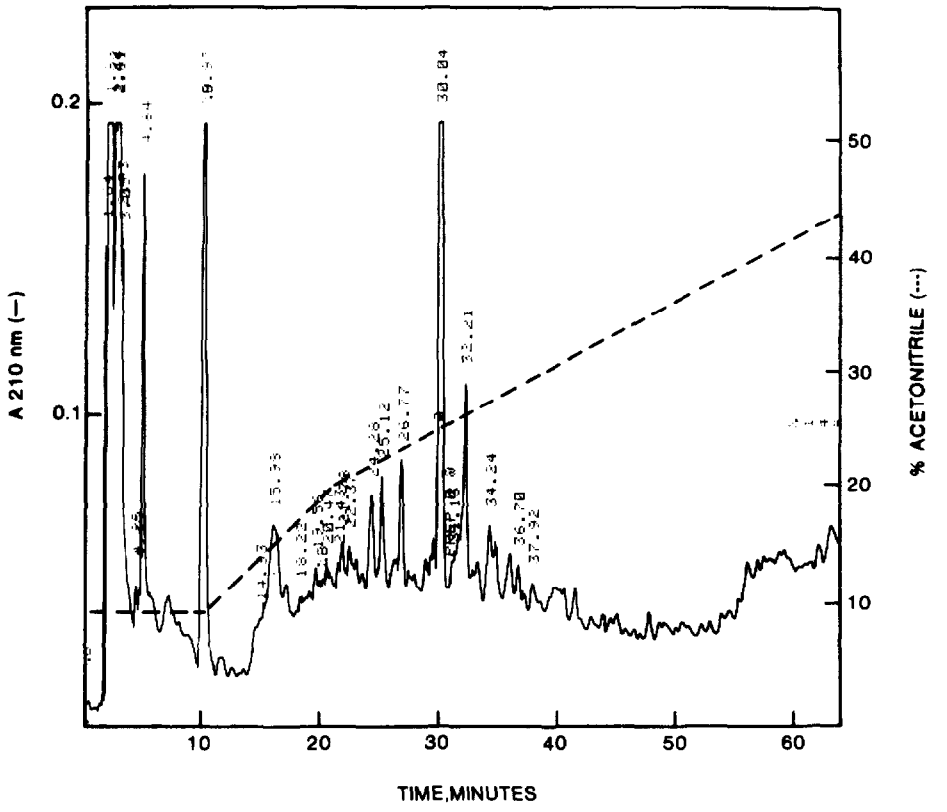

Fig. 13. HPLC analysis of murine spleen tissue extract. Conditions as in Fig. 12.

	5	10	15	20	25
β_4	Ac-Ser-Asp-Lys-Pro-Asp-Met-Ala-Glu-Ile-Glu-Lys-Phe-Asp-Lys-Ser-Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-				
β_7	___ Ala	Leu-Gly	Asn-Ser	Ala	
β_8	___ Ala	Leu-Gly	Asn-Ser	Ala	
β_9	___ Ala	Leu-Gly	Asn-Ser	Ala	
β_{10}	x Ala	Gly	Ala-Ser	Ala	
β_{10}^{arg}	x Ala	Gly	Ala-Ser	Ala	
β_{11}	x	Leu-Glu	Val-Ala-Ser	Thr	

	30	35	40
β_4	Asn-Pro-Leu-Pro-Ser-Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-Gly-Glu-Ser-OH		
β_7	___Thr	Thr	Lys -OH
β_8	___Thr	Thr	OH
β_9	___Thr	Thr	Lys -OH
β_{10}	___Thr	Thr	Ser-Glu-Ile -Ser-OH
β_{10}^{arg}	___Thr	Thr	Arg-Ser-Glu -Ile
β_{11}		Thr	Ser -OH

Fig. 14. Amino acid sequences of thymosin β_4, β_7, β_8, β_9, β_{10}, β_{10}^{arg} and β_{11}. A line indicates identity with thymosin β_4.

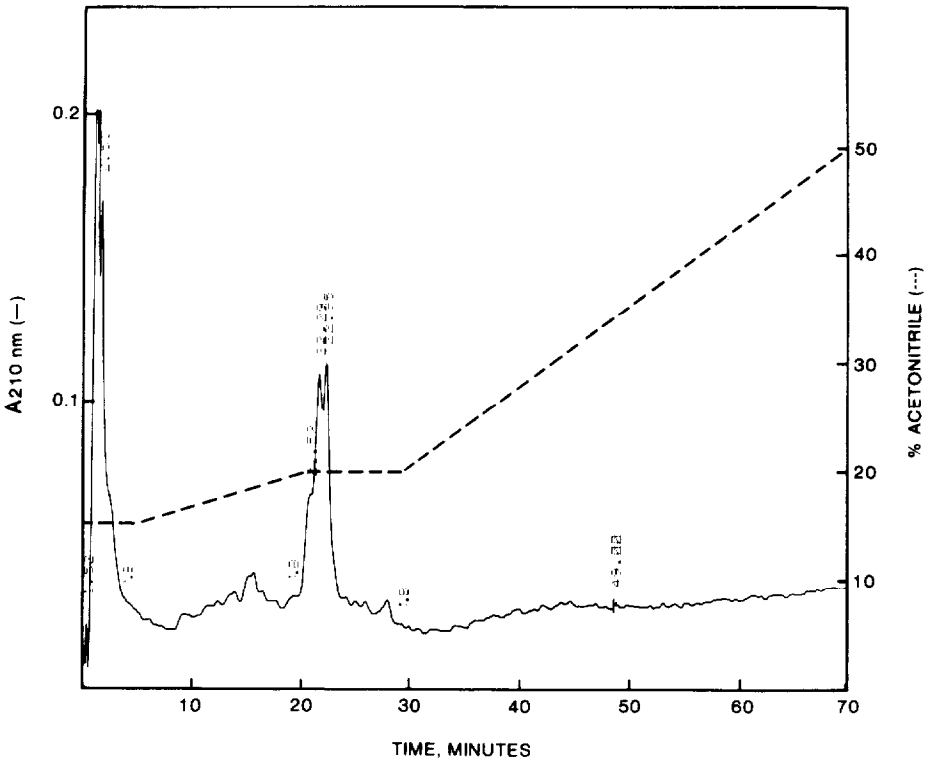

Fig. 15. HPLC analysis of oxidized porcine thymosin f5A. Conditions as in Fig. 2.

Isolation of thymosin β_4 from thymosin f5A by conventional chromatographic techniques is time-consuming and requires a large amount of starting material (at least 1 g of thymosin f5A). Using the HPLC procedures, it is possible to isolate and identify thymosin β_4 from 1–5 mg of thymosin f5A. Isolation of thymosin β_4 directly from the animal tissues is also possible. However, the β_4 preparations obtained from HPLC of tissue extracts are not as clean as those obtained from f5A.

Several small or hydrophilic tryptic peptides were poorly resolved using a μBondapak C_{18} column. In Figs. 9–12, peaks 1 appear to be a mixture of T3 and T8, and peaks 2 a mixture of T10 and T12. These peptides were eluted from the column with 0% acetonitrile before the gradient started. An attempt to separate these peptides using an Ultrasphere ODS (Altex) column did not improve the resolution. Hannappel *et al.*[14] separated tryptic peptides from β_4 by HPLC using an Ultrasphere ODS column, a fluorescence detection system and a solvent system consisting of pyridine–formic acid–acetonitrile. In their case, peptides T3 (Ser-Lys), T8 (Gln-Ala-Gly-Glu-Ser) and T10 + T12 (Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys) were retained by the column to different extents and thus were eluted individually by an acetonitrile gradient. The difference in resolution using identical columns, yet different aqueous buffers demonstrates that the ion-pairing property of 0.2 *M* pyridine–1 *M* formic acid seems to supercede that of TFA in separating small or hydrophilic peptides. Since most HPLC systems, including ours, are equipped with

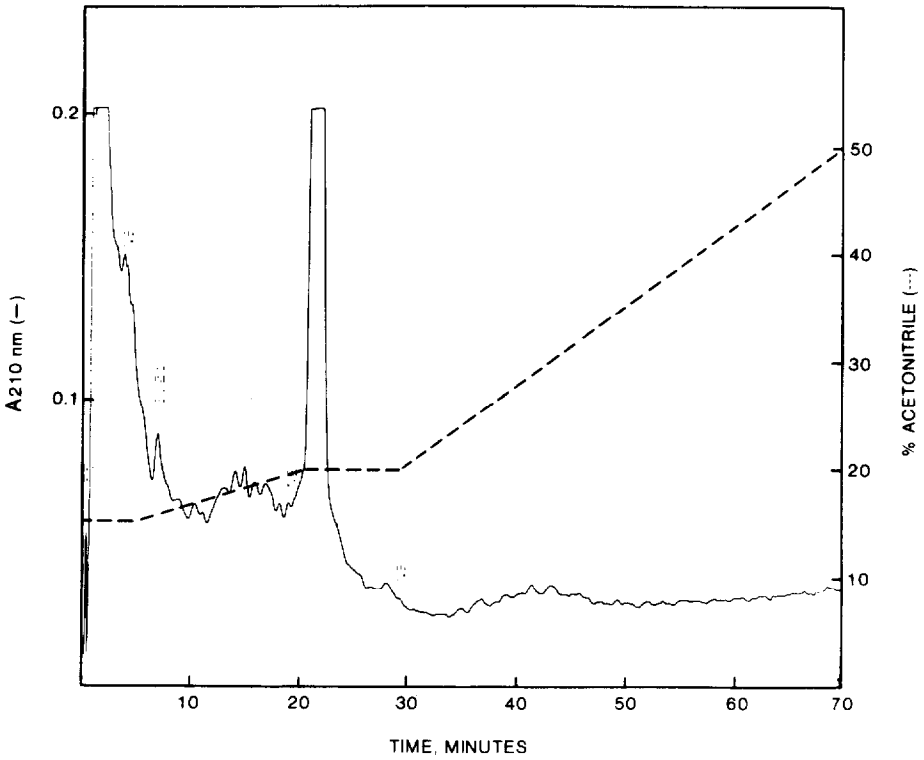

Fig. 16. HPLC analysis of oxidized ovine thymosin f5A. Conditions as in Fig. 2.

UV detectors operated at 210 nm for peptides, it is not possible for us to use pyridine–formic acid as solvent which absorbs strongly in the UV region. When hexafluorobutyric acid (HFBA) was used in place of TFA, the peptides appeared to be retarded somewhat by the column. However, the high noise background in the HPLC profile generated by the HFBA buffer offsets the usefulness of this method. A better ion-pairing capacity might be obtained by the addition of ethanolamine to TFA. Alternatively, the tryptic peptides can be separated by two-dimensional chromatography using ion-exchange HPLC followed by separation on a reversed-phase column.

The isolation of thymosin β_4 from tissue extracts and cultured cell lines was recently reported by Horecker and co-workers[11–13]. They found that β_4 was present in all vertebrate classes except bony fishes. The following peptides related to β_4 have also been reported: β_9 in calf thymus tissue extract[14]; β_{10} in human, cat, rat and mouse spleen extracts[15]; β_{10}^{arg} in rabbit thymus, spleen and liver extracts[16] and β_{11} in bony fish spleen extract[17]. Their isolation was achieved by HPLC procedures on frozen or fresh tissue extracts. We have previously reported the isolation of thymosin β_7 from calf thymosin f5A[6]. Preliminary structural studies (T. L. K. Low and A. L. Goldstein, unpublished results) indicate that β_7 has an identical amino acid sequence to β_9. Thus, this molecule can be isolated from either bovine thymosin f5A or frozen calf thymus tissue.

Our studies on the isolation and characterization of thymosin β_4 from porcine

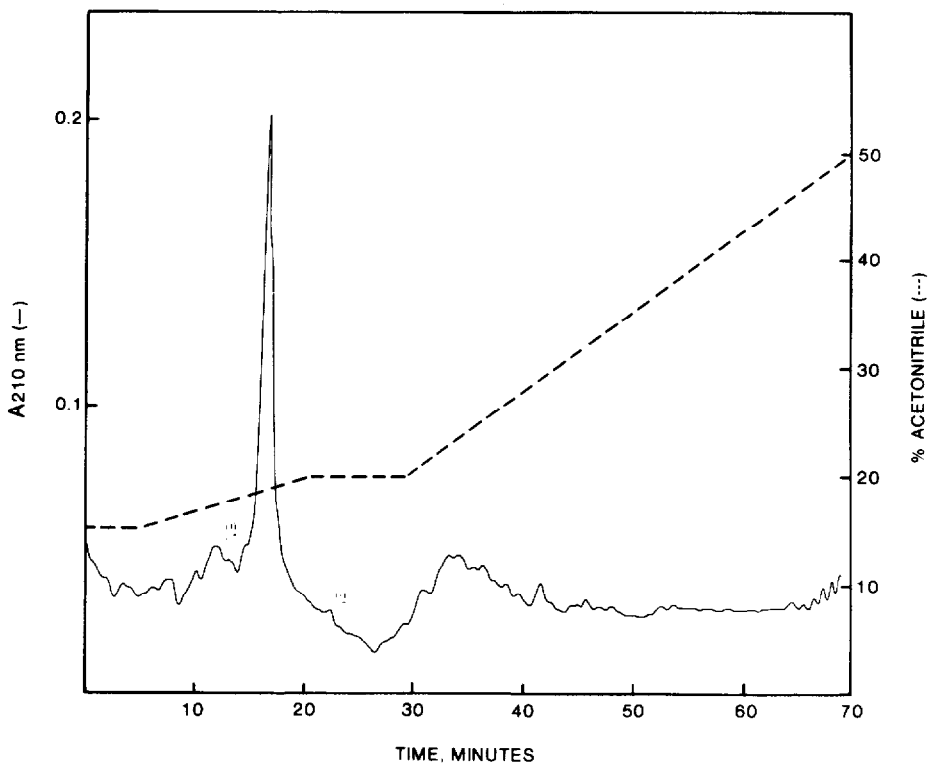

Fig. 17. HPLC analysis of oxidized murine spleen f5A. Conditions as in Fig. 2.

and ovine thymosin f5A as well as murine spleen f5A support the results of Horecker and co-workers[11–13] in that β_4 is a highly conservative protein. This peptide is also present in tissues other than the thymus gland. Based on the distribution and high concentration of this peptide in various tissues, it has been suggested[13] that β_4 plays a general biological role, possibly as a component of the cytoskeletal system. However, a computer search for a structural relationship of β_4 to the cytoskeletal proteins did not reveal any significant homology[18].

The fact that the same size of molecules is present in thymosin f5A and in tissue extracts indicates that β_4 is probably the native protein. This is further supported by the studies of Filipowicz and Horecker[19]. Using a cell-free wheat germ system, they found that the product immunoprecipitable with anti-β_4 was identical to thymosin β_4 with no indication of the formation of a larger precursor polypeptide.

Our biological studies have shown that β_4 is very active in inducing the expression of terminal deoxynucleotidyl transferase in murine bone marrow cells[20,21] and in stimulating the production of macrophage migration inhibitory factor[22]. These results indicate that β_4 is very important in modulating T-cell differentiation. Many questions remain regarding the actual biological functions as well as the site of synthesis of thymosin β_4. Nevertheless, the fact that it is possible to isolate and identify this molecule very easily by HPLC techniques would facilitate further chemical and biological investigations to document the role of this molecule in the immune system.

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