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ISOLATION OF THYMOSIN α_1 FROM THYMOSIN FRACTION 5 OF DIFFERENT SPECIES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

High-performance liquid chromatography (μBondapak C_{18} column with 0.05% trifluoroacetic acid in acetonitrile as solvent system) was used to isolate thymosin α_1 (α_1) from thymosin fraction 5 (f5) of various species (calf, pig, sheep and mouse). Each of the f5 preparations gave a protein peak similar in retention time to bovine thymosin α_1. This peak coincided with the immunoreactive peak determined by a radioimmunoassay for α_1. Chromatographic analysis of fresh thymus tissue extracts using a high-performance liquid chromatographic similar system did not reveal a detectable protein peak or immunoreactive peak at the α_1 position. Our results suggest that α_1 may be synthesized in a precursor form in animal tissues.

INTRODUCTION

The essential role of thymus in the development, growth and function of the lymphoid system and in the maintenance of immune balance has now been fully recognized[1–3]. Our previous studies demonstrated that a partially purified bovine thymic preparation termed thymosin fraction 5 (f5) could partially or fully reconstitute immune functions in immunodeficient animals[4–6] and in human subjects with primary immunodeficiency diseases[7,8], autoimmune diseases[9] and cancer[10–12]. Our ongoing studies indicate that there is a family of biologically active polypeptides within f5 that acts on T-cell populations to maintain normal immunological reactivity[13,14].

Several components of bovine thymosin f5 such as thymosin α_1, β_4 and polypeptide β_1 have been isolated and amino acid sequence determined[15–17]. As shown in Fig. 1, bovine thymosin α_1 is a polypeptide consisting of 28 amino acid residues. It is highly active in amplifying T-cell immunity[16,18] and is capable of modulating the expression of terminal deoxynucleotidyl transferase (TdT)[19,20]. Thymosin β_4 consists of 43 amino acid residues. This peptide induces expression of TdT activity both *in vivo*[19] and *in vitro*[21], and also inhibits the migration of macrophages[22]. The chemical synthesis of thymosin α_1 (ref. 23) and β_4 (ref. 24) has been accomplished.

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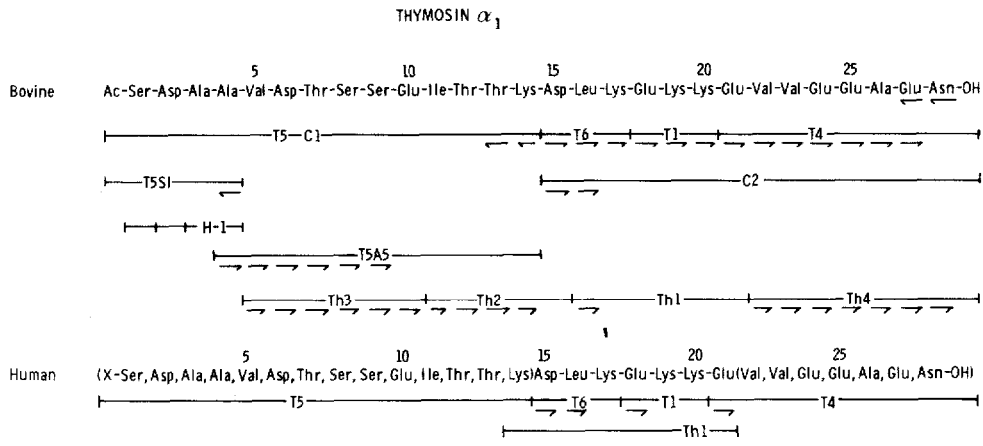

Fig. 1. Complete amino acid sequence of bovine thymosin α_1 and partial sequence of human thymosin α_1. Results show that these two sequences appear to be identical[14]. Line segments denote the peptides isolated after digestion of thymosin α_1 with trypsin (T1, etc.), chymotrypsin (C1, etc.), and thermolysin (Th1, etc.), and those from peptide T5 on digestion with subtilisin (T5S1) and α-protease (T5A5). Partial acid hydrolysis of T5S1 produced free serine, aspartic acid and the dipeptide Ala–Ala (H-1). Arrows pointing to the right indicate residues degraded sequentially by the dansyl-monitored or subtractive Edman procedures, or both; those pointing to the left denote sequences elucidated by carboxypeptidase digestion. Ac = Acetyl. Sequence of human α_1 was only partially completed. The amino acids in parenthesis are from amino acid composition, rather than sequence determination. The N-terminal blocking group in human α_1 was not identified and is designated by an X. The tryptic peptides in human α_1 were placed by homology to the tryptic peptides in bovine α_1.

In order to study the species variations of thymosin polypeptides, f5 of several species (human, calf, pig, sheep and mouse) were prepared. We have previously reported[14] the isolation and partial amino acid sequence of thymosin α_1 from human thymosin f5 achieved by conventional column techniques (ion-exchange chromatography on CM-cellulose and gel filtration on Sephadex G-50). From the partial sequence obtained for human α_1 as shown in Fig. 1, the human and bovine thymosin α_1 appear to have an identical sequence[14].

In this paper, we report the isolation of thymosin α_1 from various f5 preparations by reversed-phase high-performance liquid chromatography (HPLC). The peptides were detected by UV absorbance at 210 nm. Furthermore, the collected fractions were analyzed by a radioimmunoassay for thymosin α_1. Our results indicate that each of the f5 preparations examined contains a peptide with similar or identical structure to bovine α_1. On the other hand, chromatographic analysis of fresh thymus tissue extracts with a similar HPLC system did not reveal a detectable polypeptide peak or immunoreactive peak at the α_1 position. These observations suggest that α_1 may be synthesized in a precursor form in animal tissues.

EXPERIMENTAL

Materials

Freshly frozen bovine, porcine and ovine thymus tissues from young animals (less than 1 year old) were supplied by Max Insel Cohen (Livingston, NJ, U.S.A.) or Manassas Slaughter House (Manassas, VA, U.S.A.). Mouse thymus tissues were

pooled from mice of various ages and strains. Rat thymus tissues were from male Sprague–Dawley rats. 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 added HPLC-14 column. Guanidinium chloride (ultra-pure-reagent grade) was from Bethesda Research Labs. (Bethesda, MD, U.S.A.).

Methods

Preparation of bovine, porcine, ovine and murine thymosin f5. Thymosin f5 was prepared from frozen pig, sheep and calf thymus glands and fresh mouse thymus tissues according to the purification procedures as described previously[25]. As outlined in Fig. 2, thymus tissue was 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 am-

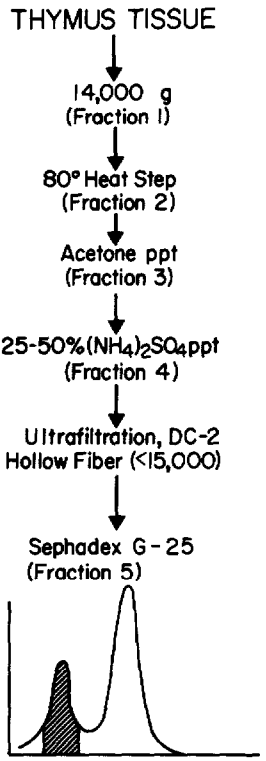

Fig. 2. Purification of thymosin fraction 5. One kilogram of thymus tissue homogenized in 3 l of sodium chloride provides the sample to initiate the procedure.

monium sulfate precipitation. The 25–50% cut was collected and further purified by ultrafiltration in an Amicon DC-2 hollow-fiber system with a molecular weight cutoff at 15,000. The filtrate was desalted on Sephadex G-25. The lyophilized protein peak was designated thymosin f5.

Preparation of thymosin f5 samples and thymus tissue extracts for HPLC analysis. (1) For f5 samples.

Thymosin f5 (5–6 μg) was suspended in 3 ml of 0.05% TFA, swirled and centrifuged. The supernatant was forced through a primed Sep-Pak cartridge with a 20-ml Luer-Lok syringe. The cartridge was washed with 10 ml 0.05% TFA and the peptides eluted with 2 ml 50% acetonitrile containing 0.05% TFA. The eluates were lyophilized.

(2) For tissue extracts. Aliquots of the frozen bovine or porcine thymus tissues (1–2 g) or fresh rat thymus glands were homogenized in 10 ml of 6 *M* guanidinium chloride or phosphate-buffered saline (pH 7.4), using a polytron homogenizer (Brinkmann Instruments, setting 6, 20–30 sec). After addition of 10 ml 0.1% TFA to each homogenate and swirling, it was centrifuged at 18,000 *g* for 30 min. The small amount of floating lipid layer was removed and the remainder was forced through the Sep-Pak cartridge and peptides eluted as described above.

High-performance liquid chromatography. Separation of peptides was performed on a Hewlett-Packard 1084B HPLC apparatus with a μBondapak C_{18} column at 35°C. The solvents used were 0.05% TFA (pH 2.3) in reservoir A and acetonitrile containing 0.05% TFA in reservoir B. Solvents were filtered through a Millipore filter apparatus (Waters Assoc.) before use. Detection of the peptides was accomplished by a variable-wavelength detector (Hewlett-Packard) set at 210 nm. The flow-rate was set at 1.5 ml/min and the chart speed at 0.3 cm/min. The lyophilized eluates from the Sep-Pak cartridge were resuspended in 200–500 μl of 0.05% TFA, and filtered through a MF-1 microfilter (Bioanalytical systems, West Lafayette, IN, U.S.A.) with a RC60 membrane (1.0 μm, regenerated cellulose). An amount of 20–50 μl was applied onto the column. Peptides were eluted from the column with 10% 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. Ten to twenty fractions were collected at 1-min intervals around the region where synthetic α_1 is eluted (26.4 min). The fractions were lyophilized and analyzed by radioimmunoassay for thymosin α_1.

Radioimmunoassay (RIA) for thymosin α_1. The RIA for α_1 was performed as described previously[26] with minor modifications. The antiserum to synthetic thymosin α_1 used in the RIA was absorbed against kidney f5 (prepared from bovine kidneys using similar procedures to the one for thymosin f5), and used at final dilutions between 1:10,000 and 1:20,000. Labeled N-Ac(Tyr^1) thymosin α_1 (^{125}I-labeled synthetic α_1 with the N-terminal serine substituted by a tyrosine) was purified by gel filtration on Sephadex G-10. The assay conditions included incubation for 24 h at 4°C of the standard or unknown with antiserum and the tracer in RIAB (phosphate-buffered saline, containing 0.01 m*M* EDTA, 0.05% NaN_3 and normal rabbit serum at 1:200 dilution). A second antibody (goat anti-rabbit IgG) was used to precipitate the immune complexes. By use of the highly purified synthetic thymosin α_1 standard, a calibration curve was generated which had a minimum detectable dose of 30–50 pg and an ED_{50} (amount of α_1 required for 50% binding) of 150–400 pg.

RESULTS AND DISCUSSION

The separation of thymic peptides was carried out by reversed-phase HPLC on a μBondapak C_{18} column with 10–45% acetonitrile in 0.05% TFA as eluent. As shown in Fig. 3, synthetic thymosin α_1 (chemically synthesized according to the sequence of bovine α_1) was eluted from the column at 26.4 min. The HPLC of bovine thymosin f5 is shown in Fig. 4. Also shown in the figure are the RIA results. The immunoreactive peak coincided with the protein peak at the retention time of synthetic thymosin α_1. As listed in Table I, the yield of thymosin α_1 from 1 mg of bovine f5 was quantitated by UV absorbance at 210 nm (9.8 μg) and by RIA (5.5 μg). These results were reasonably close to the value (0.6%, 6 μg) obtained when conventional column chromatography was used[15]. The higher values of α_1 obtained by UV absorbance indicates that not all of the UV peak at this position is attributable to thymosin α_1. It is apparent that the complex mixture present in thymosin f5 was not completely resolved in this HPLC system. An improved resolution has been achieved using a shallower gradient (10 to 50% B in 150 min) with the same column and solvent system.

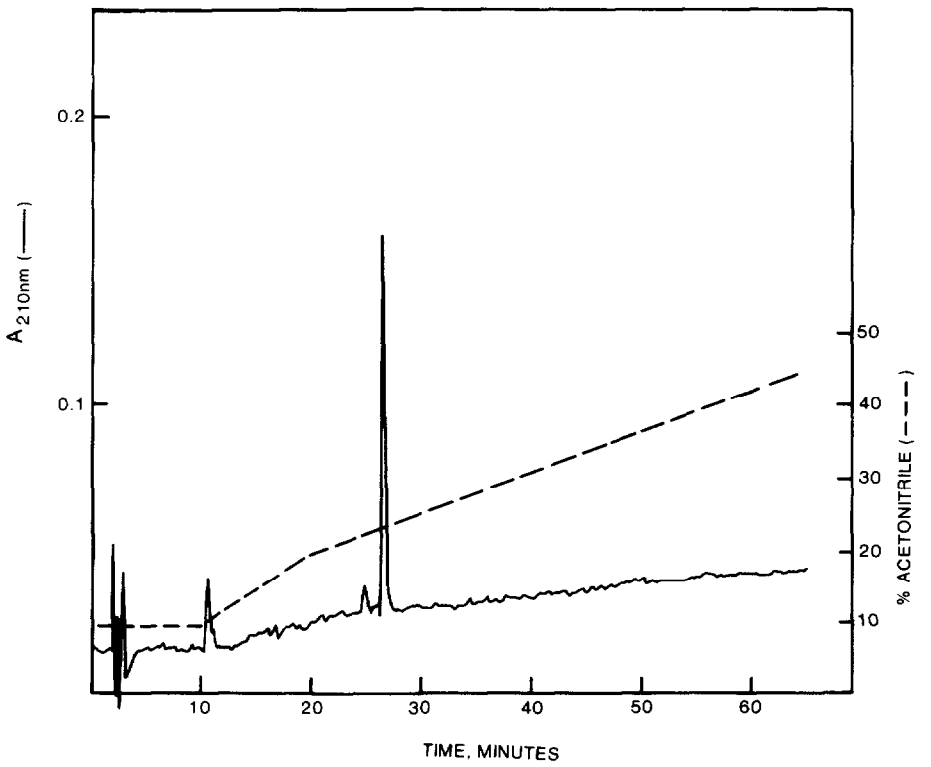

Fig. 3. HPLC analysis of synthetic thymosin α_1 (bovine) in a 30 × 0.39-cm μBondapak C_{18} column (10 μm, Waters Assoc.) at 35°C. The solvents used were: 0.05% TFA in Reservoir A and acetonitrile containing 0.05% TFA in Reservoir B. The peptides were eluted with 10% B for 10 min, followed by a linear gradient from 10 to 20% in 10 min and second gradient from 20 to 45% B in 45 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 (- - - - -).

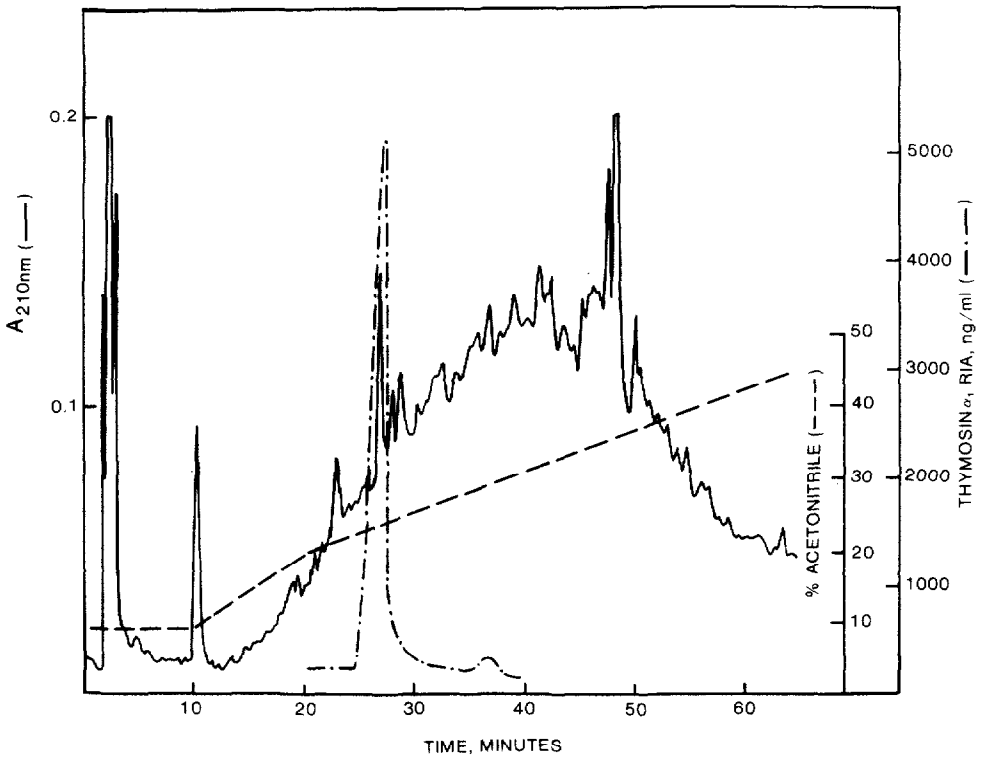

Fig. 4. HPLC separation of bovine thymosin f5. Conditions as in Fig. 3. The fractions (1-min intervals) collected were analyzed by RIA for thymosin α_1 (— · —).

The HPLC elution profiles of porcine, ovine and murine f5 are shown in Figs. 5–7. In each case, it was found that the α_1-immunoreactive peak coincided with the protein peak at the α_1-position. The recovery of α_1 from these f5 preparations is listed in Table I.

On the other hand, chromatographic analysis of some fresh thymus tissue extracts (calf, pig or rat), using a similar HPLC system revealed either no or only a very small amount of thymosin α_1. As shown in Fig. 8, when porcine thymus tissue was homogenized in 6 *M* guanidinium chloride, no protein peak or immunoreactive peak was detected at the α_1-position in the HPLC elution profile. Similar results were obtained when rat or calf thymus tissue was used (data not shown). When pig thymus tissue was homogenized in phosphate-buffered saline (PBS) instead of 6 *M* guanidinium chloride, though no protein peak was detectable at the α_1-position, an immunoreactive α_1-peak was detected (Fig. 9). However, the amount of α_1 (50 ng/g tissue) present in the PBS homogenate of pig thymus tissue is *ca.* 1 % of that expected for f5 preparation (5 μg/g tissue).

All thymosin f5 preparations or tissue homogenates were passed through Sep-Pak C_{18} cartridges prior to HPLC analysis. This serves as a clean-up step to remove some of the sample components that might be irreversibly bound to the C_{18}-bonded phase. The recovery of thymosin α_1 from Sep-Pak cartridges in different solvents has been studied. An equal amount (50 μg) of synthetic thymosin α_1 was suspended in

TABLE I

RECOVERY OF THYMOSIN α_1 FROM BOVINE, PORCINE, OVINE AND MURINE THYMOSIN f5 AND PORCINE THYMUS TISSUE

ND = Not detectable.

Preparation	*Retention time of α_1 peak (min)*	*α_1 Recovery*	
		*A_{210nm}**	*RIA***
Thymosin f5			
Bovine	26.8	9.8	5.5
Porcine	26.8	6.1	3.5
Ovine	26.5	9.4	6.2
Murine	26.6	6.0	3.2
Porcine thymus extract			
6 *M* Guanidine***	–	ND	ND
PBS §	–	ND	0.055

* Determined by absorbance at 210 nm in HPLC elution profiles using synthetic α_1 as standards. Values are expressed as μg per mg f5 for f5 samples or μg per g thymus tissue (wet weight) for tissue extracts.

** Determined by α_1 RIA in fractions eluted from the HPLC column. Units used are the same as described above in footnote*.

*** Frozen thymus tissue was homogenized in 6 *M* guanidinium chloride, followed by separation on Sep-Pak and HPLC as described under *Methods*.

§ Frozen thymus tissue was homogenized in phosphate buffered saline, followed by separation on Sep-Pak and HPLC as described under *Methods*.

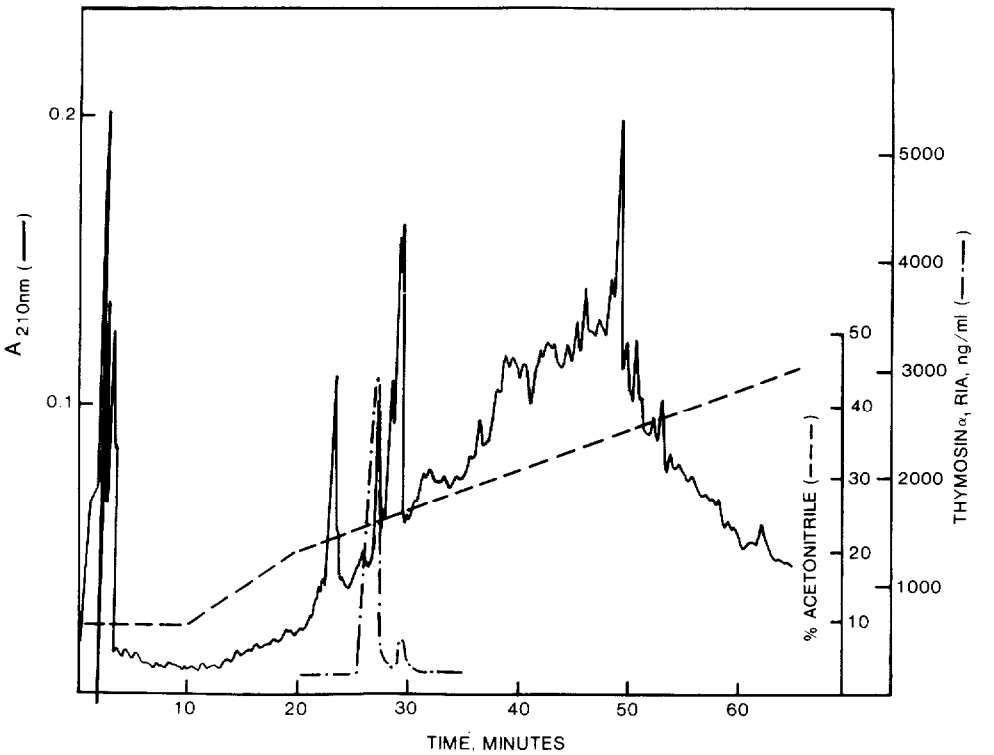

Fig. 5. HPLC separation of porcine thymosin f5. Conditions as in Fig. 3. Results from RIA for α_1 are also indicated in the figure (—.—).

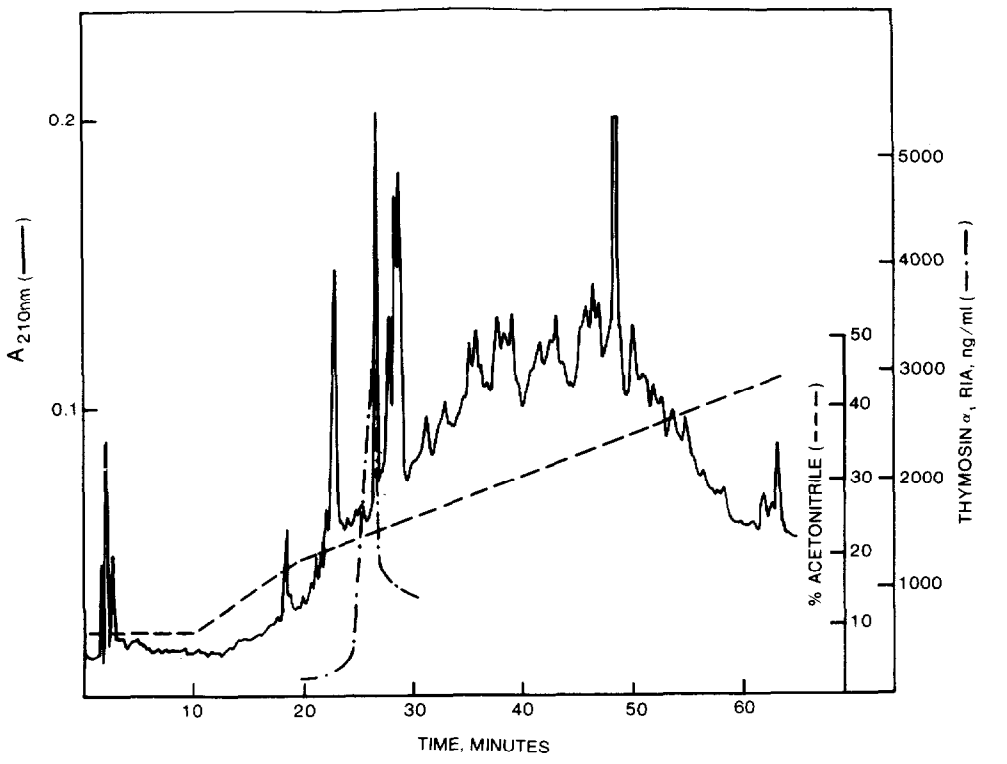

Fig. 6. HPLC separation of ovine thymosin f5. Conditions as in Fig. 3. The α_1 RIA results are also shown in the figure (— · —).

0.05% TFA, 6 *M* guanidinium chloride or PBS. The solutions were passed through Sep-Pak cartridges, washed with 0.05% TFA and then eluted with 2 ml of 50% acetonitrile, containing 0.05% TFA. The eluents were lyophilized and analyzed by HPLC. The amount of α_1 contained in each fraction was quantitated by UV absorbance. Our results showed that the recovery of α_1 from Sep-Pak in 0.05% TFA was 95–100%. On the other hand, the recovery of α_1 from Sep-Pak in 6 *M* guanidinium chloride was only *ca.* 50% and in PBS *ca.* 90%. The low recovery of α_1 from Sep-Pak in 6 *M* guanidinium chloride might be due to the denaturation of polypeptide by the reagent. Henderson *et al.*[27] found that the yield of chymotrypsin from a μBondapak C_{18} column was 80% when the protein was dissolved in 0.05% TFA. However, the yield was only 40% when the protein was first denatured in 6 *M* guanidinium chloride.

We have also found that 2 ml of 50% acetonitrile was sufficient to elute thymosin α_1 from the Sep-Pak cartridges. Subsequent elutions with 1 ml of 50% acetonitrile and a second elution with 2 ml of 50% acetonitrile, followed by HPLC analysis, did not reveal any α_1 peak in the eluates.

The fact that thymosin α_1 was not found in tissues extracted with 6 *M* guanidinium chloride suggests that thymosin α_1 may be synthesized in a precursor form in animal tissues, which supports the report of Hannappel *et al.*[28]. Freire *et al.*[29] have translated messenger RNA from fresh calf thymus and carried out synthesis in

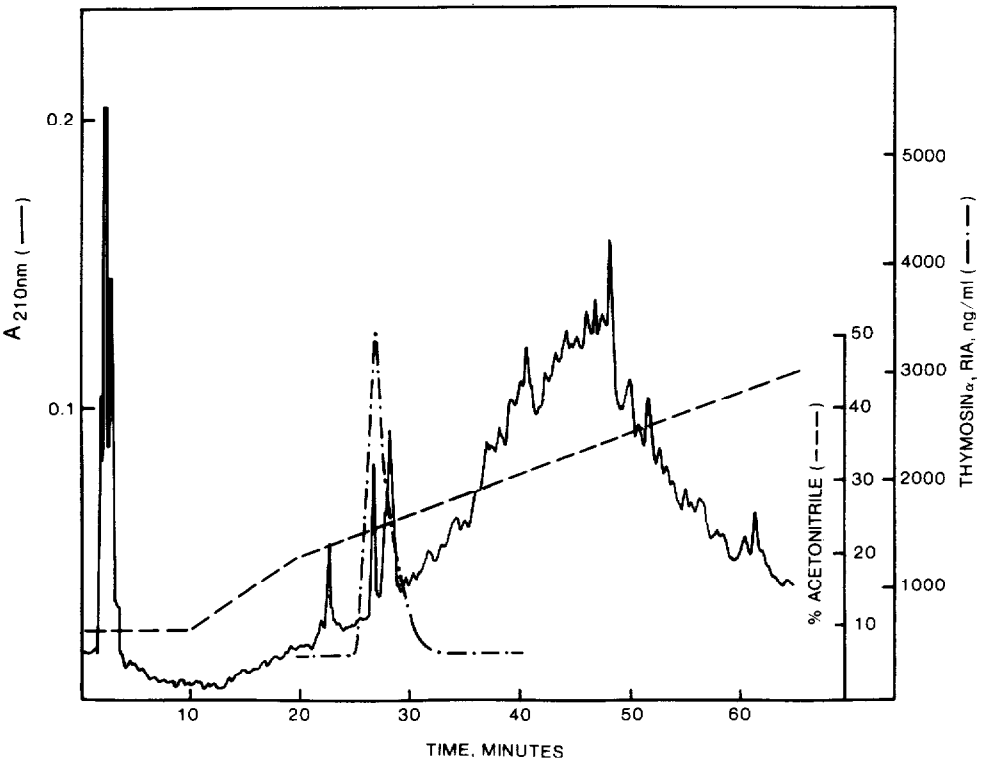

Fig. 7. HPLC separation of murine thymosin f5. Conditions as in Fig. 3. The α_1 RIA results are also shown in the figure (—·—).

cell-free wheat germ system. A peptide of 16,000 daltons that was immunoprecipitable with antisera against thymosin fractions was analyzed and found to contain tryptic peptides corresponding to fragments of thymosin α_1. Thus, the 16,000-dalton peptide appears to be a precursor of thymosin α_1. The precursor is probably processed by proteolysis outside of the thymus to produce thymosin α_1. The enzymatic cleavage of α_1 precursor is apparently not random, since only one form of α_1 is detected in the HPLC elution profile by RIA. Furthermore, our recent studies on the isolation of thymosin α_1 from human blood[30] suggest that the immunoreactive blood α_1 has physicochemical properties similar to thymosin α_1. These results indicate that α_1 precursor is activated by enzymatic cleavage when it leaves the thymus and migrates through spleen, lymph nodes and finally to the blood circulation. The fact that thymosin α_1 is immunologically active suggests that it is an important portion of the precursor molecule. In order to understand the relationship of thymosin α_1 and its precursor fully, it is necessary to isolate and characterize the precursor molecule.

The solvent system (0.05% TFA–acetonitrile) used for HPLC was originally described by Henderson *et al.*[27]. The solvents are volatile and can be removed by lyophilization. The purified thymosin α_1 appears to retain its full antigenic activity. Henderson *et al.*[27] have compared the TFA system with other more commonly used ion-pairing reagents, such as KH_2PO_4 and triethylammonium phosphate, as well as phosphoric acid at pH 2.0. They concluded that the TFA system compares favorably

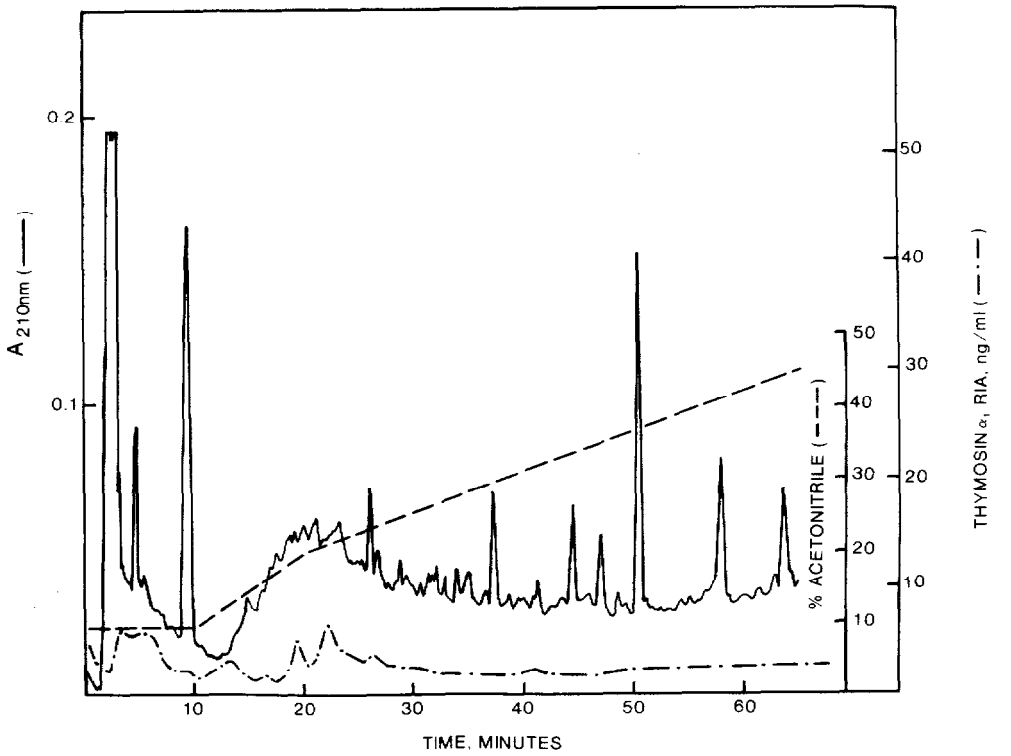

Fig. 8. HPLC separation of pig thymus extract. Thymus tissue was homogenized in 6 *M* guanidinium chloride. See *Methods* section for details. No thymosin α_1 was detected at α_1 position (*ca.* 26.4 min).

with the other systems and believe that volatile TFA buffer can replace non-volatile buffers without significant loss in resolution.

Isolation of thymosin α_1 from thymosin f5 by conventional column chromatography is time-consuming and requires a large amount of starting materials (at least 1 g of thymosin f5). Using the HPLC system and the RIA for thymosin α_1, it is possible to isolate and identify thymosin α_1 with 1–5 mg or less of thymosin f5.

The combination of HPLC and RIA provides a more reliable quantitation of biologically active components in tissues. Quantitation of biologically active compound in crude tissue extracts or physiological fluids by RIA alone often leads to results far from the true values, due to interfering substances present in the crude preparations or precursor forms which are not immunoreactive[31]. Purification of peptides by Sep-Pak cartridges, followed by HPLC, is an alternative method, which provides more reliable quantitative data. Furthermore, this method can distinguish different forms of immunoreactive components and provide information on their physicochemical properties. Efforts are now being made to isolate enough of the immunoreactive fractions for biological as well as sequence studies to characterize these molecules fully.

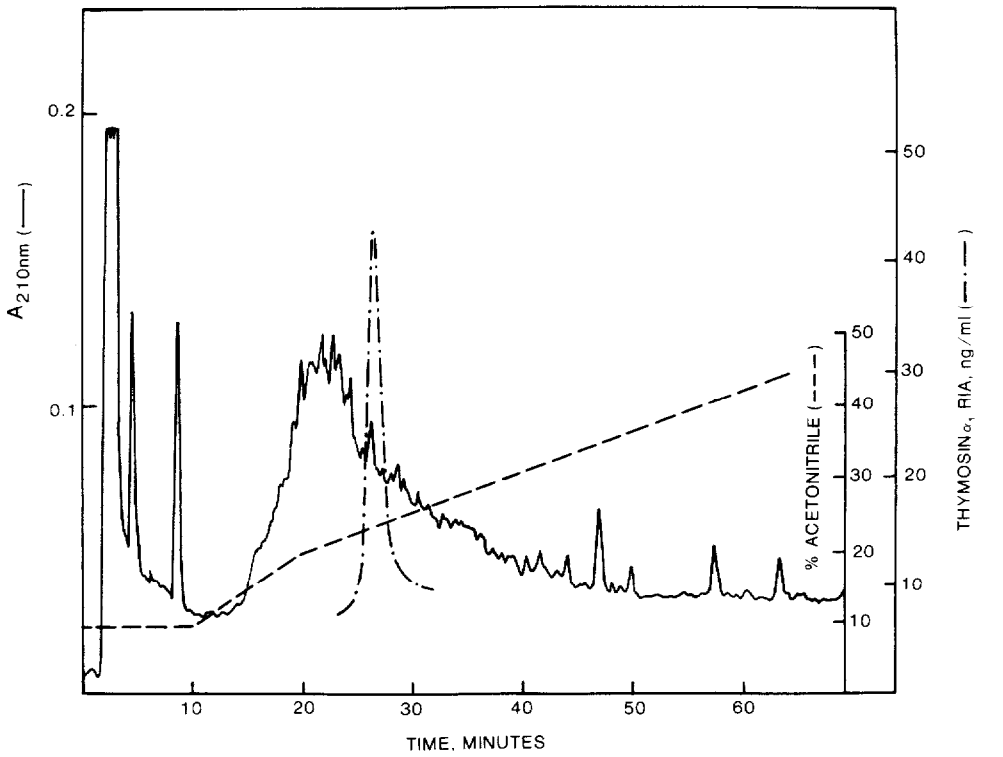

Fig. 9. HPLC of pig thymus extract. Thymus tissue was homogenized in phosphate buffered saline (pH 7.4). Small amounts of thymosin α_1 (50 ng/g tissue) were detected at the α_1-position by RIA.

ACKNOWLEDGEMENTS

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