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Immunology

Thymosin α_1: Isolation and sequence analysis of an immunologically active thymic polypeptide

(cell-mediated immunity/differentiation of thymus-dependent lymphocyte/thymic factor nomenclature/acidic polypeptide/acetylserine NH_2-terminal)

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ABSTRACT **The amino acid sequence of a biologically active polypeptide isolated from calf thymus, termed thymosin α_1, has been determined. Thymosin α_1 is a heat stable, highly acidic molecule composed of 28 amino acid residues. This peptide is one of several present in thymosin fraction 5 that may participate in the regulation, differentiation, and function of thymus-dependent lymphocytes (T cells). A nomenclature for the family of polypeptides present in thymosin fraction 5 is suggested.**

The importance of the thymus gland in the development and senescence of immunological competence in animals and man is now generally accepted. Although there is little knowledge of the molecular events by which the thymus gland exerts control over development of thymus-dependent lymphocytes (T cells), it appears that a vital part of the process occurs via a hormonal mechanism (1). The thymus produces a family of polypeptides (2) that play an important role in the maturation, differentiation, and function of T cells.

In 1966, we reported the extraction and partial purification of thymosins from calf tissue (3). In 1972 we reported the further purification and characterization of thymosins, including the isolation of one of the active components, a protein with a molecular weight of 12,600 (4). Subsequent studies have demonstrated that this protein was an aggregate of three smaller polypeptides with molecular weights of less than 4000 (5). In 1975 the isolation procedure was modified to enable us to prepare larger quantities of thymosin fraction 5 for clinical trials (5).

Thymosin fraction 5 is a potent immunopotentiating preparation and can act in lieu of the thymus gland to reconstitute immune functions in certain thymus-deprived and/or immunodeprived individuals. Thymosin has been found to induce T cell differentiation and enhance immunological functions in genetically athymic mice (6), in adult thymectomized mice (6, 7) in NZB mice with severe autoimmune reactions (6, 8) in tumor-bearing mice (9–11), and in mice with casein-induced amyloidosis (12). Ongoing clinical trials with fraction 5 suggest that thymosin is effective in increasing T cell numbers and normalizing immune function in children with thymus-dependent primary immunodeficiency diseases (13–15). It can increase T cell numbers in immunodepressed cancer patients (15–17).

Analytical polyacrylamide gel electrophoresis and isoelectric focusing have demonstrated that fraction 5 consists of 10 to 15 major components and 20 or more minor components with molecular weights ranging from 1,000 to 15,000.

The present report describes the isolation and complete structural determination of one of the purified polypeptide components in the highly acidic region of fraction 5. This peptide has been termed thymosin α_1.[§] Thymosin α_1 has been found to be 10–1000 times more active than fraction 5 in several assay systems *in vitro* and *in vivo* designed to measure T cell differentiation and function.

EXPERIMENTAL

Materials. Thymosin fraction 5 was isolated from calf thymus as described (5). Ion-exchange chromatographic media CM-22 and DE-32 were obtained from H. Reeve Angel, Inc. Sephadex G-75 was purchased from Pharmacia Chemicals, Inc. Trypsin-L-(1-tosylamido-2-phenyl)ethylchloromethyl ketone (TPCK), α-chymotrypsin, and carboxypeptidase A were purchased from Worthington Biochemicals. Thermolysin was purchased from Calbiochem and subtilisin BPN' protease from Sigma Chemical Co. All reagents used for Edman degradation were purchased from Pierce Chemical Co. Fluorescamine (Fluram) was obtained from Hoffmann-La Roche. All other chemicals were of reagent grade and were used without further purification. Sterile, pyrogen-free water used in the preparation of all buffers was purchased from Travenol Laboratories or McGaw Laboratories.

Methods. Thymosin α_1 was isolated from fraction 5 by a combination of ion-exchange chromatography and gel filtration. The procedure used for the preparation of this component is illustrated in Fig. 1.

Lyophilized thymosin fraction 5 was chromatographed on a column of carboxymethyl-cellulose in 10 mM NaOAc, 1.0 mM 2-mercaptoethanol, pH 5.0. The column was washed first with the starting buffer followed by a linear gradient of 2 liters each of starting buffer and the same buffer containing 1.0 M NaCl. The void volume was fractionated on a Sephadex G-25 column in sterile water. The second protein peak from the G-25 was applied on a DEAE-cellulose column (DE-32) equilibrated with 50 mM Tris·HCl, 1.0 mM 2-mercaptoethanol, pH 8.0. The column was washed with the starting buffer followed by a gradient of 1.3 liters each of starting buffer and the same buffer containing 0.8 M NaCl. The material from the first retained peak from the DE-32 column was further purified by passing it twice through a Sephadex G-75 column, with 10 mM Tris·HCl, pH 7.5, containing 6 M guanidine·HCl as an eluant. A single narrow cut was made from the protein pool and desalted on a Sephadex G-10 column in sterile water. The purified sample so obtained is identified as thymosin α_1.

For amino acid analysis, samples were hydrolyzed in 6 M

Abbreviation: T cell, thymus-dependent lymphocyte.

§ See *Discussion* for a description of the nomenclature used to identify this peptide.

Table 1. Amino acid composition* and NH_2-terminal residues of tryptic peptides from thymosin α_1

Amino acid	T1	T2	T3	T4	T5	T5S1¶	T5A5‖	T6
Lysine	1.94(2)	1.00(1)	0.90(1)		1.20(1)		1.00	0.92(1)
Aspartic acid				1.02(1)	2.04(2)	1.19(1)	1.23(1)	0.97(1)
Threonine					2.62(3)		2.96(3)	
Serine					2.53(3)	0.90(1)	1.96(2)	
Glutamic acid	1.06(1)		1.03(1)	3.84(4)	1.09(1)	0.15(0)	1.56(1)	
Alanine				1.01(1)	2.10(2)	1.91(2)	1.11(1)	
Valine				2.12(2)	0.94(1)		0.86(1)	
Isoleucine					0.87(1)		1.00(1)	
Leucine								1.11(1)
Total	3	1	2	8	14	4	11	3
NH_2-terminal†	Glu	Lys	Glu	Glu	Blocked	Blocked	Ala	Asp
Charge at pH 6.5‡	+1	+1	Neutral	−4	ND§	ND	ND	Neutral

* Results from 6 M HCl hydrolysates at 110° for 24 hr. The numbers of residues in the primary structure are given in parentheses.
† Determined by dansylation.
‡ Determined by electrophoretic mobilities of high-voltage paper electrophoresis at pH 6.5 by the method of Offord (25).
§ Not determined.
¶ A peptide from subtilisin digest of T5. Isolated from ion-exchange column AG 50W-X2 in H_2O.
‖ A peptide from the α-protease (Pierce Chemical Co.) digest of T5. Isolated by paper electrophoresis at pH 3.5.

HCl in evacuated sealed tubes for 24–120 hr at 110°. Beckman/Spinco Model 119 (one-column system), model 121M, and JEOL model JLC-6AH (two-column system) amino acid analyzers were used based on the method of Spackman *et al.* (18).

Gels containing 15% acrylamide were run under both basic (pH 8.3) and acidic (pH 2.9) conditions according to the methods reported previously (5). Gels for isoelectric focusing were either purchased from LKB (PAG_{plate}, pH 3.5–9.5) or prepared by a modification of methods described by Awdeh *et al.* (19) and Wilson *et al.* (20). Isoelectric focusing was conducted for 90 min using a constant power of 25 W (LKB model 2103 power supply). The gels were fixed in 15% trichloroacetic acid (TCA) (wt/vol), 25% isopropanol for 16 hr and stained with 0.05% (wt/vol) Coomassie brilliant blue in 25% isopropanol, 10% acetic acid overnight. Gels were destained in 10% acetic acid.

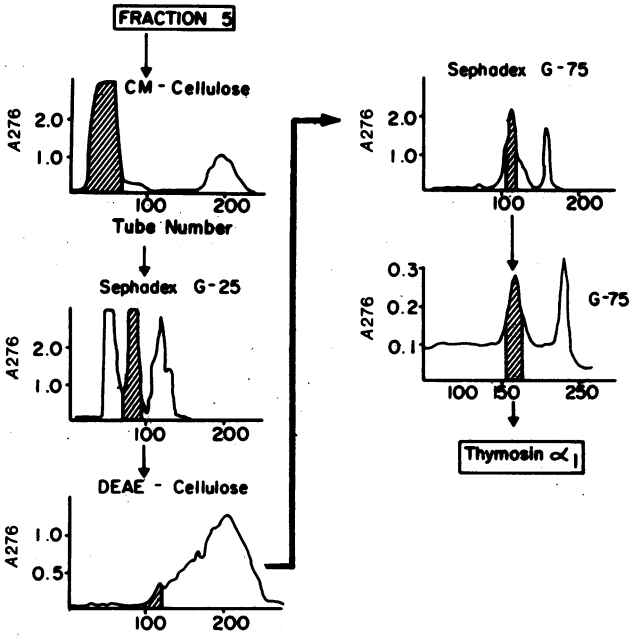

FIG. 1. Purification of bovine thymosin α_1. Flow diagram of the fractionation of thymosin α_1 from bovine thymosin fraction 5. Shaded areas in elution profiles indicate fractions pooled for purifying thymosin α_1. In these profiles, the abscissa is the tube number and the ordinate absorbance at 276 nm. For details, see *text*. CM-cellulose, carboxymethyl-cellulose.

Enzymatic digests of thymosin α_1 were separated largely by paper electrophoresis and/or chromatography. In the two-dimensional separation on paper, chromatography was done first with the solvent system of *n*-butanol:glacial acetic acid: water = 4:1:5 (vol/vol). This was followed by high-voltage electrophoresis at pH 1.9 for 30–40 min at 60 V/cm. Peptides were detected by staining with either cadmium-ninhydrin reagent (21) or fluorescamine (22).

Enzymatic digestion was performed in 1% ammonium bicarbonate at pH 8.3 or 0.05 M Tris·HCl buffer (pH 8.0) for 2–3 hr at 37°. Trypsin, chymotrypsin, thermolysin, or subtilisin was added to the protein solution to a final ratio of enzyme to substrate of 1:100 (wt/wt). The amino acid sequence of the peptides was determined by the dansyl-Edman (23), subtractive-Edman (24), procedures and carboxypeptidase digestion. Acids and amides were determined from the mobility on high-voltage paper electrophoresis at pH 6.5 according to Offord (25). Fluorescence measurements were made with excitation set at 390 nm and emission at 475 nm using an Aminco-Bowman spectrophotofluorometer (American Instrument Co.).

SEQUENCE ANALYSIS AND BIOASSAY RESULTS

Thymosin α_1 isolated according to the procedure outlined in Fig. 1 has a molecular weight of 3107, as calculated from its structure. This correlates with the value 3350 estimated from a calibrated Sephadex G-75 column in 6 M guanidine·HCl by the method of Fish *et al.* (26). The peptide migrates as a single band on analytical polyacrylamide gels at pH 8.3 or pH 2.9, and as a major band with a pI of approximately 4.2 on isoelectric focusing slab gels of pH range 3–5. The yield of thymosin α_1 from fraction 5 is about 0.6%. The preparation is free of carbohydrate and nucleotide.

Amino acid analyses on 24-, 48-, 72-, and 120-hr acid hydrolysates of thymosin α_1 showed¶: Lys 3.75(4), His 0.04(0), Arg 0.25(0), Asp 4.38(4), Thr 2.75 (3), Ser 2.81(3), Glu 6.44(6), Pro 0.50(0), Gly 0.62(0), Ala 3.44(3), ½Cys 0(0), Val 3.25(3), Met

¶ The numbers of residues in the primary structure are given in parentheses.

Table 2. Amino acid composition* and NH_2-terminal residues of thermolysin and chymotryptic peptides of thymosin α_1

Amino acid	Thermolysin peptides					Chymotryptic peptides		
	Th1	Th2	Th3	Th4	Th5	C1	C1Th1	C2
Lysine	2.94(3)	1.04(1)				0.90(1)	0.91(1)	2.71(3)
Aspartic acid		1.06(1)	1.07(1)	1.03(1)		1.97(2)		1.84(2)
Threonine		1.95(2)	0.99(1)			2.65(3)	1.71(2)	
Serine			1.77(2)			2.51(3)		
Glutamic acid	1.95(2)		1.13(1)	3.25(3)		1.09(1)		5.58(5)
Alanine				1.04(1)	1.00(1)	1.95(2)		1.15(1)
Valine			1.04(1)	1.68(2)		1.08(1)		1.73(2)
Isoleucine		0.95(1)				1.05(1)	1.03(1)	
Leucine	1.00(1)							0.82(1)
Total	6	5	6	7	1	14	4	14
NH_2-terminal†	Leu	Ile	Val	Val	Ala	Blocked	Ile	Asp
Charge at pH 6.5‡	+1	Neutral	−2	−3	Neutral	ND§	+1	ND

* Results from 6 M HCl hydrolysates at 110° for 24 hr. Numbers in parentheses are the numbers of residues in the primary structure.
† Determined by dansylation.
‡ Determined by electrophoretic mobilities of high-voltage paper electrophoresis at pH 6.5 by the method of Offord (25).
§ Not determined.

0.38(0), Ile 1.13(1), Leu 1.25(1), Tyr 0.11(0), Phe 0.12(0), and Trp 0(0) [after hydrolysis with mercaptoethanesulfonic acid (27)]. The presence of small amounts of proline, glycine, and methionine in the acid hydrolysates is attributed to minor contaminants in the preparation, which did not interfere with sequence analysis. Thymosin α_1 has a high aspartic and glutamic acid content and does not contain histidine, arginine, proline, glycine, cystine, methionine, tyrosine, phenylalanine, or tryptophan residues.

The failure of thymosin α_1 to give α-amino derivatives upon reaction with dansyl chloride or phenylisothiocyanate showed that its NH_2-terminus was blocked. The COOH-terminal sequence of thymosin α_1 was determined as -Glu-Asn-OH by carboxypeptidase experiments. Digestion of thymosin α_1 with trypsin, chymotrypsin, or thermolysin produced several peptides which also provided information on overlaps. Tables 1 and 2 list the amino acid compositions of the isolated peptides. The sequence analyses were performed independently in Galveston and Nutley, with results in complete agreement (Fig. 2).

Tryptic peptide T5 is assigned to the NH_2-terminal region of thymosin α_1 for lacking a free amino group. Peptide T4 is derived from the COOH-terminus because of its lack of a lysine residue and the agreement of its terminal sequence with the carboxypeptidase data. A subtilisin digest of T5 was applied onto a cation-exchange column packed with Bio-Rad AG 50W-X2 in H_2O. The column was washed with water and effluents assayed with fluorescamine after alkaline hydrolyses, as described by Nakai *et al.* (28). The peptide eluted with H_2O was found to be a tetrapeptide (T5S1) with a blocked NH_2-terminus (Table 1). When this peptide was hydrolyzed in 0.03 M HCl at 110° for 16 hr, serine, aspartic acid, and the dipeptide, Ala-Ala, were produced. Treatment of the peptide with carboxypeptidase A released alanine. The amino acid sequence of this peptide was, therefore, deduced as X-Ser-Asx-Ala-Ala- (see Fig 2). To confirm the sequence of T5S1, and, in particular, to confirm preliminary gas chromatographic results that the blocking group was acetyl, T5S1 was subjected to mass spectrometric analysis.‖ As observed in the mass spectrum, the NH_2-terminus of this peptide is blocked by an acetyl group. The analysis also showed the first residue to be serine. From electrophoretic mobility of aspartate- or asparagine containing synthetic preparations**, the second residue appears to be aspartic acid.

As can be seen in Table 3, thymosin α_1 is from 10 to 1000 times more active than thymosin fraction 5 in a mouse mitogen assay *in vivo* (6), a lymphokine assay *in vitro* measuring production of macrophage inhibitory factor (29), and a human E-rosette assay *in vitro* (13). Details of the biological activity of thymosin α_1 have been presented elsewhere (29).

DISCUSSION

The complete amino acid sequence of thymosin α_1 is shown in Fig. 2, along with the peptide segments isolated from proteolytic digests that provided information toward the sequence elucidation. Aside from the presence of several repeating amino acid residues along the polypeptide chain and a blocked NH_2-terminus, there are no unusual features. It was rather unexpected to find that chymotrypsin cleaved in the middle of the molecule at Lys-14 instead of at Leu-16. This suggests a conformational susceptibility at this position, but whether it is related to function remains to be investigated.

The amino terminus of thymosin α_1 is blocked by an acetyl group. This raises questions regarding its origin and functional significance. Järnvall (30) has analyzed 40 known α-amino-acetylated polypeptide chains and has postulated that acetylation occurs in an early post-translational stage and may have

Table 3. Thymosin activity in various bioassays

	MLR	MIF	E-Rosette	Mitogen*
Thymosin fraction 5 (μg)	1–10	1–5	1–10	1–10
Thymosin α_1 (μg)	N.A.†	0.01–0.1	0.001–0.01	0.01–0.1

MLR, mixed lymphocyte response; MIF, macrophage inhibiting factor.
* Fourteen daily injections *in vivo*.
† Not active.

‖ Mass spectrometric analysis was performed by Dr. K. Biemann of the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Mass. The experimental details of the analysis will be reported elsewhere.

** Details of thymosin α_1 synthesis (S. S. Wang *et al.*) will be reported elsewhere.

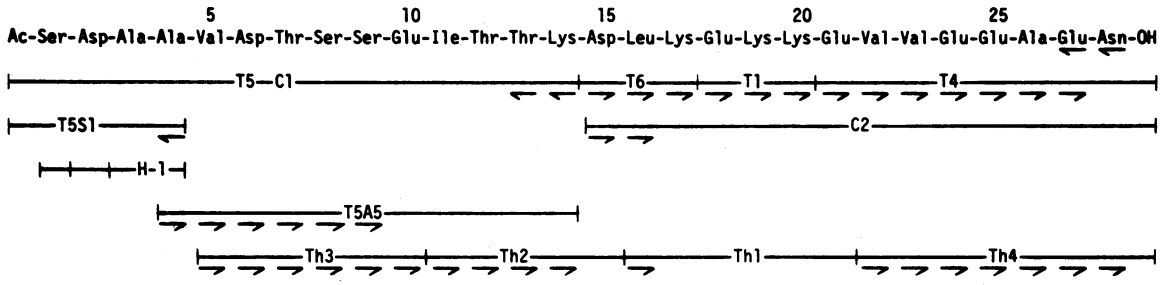

FIG. 2. Schematic diagram of the sequence analyses that led to the elucidation of the primary structure of thymosin α_1. Line segments denote the peptides isolated after digestion of thymosin α_1 with trypsin (T1, etc.), chymotrypsin (C1, etc.), and thermolysin (Th1, etc.), and those from peptide T5 on digestion with subtilisin (T5S1) and α-protease (T5A5). Dilute acid hydrolysis of T5S1 (see *text*) produced free serine, aspartic acid, and dipeptide Ala-Ala (H-1). Acids and amides were assigned according to the charges determined by high-voltage paper electrophoresis at pH 6.5 of the original enzymatic peptides or the peptides derived from stepwise manual Edman degradation. Arrows pointing to the right indicate residues degraded sequentially by the dansyl and/or subtractive Edman procedures; those pointing to the left denote the sequence elucidated by carboxypeptidase digestion. Ac, Acetyl.

universal importance. Thus, although the structural or functional significance of the NH_2-terminal acetylation of thymosin α_1 is not known, a protective function of acetylation towards proteolysis is possible.

It has been observed and reported previously (2) that the biological activity of thymosin fraction 5 resides mainly with its more acidic polypeptides. The established structure of thymosin α_1 reveals that this polypeptide is highly acidic. Using parameters for the prediction of protein conformation described by Chou and Fasman (31) it would appear that the COOH-terminal half of the molecule has a high helical potential.

Comparison of the sequence of thymosin α_1 with the published sequence of another thymic factor termed thymopoietin (32) and a molecule termed ubiquitin (33) reveals no homology. Other thymic factors that have been reported include thymic humoral factor (34) and thymic factor (35). The amino acid composition, but not the sequence has been described for thymic humoral factor (34). It appears that thymosin α_1 and thymic humoral factor, although of similar molecular weight, differ greatly in amino acid composition. There is very little known about the chemistry of thymic factor other than that it is a small molecule (molecular weight <1000) and that it is heat labile. However, a comparison of pI values [thymic factor, pI 7.5 (35); thymosin α_1, pI ca. 4.2] would suggest that they are different compounds.

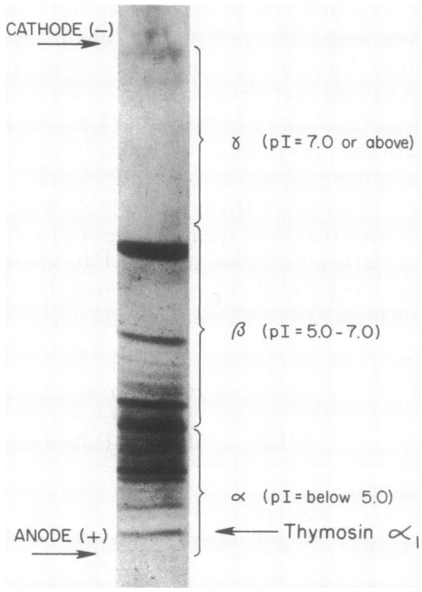

FIG. 3. Suggested nomenclature for thymosin polypeptides on the basis of a isoelectric focusing gel of thymosin fraction 5 at pH 3.5–9.5 (see *text* for details of nomenclature).

To facilitate the identification and comparison of all thymic peptides from one laboratory to another, we propose a nomenclature (Fig. 3) based on the isoelectric focusing pattern of thymosin fraction 5 in the pH range 3.5–9.5. The separated peptides are divided into three regions based upon their migration patterns. The regions are identified by the Greek letters α, β and γ. The α region consists of the peptides with isoelectric points below 5.0 (highly acidic), the β region 5.0–7.0 (acidic), and the γ region above 7.0 (basic). The subscript numbers α_1, α_2, α_3, etc. will be used to identify the peptides from that region as their complete sequence becomes known; thus, the designation of thymosin α_1 (Fig. 3) for the peptide described in this paper.

The observation that thymosin α_1 is more potent than fraction 5 in enhancing some T cell assays (E-rosettes, lymphokine, mitogen) but not others, such as the mixed lymphocyte response, suggests that more than one peptide component may be necessary to elicit full immunologic responsivity. Alternatively, a purification procedure such as the guanidine chromatography step may have altered the conformation of the molecule so as to abolish its activity in the mixed lymphocyte response assay. It may also be possible that trace amounts of guanidine·HCl in the preparation interfered with the bioassay. It appears that there is a family of biologically active peptides within fraction 5 that may act in concert, sequentially, or separately on pre-T and T cell populations to maintain normal immunological reactivity.

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