

Thymosin α_{11} : A peptide related to thymosin α_1 isolated from calf thymosin fraction 5

(thymic peptides/amino acid sequences/*Candida albicans* infection/thymosin α_1 analogues)

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ABSTRACT Two peptides related to thymosin α_1 have been isolated from preparations of calf thymosin fraction 5. One, lacking four amino acid residues at the COOH terminus, is designated des-(25-28)-thymosin α_1 . The other, named thymosin α_{11} , contains seven additional amino acid residues at the COOH terminus. The sequence of this peptide is: AcSer-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-Gly-Arg-Glu-Ala-Pro-Ala-AsnOH. Thymosin α_{11} , in doses of <300 ng per mouse, protects susceptible inbred murine strains against opportunistic infections with *Candida albicans*. It is ≈ 30 times as potent as thymosin fraction 5 and approximately equal in potency to thymosin α_1 .

A preparation from calf thymus designated thymosin fraction 5 (1, 2) has been shown to enhance parameters of immune function in several *in vivo* and *in vitro* systems (for reviews, see refs. 3 and 4). Analytical isoelectric focusing (IEF) and gel filtration chromatography of thymosin fraction 5 indicated the presence of as many as 30 distinct peptides, ranging in isoelectric points from pH 4.0 to pH 7.0 and in molecular weights from 1,000 to 15,000 (5, 6). The first peptide to be isolated from thymosin fraction 5 was thymosin α_1 ; sequence analysis showed this peptide to contain 28 amino acid residues, corresponding to a M_r of 3,107 (5). The sequence was confirmed by chemical synthesis (7). Thymosin α_1 was reported to exhibit many, but not all, of the biological activities of thymosin fraction 5 (6).

The biosynthesis of thymosin α_1 appears to involve the formation of a larger precursor polypeptide, $M_r = 16,000$, based on experiments in which calf thymus mRNA was translated *in vitro* (8, 9). The identity of proteinases involved in the processing of this putative precursor remains unknown.

We report here the isolation from calf thymus fraction 5 of two peptides structurally related to thymosin α_1 . One is a shorter peptide derived from residues 1-24 of thymosin α_1 , designated des-(25-28)-thymosin α_1 . The other, named thymosin α_{11} , is a longer peptide composed of 35 amino acid residues, the first 28 of which are identical to thymosin α_1 . The sequence of the COOH-terminal extension in thymosin α_{11} is Gly-Arg-Glu-Ala-Pro-Ala-AsnOH. We also report that thymosin α_{11} can enhance resistance to intravenous challenge with *Candida albicans* to a degree approximately equal to that observed with thymosin α_1 .

EXPERIMENTAL PROCEDURES

Materials. All chemicals and solvents employed were chromatography grade. Trypsin (TPCK-treated), *Staphylococcus aureus* V-8 protease, carboxypeptidase Y, and Polybrene were from Worthington, Miles, Sigma, and Pierce, respectively.

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Thymosin fraction 5 (batches 577 and 677), prepared by the Roche Biopolymer Department, was generously provided by Courtney McGregor. The pentapeptide Glu-Ala-Pro-Ala-AsnOH was synthesized by the solid-phase method (10).

Methods. Digestion with proteinases was carried out as described in the tables. Separation of peptides by HPLC was performed with an Ultrasphere ODS C18 column (5 μ m, 4.6 \times 250 mm, Altex, Berkeley, CA) with a fluorescamine detection system as described by Stein and Moschera (11). Amino acid analyses and the manual Edman degradation procedures were carried out as described (12), except that for the latter, Polybrene (1 mg) was added to the peptide solutions. Automated Edman degradation was by the method of Hewick *et al.* (13).

Preliminary separation of the components of thymosin fraction 5 was by preparative IEF (14, 15). After electrofocusing for 17 hr at a maximal current of 20 mA and a maximal voltage of 1.1 kV, the gel bed was divided into 30 sections with a stainless steel grid and the peptides in each fraction were eluted with 5 ml of water. The pH of each eluate was determined with a Radiometer PHM 83 Autocal pH meter.

For analysis of IEF fractions by HPLC, aliquots were lyophilized and dissolved in a small volume of buffer A (0.2 M pyridine/1.0 M HCOOH). Elution was with buffer A and a linear gradient of 1-propanol.

Fungus. The isolate of *C. albicans*, originally obtained from a human patient, was maintained on Sabouraud's dextrose agar slants at room temperature. For inoculation into mice, the yeast cells were grown in Sabouraud's dextrose broth on a shaker at 37°C for 18-20 hr.

Mouse Protection Assay. The inbred strain of mice (C₃H/HeJ) was purchased from The Jackson Laboratory. The mice were inoculated daily intraperitoneally, each with 0.5 ml of thymosin fraction 5 or one of the purified thymosins therefrom, beginning 2 days before challenge with *C. albicans* and continuing daily until the termination of the experiment. Each mouse was challenged intravenously with 4×10^4 viable cells of *C. albicans* and the numbers of fungus cells in the left kidneys were determined quantitatively at specified times after infection by grinding the tissue in sterile sand and plating increasing dilutions of the suspensions on Sabouraud's agar. The numbers of colonies of *C. albicans* were counted after incubation of the plates for 72 hr at 37°C (15).

RESULTS

Isolation of Peptides by HPLC. Thymosin α_1 was found to be present in fractions 4-7 from the IEF separations, corresponding to a pH range of 3.72-3.97. In addition to thymosin α_1 (peak b, Fig. 1), two other peptides were recovered that

Abbreviation: IEF, isoelectric focusing.

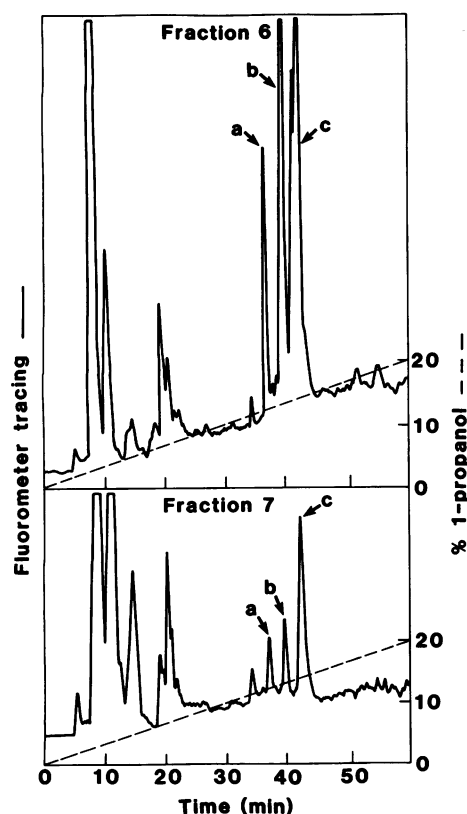


FIG. 1. Separation by HPLC of peptides in the IEF fractions. Each thymosin fraction 5 (2.0 g, batch 577) was fractionated by IEF. Each of the 30 fractions obtained was analyzed by HPLC as described (14, 15). The results are shown for IEF fractions 6 and 7 by using a linear gradient of 1-propanol in buffer A as shown (---). Peaks a, b, and c were identified as des-(25–28)-thymosin α_1 , thymosin α_1 , and thymosin α_{11} , respectively, as described in the text. Some additional thymosin α_1 was recovered from IEF fractions 4 and 5 and a small quantity of thymosin α_{11} was also present in IEF fraction 5 (data not shown).

emerged slightly before and slightly later than thymosin α_1 (peaks a and c, Fig. 1). The recoveries from 2.0 g of thymosin fraction 5 (batch 577) were 1.0 mg in peak a, 3.2 mg in peak b, and 4.2 mg in peak c, respectively. From its amino acid composition (Table 1) and analysis of tryptic fragments (data not shown), the peptide in peak b was identified as thymosin α_1 .

Table 1. Amino acid composition of peptides isolated from thymosin fraction 5

Residue*	Des-(24–25)- thymosin α_1	Thymosin α_1	Thymosin α_{11}
Asp	4.0 (3)	4.7 (4)	6.1 (5)
Thr	3.4 (3)	2.6 (3)	2.9 (3)
Ser	3.8 (3)	2.8 (3)	3.1 (3)
Glu	3.5 (4)	6.2 (6)	7.9 (7)
Gly	0 (0)	0 (0)	2.1 (1)
Ala	2.6 (2)	3.1 (3)	5.5 (5)
Val	3.0 (3)	2.4 (3)	2.5 (3)
Ile	1.0 (1)	1.0 (1)	1.0 (1)
Leu	1.0 (1)	1.0 (1)	1.1 (1)
Lys	4.0 (4)	2.9 (4)	4.0 (4)
Arg	0 (0)	0 (0)	1.2 (1)
Pro	0 (0)	0 (0)	1.0 (1)

The results are expressed as ratios to the value for isoleucine, which was taken as 1.0. The values in parentheses are those predicted from the amino acid sequences.

* Met, Tyr, Phe, and His were absent or present in only trace quantities.

Identification of the Peptide in Peak a. This peptide was characterized as a fragment derived from residues 1–24 of thymosin α_1 , based on its amino acid composition (Table 1), the absence of a tryptic peptide corresponding to residues 21–28, and the presence of a new tryptic peptide corresponding to residues 21–24 (data not shown).

Sequence Analysis of Thymosin α_{11} Purified from Peak C. The fractions in peak c were combined and rechromatographed as described in the legend to Fig. 1. The major peptide recovered (designated thymosin α_{11} , Table 1) was digested with trypsin (Fig. 2). Six fragments were recovered and identified by their amino acid composition (Table 2). Peptides T6, T3, and T1 were identical to peptides derived from residues 1–14, 15–17, and 18–20, respectively, of thymosin α_1 . Peptides T4 and T5 were similar in amino acid composition, differing only in the presence of a trace of lysine in peptide T4. Their composition indicated that they corresponded to residues 20–28 of thymosin α_1 , plus glycine and arginine. Automated sequence analysis established the structure of peptide T5 (Fig. 3). Peptide T4, which was identical to peptide T5 in amino acid composition, was found to contain aspartic acid, instead of asparagine, at position 28. The tryptic digests contained an additional peptide (T2) that was not present in tryptic digests of thymosin α_1 . This peptide contained no lysine or arginine, and must therefore have arisen from the COOH terminus of thymosin α_{11} .

Manual Edman degradation of tryptic peptide T2 yielded the sequence Glu-Ala-Pro-Ala-AsnOH. Asparagine was recovered as the free amino acid after the fourth step of the Edman procedure. The structure of peptide T2 was confirmed by its cochromatography with the synthetic pentapeptide. Localization of peptide T2 at the COOH terminus of thymosin α_{11} was confirmed by digestion of the latter with carboxypeptidase Y, which released ≈ 1 equivalent of asparagine, followed by alanine (2 equivalents) and proline (1 equivalent). The location of arginine at position 30 was confirmed by the isolation of a major fragment containing arginine after digestion of thymosin

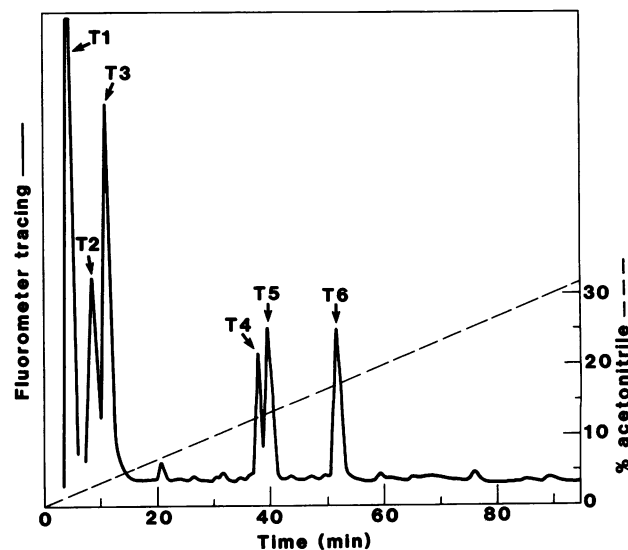


FIG. 2. Peptides recovered from the tryptic digest of thymosin α_{11} . The peptides in peak c from IEF fractions 6 and 7 (Fig. 1) were combined, lyophilized, and purified by rechromatography as described in the legend to Fig. 1. An aliquot (600 μ g) was digested with 42.9 μ g of TPCK-treated trypsin in 100 μ l of 0.4 M pyridine (pH 7.5). After 15 hr at 25°C the reaction mixture was lyophilized and the tryptic peptides were separated by HPLC using a gradient of acetonitrile in buffer A as shown (---). Fractions (0.65 ml) were collected every minute. At 6-sec intervals, 5- μ l samples were diverted to the fluorescamine detector.

Table 2. Amino acid composition of peptides isolated from tryptic and *S. aureus* V8 protease digests of thymosin α_{11}

Residue	T1 (68)*	T2 (53)*	T3 (57)*	T4 (60)*	T5 (32)*	T6 (66)*	S2 (2.6)†	S3 (0.8)†	S7 (3.7)†
Asp		0.8	1.3	1.0	1.1	2.2	1.3	1.4	1.4
Thr						2.7			
Ser						2.7			
Glu	1.0	1.0		3.4	3.3	1.1	2.4	4.8	1.7
Gly				1.0	1.0		1.4	1.1	1.9
Ala		2.0		1.0	1.0	1.9	2.6	2.7	1.0
Val				1.9	1.5	1.1		1.0	
Ile						1.0			
Leu			0.8						
Lys	2.1		1.0	0.2		1.0		2.9	
Arg				1.0	1.1		1.0	1.0	0.5
Pro		1.3					ND	ND	ND

Calculations were based on assigning a value of 1.0 for the residue shown in italics. ND, not determined.

* nmol recovered from a digest of 200 nmol of thymosin α_{11} .

† nmol recovered from a digest of 8.7 nmol of thymosin α_{11} .

α_{11} with *S. aureus* V8 protease (peptide S7, Table 2). The amino acid composition of this peptide corresponded to that predicted for residues 26–31 of thymosin α_{11} , including the last four residues of thymosin α_1 , plus the first three amino acid residues, glycine, arginine, and glutamic acid, found in the COOH-terminal extension of thymosin α_{11} . Smaller quantities of two other fragments, whose amino acid compositions corresponded to residues 19–35 (peptide S3) and 25–35 (peptide S2) of thymosin α_{11} , were also isolated from the *S. aureus* protease digests (Table 2 and Fig. 3). The results establish thymosin α_{11} as containing the thymosin α_1 sequence plus seven additional amino acids at the COOH terminus.

Mouse Protection Assay. Inbred strains of mice vary in their susceptibility to infection with *C. albicans* (16). Thus, mice of such strains as C3H/HeJ or CBA/CaJ were found to be highly susceptible to infection, whereas mice of such strains as C57BL/10SNJ or C57BL/KsJ were highly resistant to challenge. Because resistance to infection with *C. albicans* is associated with cell-mediated processes (17), and therefore with T lymphocytes, thymic hormones should have an effect on the host response. Thymosin fraction 5 and some peptides derived therefrom have been found to enhance maturation and replication of T lymphocytes (4) and, accordingly, should influence the resistance of a susceptible murine strain, such as C3H/HeJ, to infection with *C. albicans*.

Thymosin fraction 5, thymosin α_1 , or thymosin α_{11} was injected daily in graded doses into three different groups of mice, beginning 2 days before intravenous challenge with 4×10^4 cells of *C. albicans*. In comparison with control mice, at optimal doses all three preparations provided protection (Table 3). The polypeptides, thymosin α_1 , and thymosin α_{11} , were approximately equal in potency, being most active in daily doses of 160–320 ng per mouse. Because the optimal dose for thymosin fraction

5 was 5–10 μ g, the peptides were therefore about 30 times more potent than fraction 5 in their ability to induce resistance to infection with *C. albicans*.

DISCUSSION

The isolation from thymosin fraction 5 of two peptides structurally related to thymosin α_1 , one lacking four amino acids from the COOH terminus and the other containing seven additional amino acids, supports our previous suggestion that thymosin α_1 is not the native form of the thymic hormone and that peptides present in thymosin fraction 5 may be proteolytic fragments of larger peptides (18). We have previously identified thymosin β_8 , also isolated from thymosin fraction 5, as a modified form of the native peptide, thymosin β_9 , the latter containing two additional amino acid residues at the COOH terminus (14).

Both thymosin α_1 and thymosin α_{11} contain COOH-terminal asparagine residues, suggesting the existence of a proteinase with some selectivity for asparaginyl bonds. We have identified a lysosomal proteinase in rat liver that catalyzes the hydrolysis of an asparaginyl-valine bond in rabbit liver fructose-1,6-bisphosphatase (19). An internal asparaginyl-valine bond in cathepsin B also appears to be the target of a proteinase in rat liver, possibly cathepsin B itself (20).

Both thymosin α_1 and thymosin α_{11} enhanced the resistance of susceptible C3H/HeJ mice to challenge with *C. albicans*, when administered at a daily optimal dose of 160–320 ng per mouse. Increasing or decreasing the daily dosage caused a decline in the degree of host resistance.

Thymosin fraction 5, or its individual components, has been reported to enhance resistance to infection with a variety of microorganisms under a variety of host conditions. Thus, thymosin fraction 5 enhanced the cell-mediated resistance of moderately protein-malnourished mice to *Listeria monocytogenes*

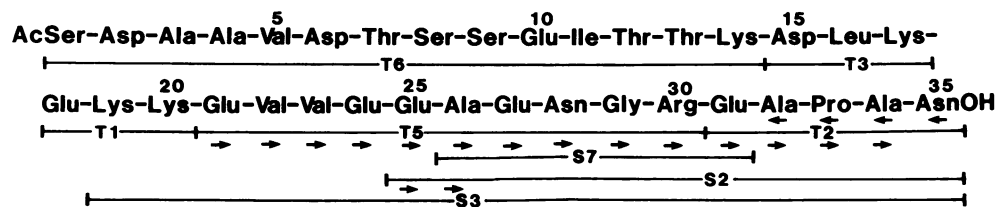


FIG. 3. The amino acid sequence of thymosin α_{11} . The sequence shown is based on the composition of the tryptic peptides (T1–T6) and of peptides generated by digestion with *S. aureus* V8 protease (S2, S3, and S7). The tryptic peptide lacking lysine and arginine, presumably derived from the COOH terminus, was analyzed by Edman degradation (→). For peptide T5, amino acids released from thymosin α_{11} by carboxypeptidase Y are indicated (←). Des-(25–28)-thymosin α_1 and thymosin α_1 would be derived from residues 1–24 and residues 1–28 of thymosin α_{11} , respectively.

Table 3. Effect of thymosin fraction 5 and thymic peptides on the growth of *C. albicans* in C3H/HeJ mice

Thymosin fraction 5		Thymosin α_1		Thymosin α_{11}	
Dose, ng per mouse	<i>C. albicans</i> cell count*	Dose, ng per mouse	<i>C. albicans</i> cell count*	Dose, ng per mouse	<i>C. albicans</i> cell count*
None	5,100				
2,560	8,500	80	5,870	80	4,200
5,120	440	160	190	160	510
10,240	320	320	780	320	320
20,480	1,600	640	1,410	640	1,260

Mice were treated daily with the indicated doses of thymosin fraction 5, thymosin α_1 , or thymosin α_{11} and challenged with 4×10^4 cells of *C. albicans* 2 days after the start of treatment (see *Methods*).

* Three mice from each set were sacrificed on days 7, 14, and 21 after infection and the values represent the average number of organisms in the left kidneys of the nine mice in each set.

(21) and of alloxan-diabetic mice to *C. albicans* (22). Also, administration of thymosin α_1 at optimal dose and schedule protected CD2F₁ mice infected with lethal doses of *C. albicans* (23) and of ddY or C57BL/6 mice infected with *C. albicans*, *Listeria monocytogenes*, or *Serratia marcescens* after immunosuppression with 5-fluorouracil (24).

It is highly important that the individual components of thymosin fraction 5 be separated, purified, and characterized. This concept is based on the observation that the action of thymosin fraction 5 is often quite variable. For example, when the effect of thymosin fraction 5 on human peripheral blood lymphocytes was determined *in vitro* in an assay employing an allogeneic "mixed lymphocyte reaction," the outcome of the response was unpredictable (25). This result was attributed to an ability of thymosin fraction 5 to stimulate both helper and suppressor T lymphocytes. In addition, administration of thymosin fraction 5 to some susceptible murine strains—i.e., C3H/HeJ—enhanced cell-mediated immunity, whereas administration to resistant murine strains—i.e., C57BL/10SNJ—decreased cell-mediated immunity (16, 26).

Thymosin α_{11} may itself represent a proteolytically modified fragment of the native thymic peptide; if so, it is a larger fragment than thymosin α_1 , closer in structure to the native peptide and retaining biological activity. The isolation of the native peptide and evaluation of its chemical and biological properties will require extraction conditions that preclude any possibility of proteolysis.

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