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Thymosins β_8 and β_9: Two new peptides isolated from calf thymus homologous to thymosin β_4

(hormone/thymic/amino acid sequence/acetylated peptide)

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ABSTRACT **Two new peptides, designated thymosin β_8 and thymosin β_9, respectively, have been isolated and their amino acid sequences established. Thymosin β_8, isolated from calf thymus fraction 5, has a mass of 4518 daltons and contains 39 amino acid residues, of which 31 are identical to the corresponding amino acid residues in thymosin β_4 isolated from the same source. The NH_2 terminus of thymosin β_8 is acetylalanine, compared with acetylserine in thymosin β_4. Thymosin β_9, isolated from fresh-frozen calf thymus by a procedure that minimizes proteolysis, is identical to thymosin β_8 except for the presence of an additional dipeptide, -Ala-LysOH, at the COOH terminus. It has a mass of 4717 daltons and 32 of its 41 amino acids are identical to those of thymosin β_4. The similarity in structures of thymosin β_4 and thymosin β_9 suggests that they may have related functions.**

Peptide hormones from the thymus gland are considered to play a role in the maturation and differentiation of T cells (for reviews, see refs. 1 and 2). A heat-stable fraction prepared from calf thymus extracts, designated thymosin fraction 5 (3), has elicited considerable interest because it has shown promise for the restoration of immune function in patients with some immunodeficiency diseases (4, 5). Thymosin fraction 5 was found to contain a number of peptides, and several of them have been isolated in pure form and subjected to sequence determination. These include thymosin α_1, an acidic peptide (pI = 4.2) containing 28 amino acid residues (6, 7), and thymosin β_4 (pI = 5.1) containing 44 amino acid residues (8). Each is modified by acetylation at the NH_2 terminus. Bovine fraction 5 has also been shown to contain significant quantities of ubiquitin (refs. 7 and 9; see also ref. 10), but this peptide is not considered to contribute to the biological activity of the preparation (7). However, ubiquitin may function as a modulator of the activities of other thymic peptides (11).

During the course of studies on the biosynthesis of these thymic peptides, which involved the characterization of a putative precursor polypeptide (12) formed during the translation of thymus mRNA *in vitro*, we undertook the isolation of thymosin β_4 for use as a reference compound. A simple procedure was developed that yielded thymosin β_4 in higher yield than previously reported. In addition a new peptide, designated thymosin β_8†, whose sequence is reported here, was also isolated. Thymosin β_8 was found to be closely homologous to thymosin β_4, differing in only eight amino acid substitutions and in the lack of four residues at the COOH terminus.

We have recently developed a method for the isolation of peptides from calf thymus that minimizes the possibility of proteolytic modification (13). Extracts prepared by this procedure contained important quantities of thymosin β_4, but thymosin β_8 could not be detected. Instead, we reported the presence of a new peptide, designated X, that emerged slightly earlier in the reverse-phase HPLC. Peptide X has now been shown to be identical to thymosin β_8 except for the presence of two additional residues, -Ala-LysOH, at the COOH terminus. We conclude that thymosin β_9 is the natural form of the peptide and

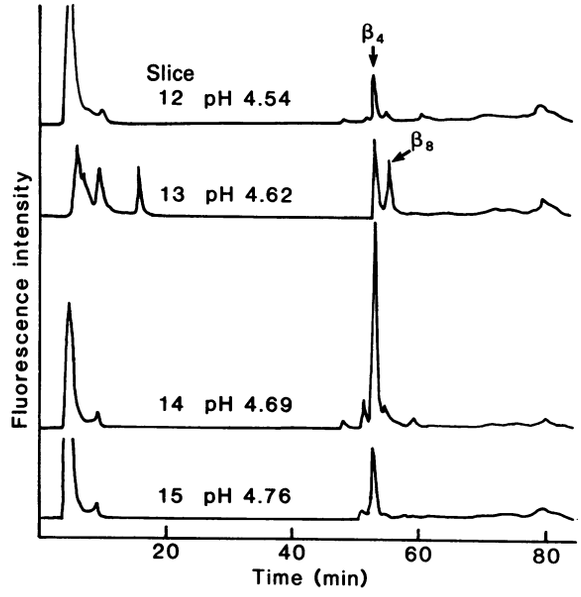

FIG. 1. HPLC analysis of fractions separated by preparative isoelectric focusing (21). A sample of thymosin fraction 5 (1.2 g) was dissolved in 100 ml of 2% Ampholine (LKB) for separation in the pH range 4–6 and filtered through a 0.45-μm Millipore HA filter. After addition of 4 g of Ultrodex the gel was cast onto a glass plate (11 × 24.5 cm) and electrofocused for 16 hr over the long direction at 8°C and 8 W (voltage, <1.5 kV; current, <20 mA). With a grid, the focused gel was cut into 30 sections, each of which was eluted on a small column with 5 ml of H_2O. The pH of each eluate was determined at 0°C. For analysis by HPLC, 5-μl aliquots of each eluate were applied to a RP18 column (Altex Ultrasphere-ODS, 5 μm, 4.6 × 250 mm) equilibrated with 0.2 M pyridine/1 M formic acid/0.05% thiodiglycol at a flow rate of 0.7 ml/min. The peptides were eluted with the same buffer containing *n*-propanol, the concentration of which was increased by 4% every 10 min to a final concentration of 40% (vol/vol). At 10-s intervals, 5-μl samples were diverted to the fluorescamine detector (22). For the preparative runs (not shown in figure), twelve 450-μl aliquots of the eluates from slices 12–15 were injected successively onto the same RP18 column used for the analytical runs and the peptides were eluted with the same buffer and *n*-propanol gradient. Fractions (0.7 ml) were collected every minute and aliquots (5 μl of a 1:25 dilution) of each fraction were analyzed by direct injection into the borate buffer line of the fluorescamine detector. Fractions found to contain the peptides corresponding to thymosin β_4 and thymosin β_8 were separately pooled and lyophilized.

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† This follows the nomenclature suggested by Goldstein *et al.* (6), based on pIs and order of isolation from fraction 5.

that it is converted to thymosin β_8 during the preparation of fraction 5. The results emphasize the need to avoid conditions that favor the action of endogenous proteinases in the isolation of peptides from thymus and other tissues.

The presence of the two very similar peptides, thymosin β_4 and thymosin β_9, in calf thymus extracts raises interesting questions regarding their possible role in the regulation of the immune response.

MATERIALS AND METHODS

Materials. Thymosin fraction 5, isolated by the procedure of Goldstein *et al.* (3), was generously provided by W. C. McGregor of the Biopolymer Laboratory of Hoffmann–La Roche. Trypsin (L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated) and carboxypeptidases A and B were from Worthington and *Staphlococcus aureus* protease V8 was from Miles. Ampholines and Ultrodex were from LKB. Enzymes for analysis of acetic acid (14) were from Sigma. All other reagents were chromatography grade.

Methods. Amino acid analyses were carried out with a Glenco amino acid analyzer model MM70 modified for derivitization with *o*-phthalaldehyde (15) and fluorescence detection. Proline was determined with an amino acid analyzer constructed at this Institute employing a fluorescamine detection system after oxidation with *N*-chlorosuccinimide (16). Amino acids released by carboxypeptidase A were analyzed by HPLC on an Altex Ultrosphere-ODS column after prelabeling with *o*-phthalaldehyde (17). Manual Edman degradation was carried out as described by Tarr (18).

Mild acid hydrolysis was carried out in 30 mM HCl at 105°C for 15 hr (19). Digestion with carboxypeptidase A was carried out at room temperature for 2 hr in 0.2 M pyridine (pH 7.4) with a ratio of peptide to carboxypeptidase of 12.5:1 (wt/wt). Digestion with trypsin and protease V8 (20) was carried out as described in the figure legends and tables.

RESULTS

Isolation of Thymosin β_4 and Thymosin β_8 from Thymosin Fraction 5. The peptides in thymosin fraction 5 were separated by preparative isoelectric focusing and purified by HPLC as described in the legend to Fig. 1. From 1.2 g of fraction 5 we recovered 16 mg of thymosin β_4 and 1 mg of thymosin β_8. Thymosin α_1 (16 mg) was also recovered from isoelectric focusing gel segment 3 (pH = 3.9) by the same HPLC procedure (data not shown).

Sequence Analysis of Thymosin β_4 and Thymosin β_8. Tryptic digests of peptides β_4 and β_8 were analyzed by HPLC. Thymosin β_8 yielded five major tryptic peptides compared to the seven previously identified in digests of thymosin β_4 (Fig. 2). Peptides Tc from thymosin β_4 and T2 from thymosin β_8 were identical with respect to retention time and amino acid composition (Tables 1 and 2) and corresponded to residues 17–25 of each peptide (see Fig. 4). Peptide T3 of thymosin β_8 contained lysine and had a COOH-terminal glutamine residue as determined by digestion with carboxypeptidase A. The terminal Lys^{38}-$Gln^{39}OH$ bond in thymosin β_8 was not cleaved by trypsin.

The peptides derived from thymosin β_4 confirmed the sequence reported by Low *et al.* (8). The sequences of peptides derived from thymosin β_8 (Fig. 3) were determined by manual Edman degradation except for the blocked NH_2-terminal peptide (peptide T5). This sequence was determined by digestion of T5 with *S. aureus* protease V8, which yielded peptide T5S2 (see Table 2 and Fig. 3); this was followed by mild acid hydrolysis which yielded 2 eq of aspartic acid and one eq of alanine, plus a dipeptide—Lys-Pro—and a tripeptide—Leu-Gly-Glu—whose sequences were established by Edman degradation (Fig. 3). Assignment of alanine to position 1 was also based on the fact that none of the other residues in T5S2 is known to occur as an acetylated NH_2-terminal residue in proteins (23) or in other peptides isolated from thymosin fraction 5.

Digestion of intact thymosin β_8 with protease V8 yielded the overlapping peptides that permitted final ordering of the tryptic peptides (Table 3 and Fig. 3).

Identification of the NH_2-Terminal Acetyl Group. This was carried out as described by Fasold (24). Aliquots of β_4 (196 nmol) and β_8 (200 nmol) hydrolyzed with 10 μl of concentrated H_2SO_4 under an atmosphere of N_2 yielded 155 nmol of acetic acid from β_4 and 122 nmol from β_8, corresponding to 78% and 62%, respectively, of theoretical yields.

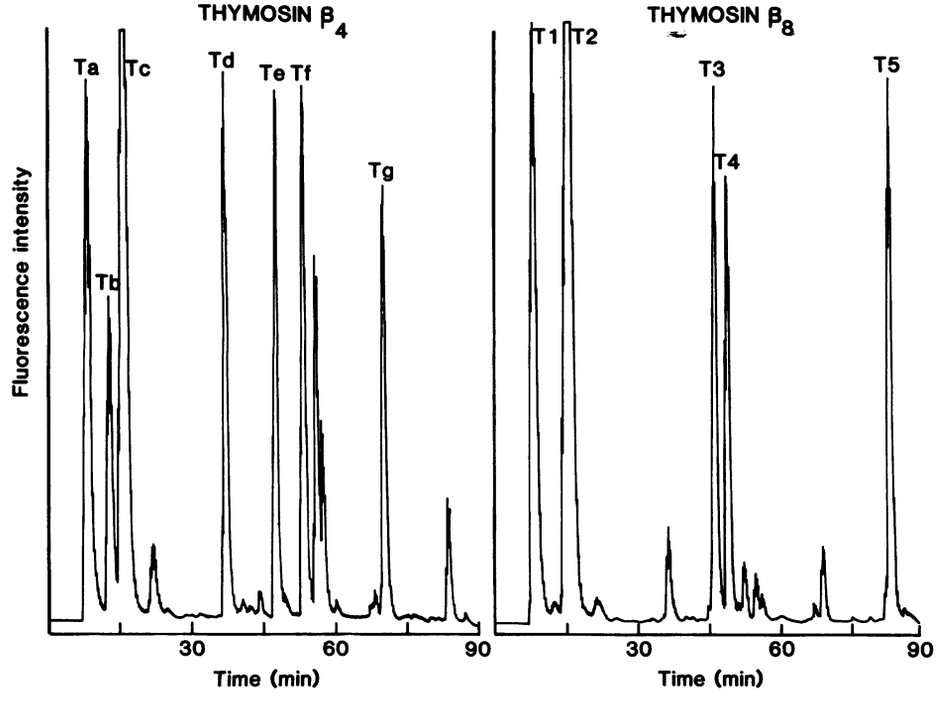

FIG. 2. HPLC separation of tryptic peptides from thymosin β_4 and thymosin β_8. Digestion with trypsin was carried out in 200-μl volumes containing 400 μg of thymosin β_4 (*Left*) or 312 mg of thymosin β_8 (*Right*) and 20 μg of trypsin in 0.4 M pyridine (pH 7.5). After 14 hr at room temperature the reactions were terminated by the addition of 15.2 μl of concentrated HCOOH and injected onto the Ultrasphere-ODS column (described in legend to Fig. 1) followed by addition of 0.2 M pyridine/1 M formic acid at a flow rate of 0.37 ml/min. Elution was with a gradient of CH_3CN, increasing from 0 to 40% (vol/vol) over a period of 2 hr. Aliquots (5 μl) were diverted to the fluorescamine detection system every 20 s. Fractions were collected every 2 min. The major peaks are labeled (see text); most of the minor peaks were identified as products of incomplete digestion.

Table 1. Amino acid compositions of thymosin β_4 and the peptides isolated from tryptic digests

		Tryptic peptides						
Residue	β_4	Ta (15–16)	Tb (39–43)	Tc (17–25)	Td (12–14)	Te (32–38)	Tf (26–31)	Tg (1–11)
Asp	3.7 (4)				1.0 (1)		1.0 (1)	2.0 (2)
Thr	3.1 (3)			2.0 (2)		0.8 (1)		
Ser	3.9 (4)	0.9 (1)	0.8 (1)				1.0 (1)	0.8 (1)
Glu	10.4 (11)		2.0 (2)	3.1 (3)		4.1 (4)		2.2 (2)
Pro	3.2 (3)						ND (2)	ND (1)
Gly	1.5 (1)		1.1 (1)					
Ala	2.2 (2)		1.1 (1)					0.9 (1)
Cys	0 (0)							
Val	0.2 (0)							
Met	1.0 (1)							1.0 (1)
Ile	1.9 (2)					0.9 (1)		1.0 (1)
Leu	2.2 (2)			0.9 (1)			0.9 (1)	
Tyr	0.2 (0)							
Phe	1.0 (1)				1.0 (1)			
Lys	7.9 (9)	1.1 (1)		2.9 (3)	1.0 (1)	1.2 (1)	1.1 (1)	2.1 (2)
His	0.1 (0)							
Arg	0.4 (0)							

Numbers in parentheses are the values and assigned positions in the sequence reported by Low *et al.* (8). Peptides were those recovered from HPLC as described in legend to Fig. 2. Values are mol/mol of peptide. ND, not determined.

Isolation and Sequence Analysis of Thymosin β_9. Thymosin β_9 was isolated directly from frozen calf thymus by using extraction of the frozen tissue with 6 M guanidinium chloride to prevent proteolytic activity (13). Thymosin β_9, which emerges immediately after thymosin β_4 (see figure 1 of ref. 13), was separated from the latter by HPLC (see figure 3 of ref. 13) after conversion of thymosin β_4 to the sulfoxide form (14). The yield from 1 g of fresh thymus was 35–80 μg of thymosin β_4 and 8–18 μg of thymosin β_9.

Digestion of thymosin β_9 with trypsin and separation of the tryptic peptides by HPLC yielded a pattern indistinguishable from that obtained with thymosin β_8. However, acid hydrolysis of peak T1 derived from thymosin β_9 yielded the composition Ala (1.6), Lys (2.4), Glu (1.0), indicating the presence of alanine, lysine, and glutamate residues that were not present in peak T1 derived from thymosin β_8. Digestion of thymosin β_9 with protease V8 yielded a tetrapeptide—Glu (1.0), Lys (1.8), Ala (1.1)—instead of the dipeptide Lys-Glu. Digestion of thymosin

Table 2. Amino acid compositions of thymosin β_8 and the peptides isolated following digestion with trypsin

		Tryptic peptides								
Residue	β_8	T1* (15–16)§	T2* (17–25)	T3* (32–39)	T4* (26–31)	T5* (1–14)	T5S1† (9–14)	T5S2† (1–8)	T5S2H1‡	T5S2H2‡
Asp	4.6 (5)				0.9 (1)	3.8 (4)	1.8 (2)	1.7 (2)		
Thr	3.8 (5)		1.7 (2)	0.9 (1)	2.0 (2)					
Ser	1.7 (1)					0.8 (1)	0.9 (1)			
Glu	9.0 (9)		3.0 (3)	5.1 (5)		1.2 (1)		1.1 (1)		0.9 (1)
Pro§	2.2 (2)				ND (1)	ND (1)		ND (1)	0.9 (1)	
Gly	1.3 (1)					1.0 (1)		1.0 (1)		1.0 (1)
Ala	2.2 (2)	1.0 (1)				1.1 (1)		1.3 (1)		
Cys	0 (0)									
Val	0.3 (0)									
Met	0.2 (0)									
Ile	1.8 (2)			0.9 (1)		1.0 (1)	0.9 (1)			
Leu	2.9 (3)		1.0 (1)		1.0 (1)	1.0 (1)		0.9 (1)		1.1 (1)
Tyr	0.2 (0)									
Phe	1.0 (1)					1.0 (1)	1.1 (1)			
Lys	8.3 (8)	1.0 (1)	3.3 (3)	1.2 (1)	1.2 (1)	2.2 (2)	1.2 (1)	1.1 (1)	1.1 (1)	
His	0.1 (1)									
Arg	0.3 (0)									
Recovered, nmol		45.2	50.2	42.3	57.8	39.2				

Numbers in parentheses are the values and assigned positions based on the sequence reported here. Values are shown as mol/mol of peptide. ND, not determined.

* Tryptic peptides recovered from HPLC as described in legend to Fig. 2.

† Tryptic peptide T5 was digested with protease V8 as described in the footnote to Table 3. The peptides were separated by HPLC as described in legend to Fig. 2.

‡ Peptide T5S2 was hydrolyzed with 30 mM HCl at 105°C for 15 hr (see *Methods*). The peptides were separated by HPLC as described in legend to Fig. 2. Free aspartic acid and free alanine were also found to be present by direct amino acid analysis.

§ Proline was not determined in the amino acid analyses but was found during Edman degradation.

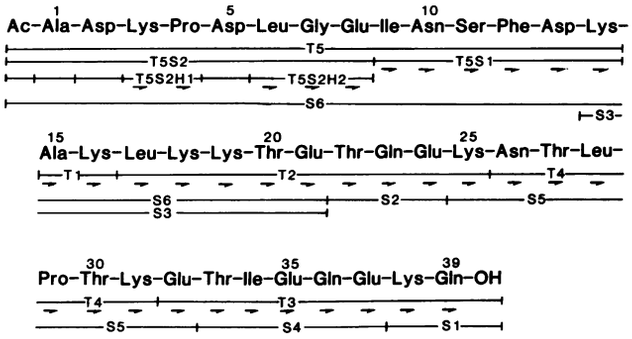

FIG. 3. Sequence determination of thymosin β_8. The peptides were derived from digestion with trypsin (T1–T5) or with protease V8 (S1–S4).

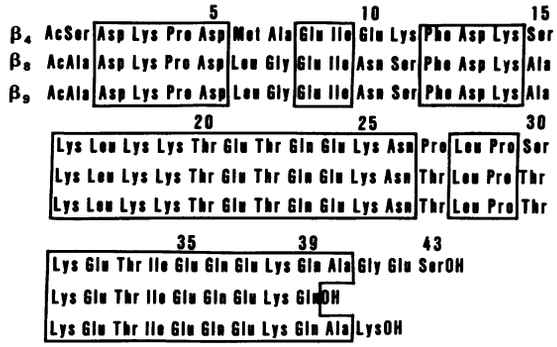

FIG. 4. Comparison of amino acid sequences of thymosin β_4 (8), thymosin β_8, and thymosin β_9. Identical sequences are in boxes.

β_9 with carboxypeptidase B yielded 1.2 eq of lysine, and subsequent addition of carboxypeptidase A yielded Ala (1.2 eq/mol), Lys (1.8 eq/mol, total), Gln (1.3 eq/mol), and Glu (0.2 eq/mol). Peak T1 derived from the tryptic digests thus contained both the tripeptide Glu-Ala-Lys from the COOH terminus and the dipeptide Ala-Lys from positions 15 and 16 (see Fig. 3). The results establish the terminal sequence of thymosin β_9 as shown in Fig. 4.

DISCUSSION

The two-step isolation procedure reported here is rapid and convenient for the isolation of peptides from thymosin fraction 5. However, two peptides present in fraction 5—thymosin α_1 and thymosin β_8—were not detected when the frozen tissue was extracted directly with 6 M guanidinium chloride. Instead of thymosin β_8, we obtained a peptide—thymosin β_9—containing two additional amino acids at the COOH terminus, and we conclude that thymosin β_8 is an artifact generated from thymosin β_9 during the preparation of fraction 5. The absence of thymosin α_1 in the guanidinium chloride extracts (see also ref. 13) suggests that it is also derived from a larger as yet unidentified peptide present in the thymus gland.

Table 3. Amino acid composition of peptides formed from thymosin β_8 by digestion with protease V8

	Peptides isolated					
Residue	S1 (38–39)	S2 (22–24)	S3 (14–21)	S4 (33–37)	S5 (25–32)	S6 (1–21)
Asp					1.0 (1)	4.3 (4)
Thr		0.9 (1)	0.8 (1)	0.9 (1)	1.6 (2)	0.6 (1)
Ser						1.2 (1)
Glu	0.9 (1)	2.1 (2)	1.1 (1)	2.9 (3)	1.0 (1)	2.1 (2)
Gly						1.4 (1)
Ala			0.9 (1)			2.1 (2)
Ile				1.2 (1)		1.2 (1)
Leu			1.3 (1)		1.3 (1)	2.1 (2)
Phe						1.2 (1)
Lys	1.1 (1)		3.9 (4)		2.1 (2)	4.7 (5)
Recovered, nmol	4.6	5.2	1.9	4.6	4.6	4.1

Digestion with protease V8 was carried out in 40-μl reaction mixtures containing 0.1 M NH_4HCO_3 (pH 7.8), 2 mM EDTA, and a 30:1 ratio of peptide to protease (wt/wt). After incubation for 14 hr at 25°C the solutions were diluted to 400 μl with 0.2 M pyridine/1 M formic acid and the peptides were separated on an RP18 column as described in legend to Fig. 2. In this experiment 6.98 nmol of peptide was digested. Numbers in parentheses are the values and assigned positions based on the amino acid sequence reported here.

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