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ISOLATION OF PEPTIDES FROM CALF THYMUS

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SUMMARY: A procedure that minimizes the possibility of proteolytic modification has been developed for the isolation of peptides from calf thymus. Separation of the peptide fraction by reverse phase high performance liquid chromatography revealed the presence of a major peak identified as thymosin β_4 [Low *et al* (1981) Proc. Nat. Acad. Sci. USA *78*, 1162-1166], but thymosin α_1 [Low *et al* (1979) J. Biol. Chem. *254*, 981-986] was absent or present in only trace amounts. The absence of thymosin α_1 and other peptides of similar size suggests that their presence in thymosin Fraction 5 may be the result of proteolytic modification of larger thymic peptides or proteins.

Peptides isolated from the thymus gland have attracted considerable attention because of their possible role in the development and maintenance of the immune response (for reviews see 1-3). Of particular interest is a preparation designated thymosin Fraction 5 (4), which has been employed in clinical trials for the treatment of immunodeficiency diseases (5) and for the restoration of cellular immunity in cancer patients receiving chemotherapy (6). Thymosin Fraction 5 has been reported (7) to contain a number of peptides, several of which, including thymosin α_1 (8,9) and thymosin β_4 (10), have been isolated and their sequence established.

We have now developed a procedure for the isolation of peptides from calf thymus that greatly reduces the possibility of proteolytic digestion. We find that calf thymus has few peptides in the size class represented by thymosin α_1 (28 residues) and thymosin β_4 (43 residues). Thymosin β_4 was the major peptide present in the extracts and was isolated in much larger quantities than predicted

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from its content in Fraction 5. Thymosin α_1, on the other hand, was absent or present in only trace quantities.

MATERIALS AND METHODS

Materials. Calf thymus glands collected at the slaughterhouse were frozen immediately in liquid N_2 and stored in liquid N_2. All chemicals and solvents were chromatography grade.

Preparation of extracts and concentrated peptide solutions. Pieces of frozen calf thymus (60 g) were pulverized with dry ice in a Waring Blender and the still frozen powder added with stirring to 1140 ml of 6 M guanidinium chloride. The suspension was then returned to the blender and blended at high speed to shear DNA. Nucleic acids were removed by the addition of 0.5 volumes of EtOH, storage at -20°C overnight and centrifugation. The supernatant solution was filtered successively through Whatman No. 541 and Whatman No. 1 filters and pumped through a hollow fiber concentration system (Amicon DC2, HP10 Cartridge) having a cut-off of 10,000 daltons. The ultrafiltrate was collected and the solution retained by the filters was treated with 200 ml of guanidinium chloride and the ultrafiltration procedure repeated twice. The combined ultrafiltrates were diluted with 2 volumes of 0.2 M pyridine-1 M HCOOH (volume = 4.4 ℓ) and aliquots (2 ml) removed for direct analysis by reverse phase hplc (see below) to evaluate recovery of peptides in the succeeding steps.

The bulk of the solution was then pumped on to a RP8 column [Lobar prepackaged column, Size B (310-25) Lichroprep™ RP8, 40-63 μm, EM Reagents] and the filtrate analyzed by hplc to ensure that all peptides were bound. The column was washed with 200 ml of 0.2 M pyridine-1 M HCOOH and the peptides eluted with 200 ml of 20% propanol in the same buffer. The larger peptides and proteins were then eluted with 200 ml of 40% propanol in 0.4 M pyridine-0.5 M HCOOH, pH 4.0. The rate of application and elution was approximately 9 ml/min.

RESULTS

Separation by hplc of peptides from the extracts and Lobar column eluates.

The major peptide present in the original guanidinium chloride ultrafiltrate was thymosin β_4 (Fig. 1, peak 2), except for the larger unidentified peptides that eluted late in the analytical hplc. No peptide was detected at the location of thymosin α_1, indicated by the arrow in Fig. 1. Immediately following thymosin β_4 was a peak (peak 3) containing an unknown peptide which was separated from thymosin β_4 in the preparative experiments as described below.

The 20% propanol eluates from the Lobar RP8 column contained 80% of the thymosin β_4 present in the original ultrafiltrate (Fig. 2A). Only an additional trace was present in the 40% propanol eluate (Fig. 2B). The latter contained only the larger peptides that eluted late (with 36-40% propanol) from the analytical RP18 columns (compare Fig. 1 and Fig. 2B).

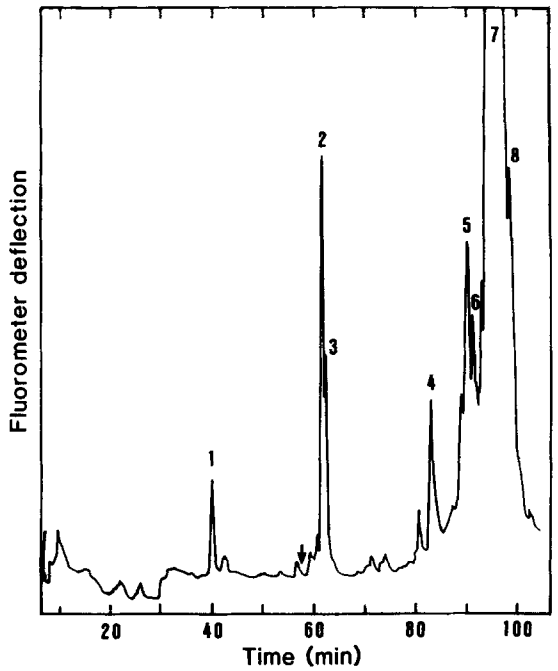

Fig. 1: Separation of peptides in the guanidinium chloride extract from calf thymus. A 2 ml aliquot of the combined guanidinium chloride filtrates (corresponding to 30 mg of calf thymus, see text) was applied to an Altex Ultrosphere-ODS C-18 column (5 μ 4.6 x 250 mm) and eluted at a flow rate of 0.7 ml/min with 0.2 M pyridine-1 M HCOOH buffer, to which 4% propanol was added after 5 min and increased to 40% in 4% increments every 10 min. At 7 s intervals 5 μl samples were diverted to the fluorescamine peptide detector (11). The arrow indicates the position of elution of a sample of synthetic thymosin α_1 (gift of Dr. Arthur Felix, Hoffmann-La Roche, Inc.).

Purification and characterization of thymosin β_4. To separate thymosin β_4 from the adjacent unknown peptide, advantage was taken of the presence of methionine in the former (10). The 20% and 40% propanol eluates from the Lobar RP8 column were combined and lyophilized, dissolved in 5 ml of 0.2 M pyridine-1 M HCOOH and oxidized for 45 min at room temperature with one-half volume of 30% H_2O_2. The sulfoxide form of thymosin β_4 was then recovered by reverse phase hplc (Fig. 3A, peak 3), which also yielded the unknown peptide (Fig. 3A, peak X). This peptide was found to be similar to thymosin β_4 in amino acid composition, but it did not contain methionine. Its structure is now under investigation.

Reduction of oxidized thymosin β_4 was carried out with 20% mercaptoethanol under argon for 24 h at 37°C. The recovery of the reduced peptide, now free of contaminating peptides, was 70% (Fig. 3B). The structure of thymosin β_4

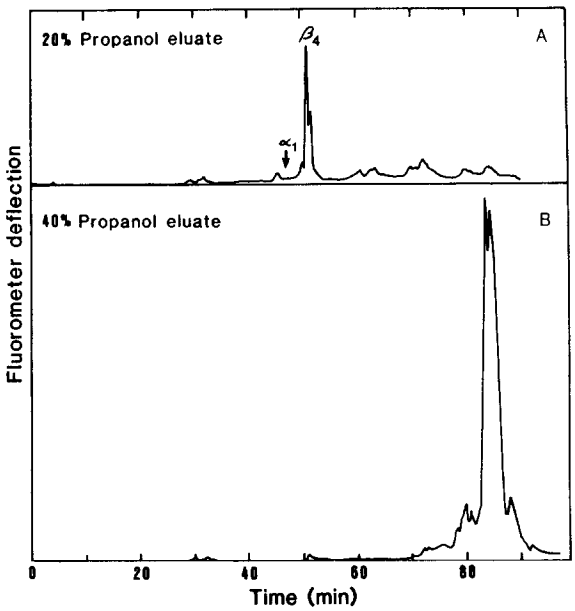

Fig. 2: Separation of peptides present in the eluates from the preparative RP8 columns. A. Fractions eluted with 20% propanol. B. Fractions eluted with 40% propanol. Analytical reverse phase hplc was carried out with aliquots corresponding to 60 mg (1%) of the original tissue sample as described in the legend to Fig. 1 except that elution was started at 0 min with 4% propanol, increased by 4% increments every 10 min. The flow rate was 0.7 ml/min. The fluorometer sensitivity was 0.1 compared to that shown in Fig. 1.

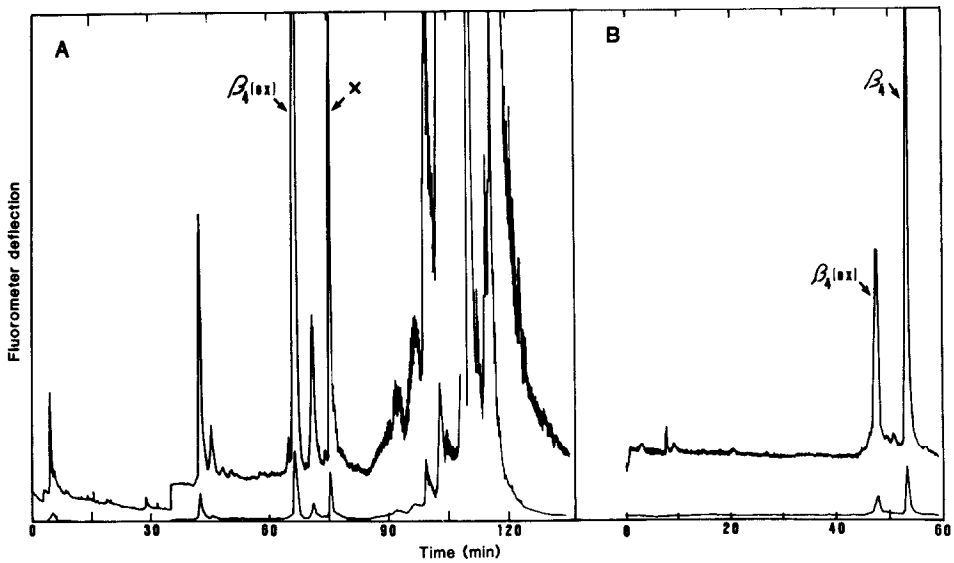

Fig. 3: Purification of thymosin β_4 by hplc. A. The combined 20 + 40% propanol eluate, after oxidation as described in the text, was chromatographed as described in the legend to Fig. 1 with 4% propanol added at 0 min and increased by 4% increments every 15 min. The flow rate was 0.5 ml/min. The sulfoxide form of thymosin β_4 is indicated by β_4(ox) and the new uncharacterized peptide that emerges immediately after thymosin indicated by X. B. The peptide recovered from the fraction corresponding to that designated β_4(ox) in A was reduced as described in the text and chromatographed on the same column with 4% propanol added at 0 min and increased by 4% increments every 10 min. The flow rate was 0.7 ml/min. Two peaks, corresponding to thymosin β_4 and a smaller quantity of the sulfoxide form, were recovered.

TABLE I

Amino Acid Composition of Purified Thymosin β_4 and Tryptic Peptides[a]

Amino acid	Composition	T1 (15-16)	T2 (39-43)	T3 (17-25)	T4 (12-14)	T5 (32-38)	T6 (26-31)	T7 (1-11)
Asp	4.6 (4)				1.1 (1)		1.1 (1)	2.3 (2)
Thr	3.0 (3)			1.5 (2)		0.9 (1)		
Ser	3.7 (4)	0.9 (1)	1.0 (1)				0.9 (1)	1.1 (1)
Glu	10.6 (11)		2.2 (2)	2.8 (3)		4.2 (4)		1.9 (2)
Pro	nd (3)						nd (2)	nd (1)
Gly	1.2 (1)		1.0 (1)					
Ala	2.1 (2)		1.0 (1)					0.9 (1)
Vla	0.1 (0)							
Met	0.7 (1)							0.3 (1)
Ile	2.0 (2)					1.0 (1)		0.8 (1)
Leu	2.2 (2)			1.7 (1)			1.0 (1)	
Tyr	0.1 (0)							
Phe	1.1 (1)				1.1 (1)			
Lys	8.5 (9)	1.1 (1)		3.0 (3)	1.0 (1)	1.1 (1)	1.0 (1)	1.7 (2)
His	0 (0)							
Arg	0.1 (0)							

[a]Digestion with trypsin was carried out in 200 µl volumes containing 400 µg of thymosin β_4 and 20 µg of trypsin in 0.4 M pyridine, pH 7.5. After 14 h at room temperature the reactions were terminated by the addition of 15 µl of concentrated HCOOH and injected on to the Ultrosphere-ODS column. Elution was described in the legend to Fig. 1. Amino acid analyses were carried out on a Glenco Amino Acid Analyzer Model MM 70 modified for derivitization with *o*-phthalaldehyde and fluorescamine detection. Samples for analysis were hydrolyzed in 6 M HCl in sealed evacuated tubes at 155°C for 45 min. Proline was not determined (nd). The values in parentheses were those predicted from the sequence reported by Low *et al* (10).

recovered from the last column was confirmed by its amino acid composition and by the separation and analysis of tryptic peptides (Table I).

Quantitative considerations. Based on the results of the direct analysis of aliquots from combined ultrafiltrates of several preparations, the quantity of thymosin β_4 present in calf thymus would be in the range of 35-80 µg/g tissue. This is to be compared with a value of 4.1 µg of thymosin per gram of thymus gland calculated from the quantities recovered from thymosin Fraction 5 as reported by Low *et al* (10).

The quantity of thymosin α_1 in calf thymus, estimated from the quantity that would have been detected in the combined guanidinium chloride filtrates would be less than 0.5 µg/g of calf thymus. This is less than 10% of the quantity expected from the recovery reported by Low *et al* from Fraction 5 (9). The lower value reported here was confirmed by radioimmune assay (unpublished procedure) of the same ultrafiltrate carried out by J. Symington and H. Spiegel of Hoffmann-La Roche, Inc. This assay yielded a value corresponding to 0.3 µg/g of calf thymus.

DISCUSSION

The results reported here suggest that calf thymus contains a relatively small number of peptides, most of which are considerably larger than thymosin β_4, based on their behavior in reverse phase hplc. Thymosin β_4 appears to be a major component, but thymosin α_1 is present in very small quantities, if at all. The presence of thymosin α_1 in thymosin Fraction 5 must therefore be attributed to its formation from larger precursors during the preparation of Fraction 5. We have previously reported evidence for a 16,000 dalton polypeptide containing sequences characteristic of thymosin α_1, synthesized *in vitro* in a translation system containing thymus mRNA (12,13). It will be of interest to determine the relation of this putative precursor to peptides in the thymus gland that can give rise to thymosin α_1.

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