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Biochemistry

Prothymosin α: Isolation and properties of the major immunoreactive form of thymosin α_1 in rat thymus

(radioimmunoassay/thymic polypeptide/protection against opportunistic infections)

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ABSTRACT **A polypeptide containing ≈112 amino acid residues, with the thymosin α_1 sequence at its NH_2 terminus, has been isolated from rat thymus by using a radioimmunoassay with an antibody prepared against synthetic thymosin α_1. The new polypeptide, named "prothymosin α," was found to be the major substance crossreacting with thymosin α_1 antiserum in rat thymus extracts; peptides corresponding to thymosin α_1 or thymosin α_{11} were not detected. In gel filtration at pH 2.8, prothymosin α emerged as a single symmetrical peak corresponding to an apparent molecular weight of 32,000, approximately 3 times larger than the minimum molecular weight calculated from its amino acid composition. On the same gel filtration columns, synthetic thymosin α_1 (calculated M_r = 3108) emerged at a position corresponding to a molecular weight of 10,000–11,000. Thus, both prothymosin α and thymosin α_1 appear to exist in solution as oligomers, possibly as trimers. Prothymosin α and synthetic thymosin α_1 also were separated readily in reverse-phase HPLC and in isoelectric focusing; the isoelectric point of prothymosin α determined by the latter procedure was found to be 3.55, consistent with an unusually high content of glutamic and aspartic acids based on amino acid analyses. Prothymosin α appears to represent the native polypeptide from which thymosin α_1 and other fragments are generated during the isolation of thymosin fraction 5.**

Thymosin α_1, a peptide containing 28 amino acid residues, was isolated by Goldstein and coworkers (1) from thymosin fraction 5, a mixture of peptides from calf thymus (2). Thymosin fraction 5 had been reported earlier to restore parameters of immunocompetence in neonatally thymectomized mice (3). Thymosin α_1 was found to be active in some of the *in vitro* tests used for thymosin fraction 5 (4), and it was considered to be one of the factors that modulated steps in the maturation of T cells (5).

We have reported (6) our inability to detect thymosin α_1 in guanidinium chloride extracts of calf thymus. The suggestion that thymosin α_1 might represent a proteolytic fragment of a larger native polypeptide was supported by the finding that preparations of calf thymosin fraction 5 contained at least two other related peptides (7). One of these, designated des-(25–28)-thymosin α_1, contained only the first 24 amino acid residues; the other, named thymosin α_{11}, contained the sequence of thymosin α_1 plus seven additional residues at the COOH terminus.

In an effort to isolate the native thymic polypeptide from which these fragments appeared to be derived, we developed a radioimmunoassay based on an antibody prepared against synthetic thymosin α_1. With this assay and a procedure designed to eliminate any possibility of proteolytic modification, we isolated a major polypeptide, ≈112 amino acid residues long, that contains the thymosin α_1 sequence at its NH_2 terminus. We named this polypeptide prothymosin α because it appears to be the source of the thymosin α_1-related peptide fragments found in preparations of thymosin fraction 5.

MATERIALS AND METHODS

Rat thymuses from male Charles River CD rats, 5 weeks old, were excised immediately after sacrifice of the animals by decapitation, quickly frozen in liquid nitrogen, and stored at −70°C. Synthetic thymosin α_1 (8) was provided by A. Felix of Hoffmann–La Roche. Trypsin (L-1-tosylamido-2-phenylmethyl chloromethyl ketone-treated) and *Staphylococcus aureus* V8 protease were from Worthington and Miles Laboratories respectively. Fluorescamine was a gift of W. E. Scott of Hoffmann–La Roche. Sephacryl S-200 (superfine) was purchased from Pharmacia. Other reagents and solvents were chromatography-grade commercial preparations; the solvents were redistilled as required.

For preparation of the antibody, rabbits were injected with synthetic thymosin α_1 coupled to keyhole limpet hemocyanin (Calbiochem–Bohring). The details of the immunization procedure and the characterization of the antibody, which was shown to recognize the NH_2-terminal sequence of thymosin α_1, will be described elsewhere. For the radioimmunoassay, we prepared a derivative of thymosin α_1 labeled with tritium by reaction of the lysyl residues with formaldehyde and reduction of the *N*-methylene groups with sodium boro[^{3}H]hydride (Amersham, 8.5 Ci/mmol; 1 Ci = 37 GBq).

The quantitative radioimmunoassay was standardized with unlabeled synthetic thymosin α_1, and the results are expressed as thymosin α_1 equivalents. The method was capable of detecting as little as 2 pmol of thymosin α_1 and yielded consistently reproducible results in the range from 3 to 40 pmol of thymosin α_1.

Details of procedures for gel filtration, HPLC, and isoelectric focusing are described in the text. The HPLC experiments were carried out with an Altex Ultrasphere ODS C_{18} column using an apparatus equipped with a Waters Associates model 720 Systems Controller and model 710B Intelligent Sample Processor, adapted for fluorescence detection after derivitization with fluorescamine (9). Protein was determined by the method of Lai (10).

RESULTS

Isolation of Prothymosin α. To prevent the formation of smaller immunoreactive peptides, we found it necessary to inactivate proteolytic enzymes before the frozen tissue was allowed to thaw. In the method finally selected, the frozen tissue was pulverized under liquid nitrogen with a chilled mortar and pestle. Batches of powdered frozen thymus (7 g

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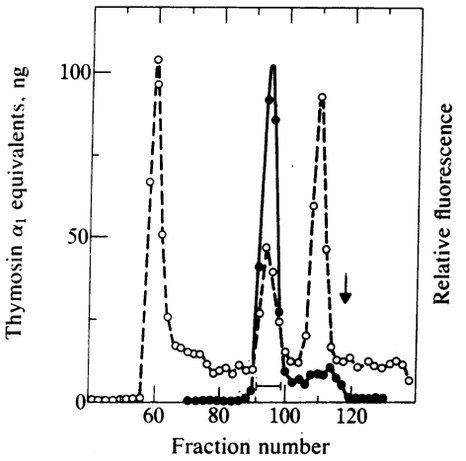

FIG. 1. Separation on Sephacryl S-200 of peptides extracted from boiled rat thymus tissue. Aliquots (0.8 ml) of the lyophylized material eluted from the Sep-Pak C_{18} cartridges, after solution in buffer A, were applied to a column of Sephacryl S-200 (1.5 cm × 89 cm) previously equilibrated with buffer A. The column was developed with the same buffer at a flow rate of 8.4 ml/hr, and 0.84-ml fractions were collected. For the radioimmunoassay, aliquots (7 μl) of each fraction were dried in a Speed Vac concentrator (Savant). The assay mixture (500 μl) contained 10^4 cpm of tritium-labeled thymosin α_1, 20 μl of preimmune serum and 5 μl of antiserum, sufficient to precipitate ≈50% of the radioactivity, in 0.2 M sodium phosphate buffer (pH 7.0). After 2 hr at room temperature, an equal volume of saturated $(NH_4)_2SO_4$ was added; 30 min later the solution was centrifuged, the pellet was dissolved in 0.2 ml of 90% HCOOH and transferred to 10 ml of Aquasol (New England Nuclear), and the radioactivity was determined. The assay was standardized with 0.5–500 ng of thymosin α_1, and the results are expressed as ng of thymosin α_1 equivalents (—). To locate peptide peaks (---) aliquots (10 μl) were dried, hydrolyzed with alkali, and analyzed with fluorescamine as described by Lai (10). The elution position of synthetic thymosin α_1 on the same column is shown by the arrow. For subsequent purification by HPLC, the fractions indicated by the bar were pooled and combined with similar fractions from three other gel-filtration separations.

each) were quickly dispersed into 100-ml portions of boiling 0.1 M sodium phosphate buffer (pH 7.0), and boiling was continued for 5 min. The suspensions were then cooled in ice. Four such suspensions were combined and homogenized with three 30-sec bursts at top speed with a Polytron homogenizer (Brinkman type P710/35). The resulting homogenate was centrifuged for 30 min at 12,000 × *g*. Subsequent operations were carried out at room temperature. The clear supernatant solution (347 ml) was diluted with an equal volume of buffer A (1 M HCOOH/0.2 M pyridine, pH 2.8) and forced through banks of three Sep-Pak C_{18} cartridges (Waters Associates) mounted in series. For the extract derived from 28 g of tissue, 28 such sets of three cartridges (84 total) were required. The cartridges containing the adsorbed peptides were washed with buffer A (20 ml for each set of three cartridges), and each set was eluted with 10 ml of the same buffer containing 20% 1-propanol (11). The recovery of immunoreactive material in the combined eluates was 63% of that present in the solution applied to the Sep-Paks.

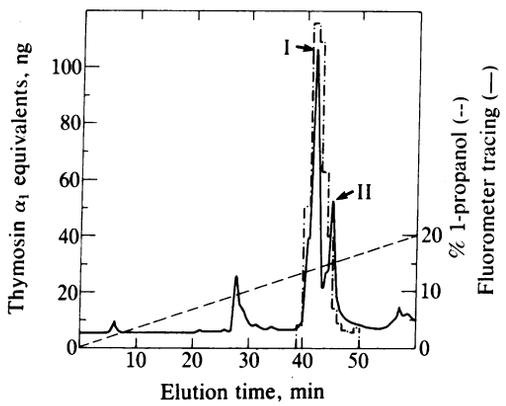

FIG. 2. Separation of immunoreactive peptide(s) on reverse-phase HPLC. The fractions comprising the immunoreactive peaks (pooled as described in the legend to Fig. 1) were lyophylized, and the residue was dissolved in 900 μl of buffer A. The HPLC experiments were carried out with 150-μl aliquots of this solution. Elution was with a gradient of 0–20% 1-propanol in buffer A (---). Fractions (0.6 ml) were collected, and 6-μl aliquots taken for radioimmunoassay (—·—) (see legend to Fig. 1). For analysis with fluorescamine (—), 5-μl aliquots were diverted every 6 sec. In the experiment shown, fractions 42 and 43 (peak I) were pooled and combined with similar fractions from five other HPLC separations.

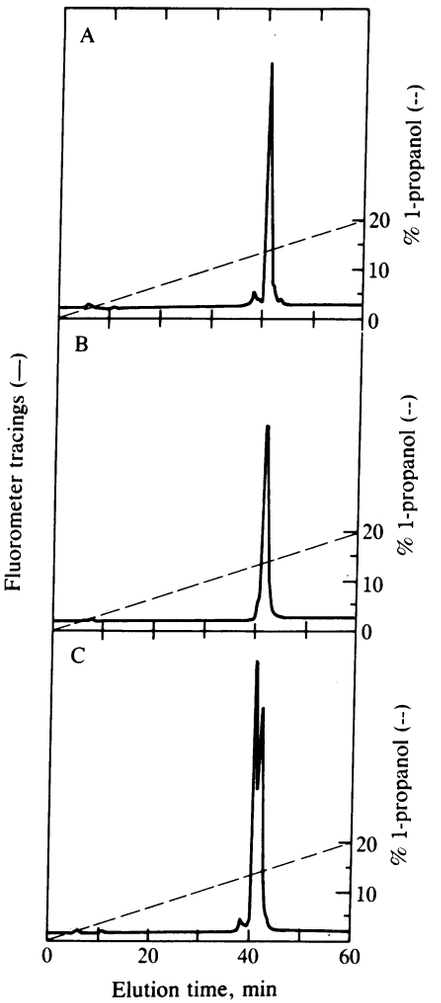

FIG. 3. Reverse-phase HPLC of a mixture of purified prothymosin α and synthetic thymosin α_1. The experiments were carried out as described in the legend to Fig. 2 with 13.7 μg of synthetic thymosin α_1 (*A*) or 35.2 μg of purified prothymosin α from peak I in Fig. 2 (*B*). A mixture of the two peptides analyzed on the same HPLC column yielded two peaks at the expected positions (*C*).

The combined Sep-Pak eluates were lyophilized, and the viscous residue was dissolved in 2.4 ml of buffer A (final volume, 3.2 ml). Aliquots (0.8 ml) containing peptides recovered from 7 g of thymus tissue were chromatographed on a column of Sephacryl S-200 (Fig. 1). The immunoreactive peptide(s) emerged in a single sharp peak with an elution position corresponding to a molecular weight of ≈32,000.

The peptides recovered from the Sephacryl S-200 column were separated and purified by HPLC (Fig. 2). A major pep-

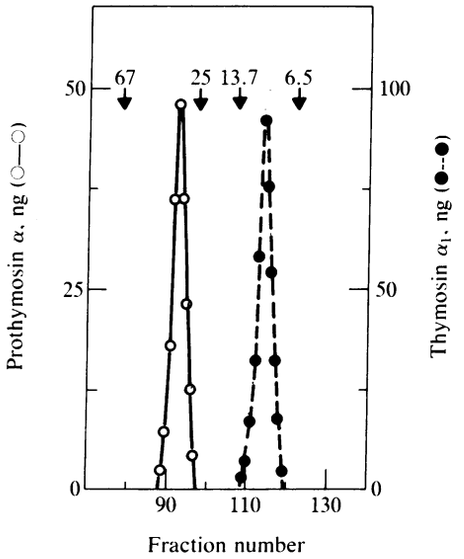

FIG. 4. Analysis on Sephacryl S-200 of purified prothymosin α and synthetic thymosin α_1. Chromatography was carried out with the same column used for the experiment described in Fig. 1. For prothymosin α (—), 17.6 μg of purified peptide from peak I (see Fig. 2) was chromatographed at a flow rate of 8.8 ml/hr, and 0.88-ml fractions were collected. Aliquots (300 μl) were taken for radioimmunoassay, and the results are expressed as ng of thymosin α_1 equivalents. A sample (3.4 μg) of synthetic thymosin α_1 (---) was chromatographed in the same way. Aliquots (100 μl) were taken for radioimmunoassay. The results from the separate experiments were plotted together to show the relative elution position. For calculation of the apparent molecular weights, the same column was standardized with bovine serum albumin (M_r = 67,000), chymotrypsinogen A (M_r = 25,000), ribonuclease A (M_r = 13,700), and trypsin inhibitor (M_r = 6,500) (all shown $\times 10^{-3}$, arrows).

tide peak (peak I) containing the bulk of the immunoreactivity was followed by a smaller peak (peak II) containing a second peptide that appeared to be weakly immunoreactive. From 28 g of rat thymus, we recovered after HPLC 1.6 mg of peptide in peak I, based on amino acid analysis of an aliquot hydrolyzed in 5.7 M HCl.

Table 1. Amino acid composition prothymosin α

	Prothymosin α	Thymosin α_1	Thymosin α_{11}
Asx	25.6 ± 0.8 (26)	4	5
Thr	6.0 ± 0.2 (6)	3	3
Ser	3.2 ± 0.2 (3)	3	3
Glx	39.6 ± 2.7 (40)	6	7
Gly	5.3 ± 0.3 (5)	0	1
Ala	10.3 ± 0.7 (10)	3	5
Val	5.9 ± 0.2 (6)	3	3
Ile	1.0 ± 0.0 (1)	1	1
Leu	1.0 ± 0.1 (1)	1	1
Lys	9.8 ± 0.3 (10)	4	4
Arg	2.3 ± 0.2 (2)	0	1
Pro	2.4 ± 0.6 (2)	0	1
Total	(112)	28	35

The values shown are the means and standard deviations from analyses of four samples of prothymosin α hydrolyzed with redistilled 5.7 M HCl at 150°C for 1 hr and analyzed with a Glenco MM-70 amino acid analyzer adapted for use of *o*-phthaldialdehyde and fluorescence detection as described by Benson and Hare (12). Proline was analyzed in an apparatus providing for oxidation of proline with *N*-chlorosuccinimide (13). The values are calculated by assuming a value of 1.0 for isoleucine. The nearest integral numbers are shown in parentheses. Tryptophan detection was carried out as described by Simpson *et al.* (14). Cysteine detection was carried out by the method of Hirs (15). Neither of these residues was present in prothymosin α. Phenylalanine, tyrosine, histidine, and methionine also were not detected.

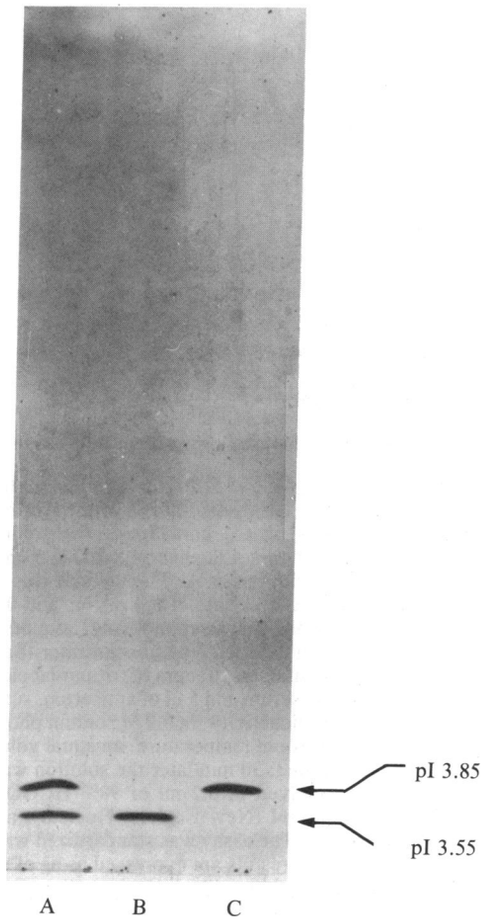

FIG. 5. Analytical isoelectric focusing of prothymosin α and synthetic thymosin α_1. Aliquots of prothymosin α (28 μg) and synthetic thymosin α_1 (21 μg) were applied to half of an LKB Ampholine PAGplate (1804-102; pH range 4.0–6.5) and electrofocused for 2.5 hr with an LKB (Biochrome) 2103 power supply on a LKB (Bromma) 2117 Multiphor cooled to 10°C. For the half-width plate, the initial conditions were 200 V and 12.5 mA, and the final conditions were 1500 V and 8.5 mA. The electrofocused slabs were fixed for 1 hr in 11.5% trichloracetic acid/3.45% sulfosalicylic acid. Staining was with 0.1% Coomassie brilliant blue solution for 1 hr in 25% ethanol/8% acetic acid, followed by 0.1% Coomassie blue in 25% 2-propanol/10% acetic acid for 1 hr. Destaining was with 25% ethanol/8% acetic acid. Lanes: A, prothymosin α/thymosin α_1; B, prothymosin α; C, thymosin α_1.

Rechromatography of an aliquot of the peptide recovered in peak I yielded a sharp peak (Fig. 3*B*) with an elution time of 42–43 min—slightly later than the elution time of thymosin α_1, which emerged almost precisely at 41 min under the conditions used (Fig. 3*A*). When a mixture of the two peptides was analyzed, each emerged at the expected position and the two were clearly separated (Fig. 3*C*). The new immunoreactive peptide was named prothymosin α.

The elution pattern of the major immunoreactive peptide on HPLC was consistent with that of a small peptide resembling thymosin α_1. On the other hand, its behavior on the Sephacryl S-200 column suggested a molecular weight in excess of 30,000 or, alternatively, binding of the peptide to a larger polypeptide carrier. In order to distinguish between these alternatives, the peptide recovered from the HPLC column in peak I was rechromatographed on the same Sephacryl S-200 column (Fig. 4). The purified peptide emerged as a single sharp peak with the same elution volume as be-

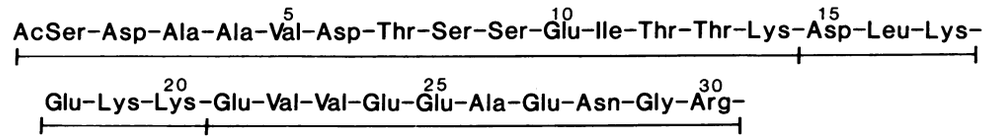

FIG. 6. NH_2-terminal sequence of prothymosin α. The sequence shown includes that reported by Goldstein *et al.* (1) for thymosin α_1 (residues 1–28) and also by Caldarella *et al.* (7) for residues 1–30 of thymosin α_{11}. The peptides identified in tryptic digests of prothymosin α are indicated by the bars.

fore. On the same column, a sample of synthetic thymosin α_1 emerged somewhat later, at a position corresponding to an apparent molecular weight of ≈11,000. As discussed later, both peptides showed a tendency to form oligomers.

Properties of Prothymosin α. The purified prothymosin α preparations yielded a single band of pI = 3.55 in analytical isoelectric focusing (Fig. 5). On the same gels, synthetic thymosin α_1 focused at a position corresponding to pI = 3.85.

Amino acid analysis (Table 1) showed prothymosin α to be unusually rich in glutamic and aspartic acids, which accounted for one-third and nearly one-fourth of the total amino acids, respectively. Cysteine, methionine, histidine, and the aromatic amino acids were not detected. The minimum chain length, calculated by assuming one residue each of leucine and isoleucine, was ≈112; the minimum molecular weight calculated from the amino acid composition was 12,600. This value was confirmed by the results of tryptic digestion, which yielded only one peptide containing leucine and one containing isoleucine (see below).

The NH_2 terminus of prothymosin α was found to be blocked, supporting the conclusion that this part of the molecule carries the immunoreactive thymosin α_1 sequence. Tryptic digests of prothymosin α yielded peptides whose elution position in HPLC and amino acid compositions indicated identity with peptides 1–14 (containing the single isoleucine residue), 15–17 (containing the single leucine residue), and 18–20 of thymosin α_1 (Fig. 6) and also with peptide 21–30 (containing the single arginine residue) of thymosin α_{11}. The tryptic peptide corresponding to residues 1–14 also was detected with the anti-thymosin α_1 antibody. Digestion with *S. aureus* V8 protease, which hydrolyzes glutamyl bonds (16), yielded a blocked peptide that could not be detected with fluorescamine but was immunoreactive (data not shown). The amino acid composition of this peptide ($Ser_3Asp_2Ala_2Val_1Thr_1Glu_1$) confirmed that it corresponded to the blocked NH_2-terminal decapeptide of thymosin α_1 (see Fig. 6). No carbohydrate was detected by HPLC analysis (17) of acid hydrolysates of prothymosin α.

DISCUSSION

Prothymosin α appears to be the native polypeptide from which the smaller fragments containing the thymosin α_1 sequence, including thymosin α_1 itself, are probably generated during the isolation of the preparation called thymosin fraction 5. The NH_2-terminal sequence of prothymosin α appears to be identical to that of thymosin a_1; the COOH-terminal extension, ≈85 amino acid residues long, is rich in acidic amino acids, accounting for the low isoelectric point, pI = 3.55, which is even lower than the value of 3.85 found for thymosin α_1.

Both prothymosin α and thymosin α_1 appear to form oligomers in aqueous solution. In gel filtration at pH 2.8, both of these peptides behave as discrete oligomers, possibly trimers. In similar gel filtration experiments carried out at pH 7.0, they emerge as sharp peaks corresponding to molecular weights of 61,000 for prothymosin α and 15,000 for thymosin α_1 (unpublished observations), possibly corresponding to hexamers. Intermediate forms were not detected. Binding to a larger carrier polypeptide in the gel filtration experiment is excluded by the identical behavior of prothymosin α after purification by HPLC. This tendency to oligomerize may be a consequence of the amphoteric nature of these peptides.

We have observed that prothymosin α is readily converted to smaller fragments unless special precautions are taken to inactivate proteinases before or during extraction of the tissue. The formation of these fragments may be related to its biological activity. Both thymosin α_1 (7, 18, 19) and thymosin α_{11} (7) have been shown to protect sensitive strains of mice against opportunistic infections. Preliminary observations (unpublished data) indicate that prothymosin α is also active in these tests. Whether this activity is due to its conversion *in vivo* to the smaller fragments remains to be established.

The amino acid composition and low pI of prothymosin α suggests an unusual acidic structure. Recently, a glutamic acid-rich acidic peptide with a pI of 4.6 has been isolated from rat brain (20). The physiological role of these highly acidic polypeptides remains to be determined.

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