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Distribution of prothymosin α in rat tissues

(radioimmunoassay/thymosin α_1/peptide isolation/HPLC)

A. A. HARITOS*†, O. TSOLAS‡, AND B. L. HORECKER*

*Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110; and ‡Department of Biological Chemistry, University of Ioannina Medical School, GR-453 32 Ioannina, Greece

Contributed by B. L. Horecker, December 7, 1983

ABSTRACT **A radioimmunoassay, using a rabbit antiserum directed against thymosin α_1, was employed to detect the presence of crossreacting peptides in rat tissues. Highest concentrations were present in thymus, but thymosin α_1 crossreacting material was also detected in brain, liver, kidney, lung, and spleen, in amounts ranging from 15% to 65% of the quantities found in thymus. In each case, the major immunoreactive peptide, after extraction and purification by a procedure that avoids proteolytic modification, was identified as prothymosin α, a peptide containing ≈112 amino acid residues. Prothymosin α is believed to be the endogenous peptide from which thymosin α_1 and other fragments are formed by proteolytic modification during the preparation of thymosin fraction 5. No peptides corresponding in size and chromatographic behavior to thymosin α_1 were detected with the extraction procedure employed.**

Peptides isolated from the thymus gland have attracted considerable interest because of their ability to restore parameters of immunity in a variety of animal models and *in vitro* assay systems (for reviews see refs. 1 and 2). Prominent among these is a preparation from calf thymus, designated thymosin fraction 5, which was reported to contain a number of peptides ranging in molecular weight from 1000 to 10,000 and in isoelectric points from pH 4.0 to pH 7.0 (3, 4). Peptides having low isoelectric points, below pH 5.0, were designated as members of the thymosin α family, with subscripts added according to their order of isolation from calf thymosin fraction 5. The first of these peptides, thymosin α_1, was isolated and its sequence determined in 1977 (3). Thymosin α_1 was reported to possess several of the biological activities of thymosin fraction 5 (5).

We have previously reported (6) that thymosin α_1 could not be detected in guanidinium chloride extracts of fresh calf thymus, a procedure employed to minimize the modification of native polypeptides by endogenous proteinases. We suggest that thymosin α_1 might be a fragment derived from a larger polypeptide during the preparation of fraction 5. More recently, two other fragments related to thymosin α_1 were isolated from batches of calf thymosin fraction 5, one, designated "(des 25–28) thymosin α_1," lacking four amino acid residues at the COOH terminus, and the other, named thymosin α_{11}, with a COOH-terminal extension containing seven additional residues (7). The presence of three related peptides, each containing the same NH_2-terminal sequence and differing only in length, supported the view that these peptides were derived from a larger native polypeptide.

We have recently succeeded in isolating a polypeptide that appears to fulfill this prediction (8). For the purification of this peptide we employed a radioimmunoassay based on a rabbit antiserum prepared against synthetic thymosin α_1. In extracts of rat thymus prepared by a procedure that prevents proteolytic modification, all of the immunoreactive thymosin α_1-like material was recovered as a larger peptide, with no evidence for the presence of thymosin α_1 or any of the related fragments previously isolated from thymosin fraction 5. The native peptide, purified by HPLC, was estimated to contain 112 amino acid residues. Its isoelectric point was 3.55, significantly more acidic than thymosin α_1. It was named prothymosin α because it appeared to be the precursor of the previously characterized fragments.

Other workers have employed similar radioimmunoassays to measure the levels of immunoreactive thymosin α_1 in preparations of thymosin fraction 5 from bovine tissues (9) and also in human plasma or serum (9–13). Immunochemical methods have also been employed to evaluate the content of immunoreactive thymosin α_1 in human thymic epithelial tissue (14, 15) and on circulating T lymphocytes (16).

In the present report we show that material crossreacting with rabbit anti-thymosin α_1 antiserum is present in a number of rat tissues, including spleen, lung, kidney, liver, and brain, in quantities approaching those found to be present in rat thymus. The crossreacting material was identified in each case as prothymosin α; peptides corresponding to thymosins α_1 and α_{11} were not detected. Our results suggest that previous reports of the presence of thymosin α_1 in serum or plasma (9–13) or in rat tissue preparations (17) require reevaluation.

MATERIALS AND METHODS

The preparation of the antiserum and the radiolabeled antigen as well as the details of the radioimmunoassay were as described (8).

Rat tissues were removed immediately after sacrifice of the animals by decapitation, quickly frozen in liquid nitrogen, and stored at −70°C. Other reagents, solvents, and methods employed were as described (8).

RESULTS

Isolation of Immunoreactive Thymosin α_1 from Rat Tissues. Extracts prepared from several frozen rat tissues were all found to contain significant immunoreactive thymosin α_1 ranging in quantities from 21 μg of thymosin α_1 equivalents/g of tissue in thymus to 3 μg of thymosin α_1 equivalents/g of tissue in brain (Table 1). However, because prothymosin α is approximately four times larger than thymosin α_1, and because it is only 20% as reactive in our radioimmunoassay (unpublished observation), the true quantities of prothymosin α may be severalfold larger than those listed as thymosin α_1 equivalents in Table 1. We have previously reported that the recovery of homogeneous prothymosin α from rat thymus, based on quantitation by amino acid analysis, was 57 μg/g of thymus.

The purification of prothymosin α from rat thymus extracts is essentially a three-step procedure, including (*i*) desalting on disposable Sep-Pak cartridges, (*ii*) size-exclusion chromatography on Sephacryl S-200, and (*iii*) final separa-

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†On leave from the Zoological Laboratory, Faculty of Science, University of Athens, Athens 621, Greece.

Table 1. Levels of crossreacting material in rat tissues

Tissue	Total immunoreactivity, μg of thymosin α_1 equivalents	Thymosin α_1 equivalents, μg/g of tissue
Thymus	149	20.7
Spleen	97	13.5
Lung	54	7.7
Kidney	46	6.3
Liver	24	3.4
Brain	21	2.9

Tissues 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. The frozen tissues from several animals (7 g in each case) were pulverized under liquid nitrogen in a chilled mortar and pestle. The powders were quickly dispersed into 100-ml quantities of boiling 0.1 M sodium phosphate buffer (pH 7.0), and boiling was continued for 5 min. After cooling in ice, each suspension was homogenized with three 30-sec bursts at top speed with a Polytron homogenizer (Brinkman type PT10/35). The resulting homogenates were centrifuged for 30 min at 12,000 × *g*. Aliquots of 60 μl from each clear supernatant solution were used for the radioimmunoassays, and the results were expressed as μg of α_1 equivalents (8). Aliquots of 10 μl of the same supernatant solution were used for the estimation of protein according to Lai (18) with bovine serum albumin as the standard. Triplicate analyses were carried out and the mean values are reported. The maximal standard deviation from the mean was in the range of 10% for brain and less for the other tissues.

tion by HPLC (8). In the Sephacryl S-200 step, each of the tissue extracts examined yielded a very similar pattern (Fig. 1) with the bulk of the immunoreactivity eluting at a position corresponding to a M_r of ≈30,000, similar to that previously reported for rat thymus extracts (8). No immunoreactive peak was found in the elution position of synthetic thymosin α_1. In each case, except for the brain extracts, the immunoreactive peak coincided with a major well-defined protein peak.

When the fractions from the Sephacryl S-200 columns were analyzed by HPLC, each yielded a major immunoreactive peak (Fig. 2) eluting at the concentration of 1-propanol expected for prothymosin α (compare figure 2 of ref. 8).

Amino Acid Compositions of Immunoreactive Peptides. The amino acid compositions of the peptides recovered from the peak immunoreactive fractions showed the high content of glutamic and aspartic acids previously found for prothymosin α from rat thymus (Table 2). On the assumption that each contained only one isoleucine residue, the total number of residues in each peptide ranged from 106 to 118, similar to the value of 112 previously reported for prothymosin α.

DISCUSSION

The results reported here identify the major immunoreactive form of thymosin α_1 in several rat tissues as prothymosin α. Peptides corresponding in size to thymosin α_1 were not detected in any of the extracts, including those obtained from thymus. Prothymosin α has been characterized previously (8) as a larger peptide, containing ≈112 amino acid residues and unusually rich in aspartic and glutamic acids. This high content of acidic amino acid residues is consistent with its unusually low isoelectric point.

Previous reports of immunoreactive thymosin α_1 in human and bovine tissues (9, 14–17) are likely to reflect the presence of prothymosin α in these tissues. In view of our finding (unpublished observation) that prothymosin α is approximately one-fifth as active as thymosin α_1 in the radioimmunoassays, the true values for the levels of prothymosin α are probably significantly higher than those calculated on the assumption that the endogenous immunoreactive material was thymosin α_1.

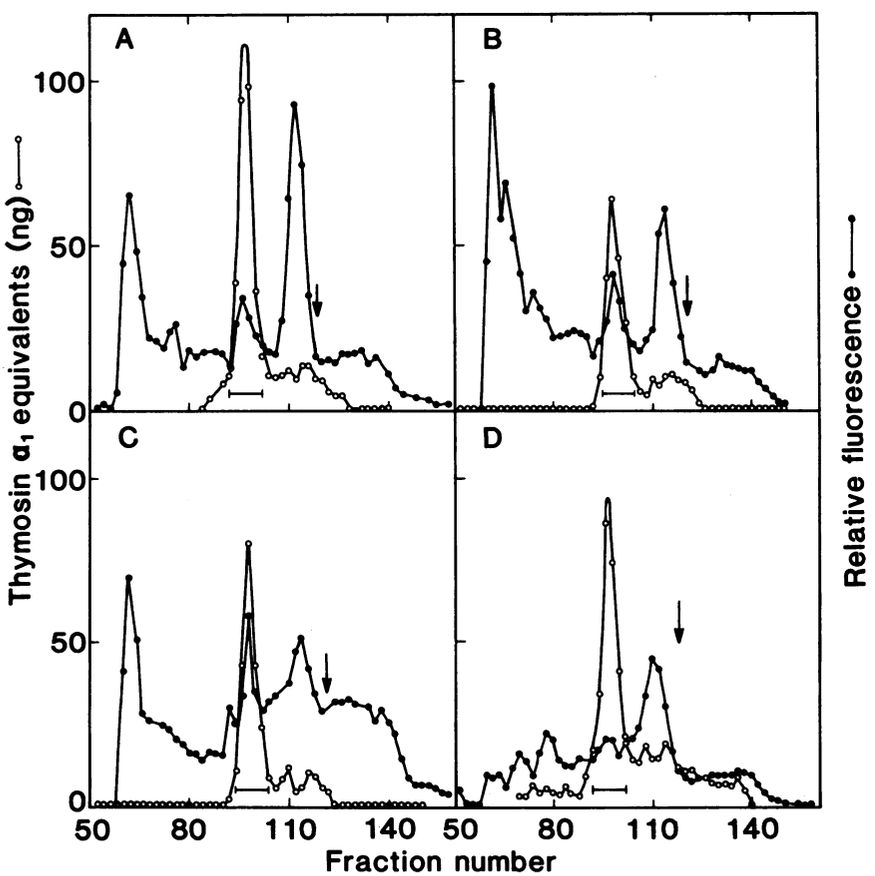

FIG. 1. Separation on Sephacryl S-200 of peptides extracted from rat tissue. (*A–D*) Spleen, lung, kidney, and brain, respectively. The tissue extracts were diluted with equal volumes of buffer A (1 M HCOOH/0.2 M pyridine, pH 2.8) and desalted on Sep-Pak cartridges as described (8). The recoveries of immunoreactive material in the Sep-Pak eluates were 79% for spleen, 81% for lung and kidney, and 88% for brain. After lyophilization, the residues were dissolved in 0.6-ml volumes of buffer A (final volumes = 0.8 ml) and applied onto a column of Sephacryl S-200 superfine (1.5 × 89 cm), previously equilibrated with buffer A. The column was developed with buffer A at flow rates of 8.33, 8.18, 8.10, and 8.36 ml/hr for *A–D*, respectively. Fractions were collected every 6 min. For radioimmunoassay (○) aliquots (10, 25, 25, or 100 μl of fractions from *A–D*, respectively) were dried and analyzed as described (8). The relative peptide concentrations (●) of each fraction were determined by analysis of 10-μl aliquots with fluorescamine after alkaline hydrolysis (18). The elution positions of synthetic thymosin α_1 are indicated by the arrows. Fractions indicated by the bars were pooled for purification by HPLC. Similar patterns were obtained with thymus extracts (8) and with liver extracts (not shown).

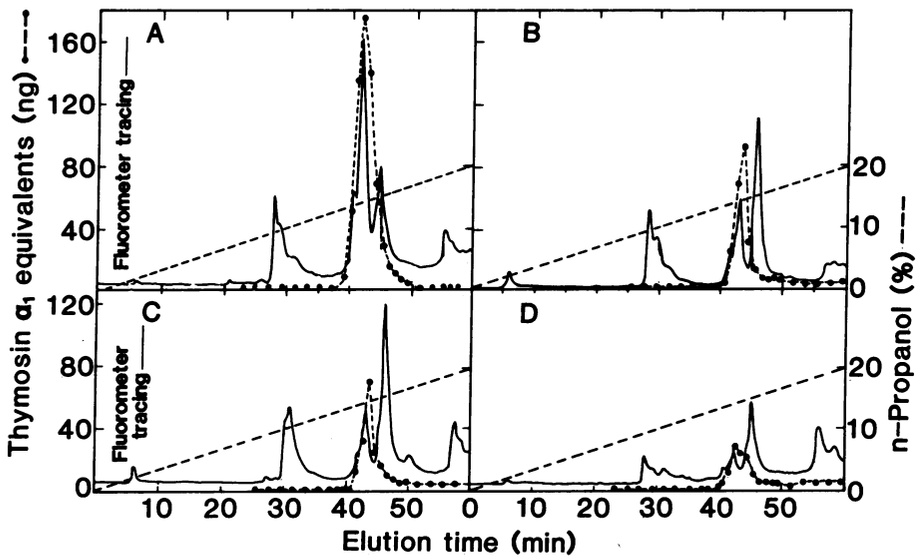

FIG. 2. Reverse-phase HPLC of fractions from gel filtration on Sephacryl S-200. (*A–D*) Spleen, lung, kidney, and brain, respectively. The fractions containing the immunoreactive material (pooled as described in the legend to Fig. 1) were lyophilized and each residue was dissolved in 320 μl of buffer A. The HPLC experiments were carried out with 150-μl aliquots of this solution as described (8). Elution was with a gradient of 0–20% 1-propanol in buffer A as shown. Fractions (0.6 ml) were collected and 15-μl aliquots were taken for radioimmunoassays (●). For analysis with fluorescamine, 5-μl aliquots were diverted to the detector system every 6 sec.

The presence of relatively high concentrations of prothymosin α in tissues such as lung, liver, kidney, and brain may require reevaluation of the putative role of thymosin α_1 as a thymic hormone (1, 2, 10, 11). Our results do not exclude the formation *in vivo* of small quantities of thymosin α_1, but the failure to detect traces of this peptide with our sensitive radioimmunoassay makes this appear unlikely. Based on the present results, it becomes essential to isolate and identify the immunoreactive material detected in human serum, in order to properly interpret the significance of reported changes (10, 11) in the serum content of the immunoreactive thymosin α_1-like substances.

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Table 2. Amino acid composition of immunoreactive peptides after purification by HPLC

Residue	Thymus	Spleen	Brain	Lung	Liver	Kidney
Asp	25.4	25.0	25.2	24.5	24.6	24.9
Thr	6.4	5.9	5.8	5.8	5.7	6.3
Ser	3.3	3.6	3.2	4.1	3.7	3.8
Glu	37.3	39.7	38.5	37.4	39.6	39.6
Gly	5.3	6.0	6.1	7.3	5.8	5.5
Ala	9.1	10.7	10.3	10.3	11.9	12.0
Val	5.6	6.0	5.7	5.8	5.9	5.9
Ile	1.0	1.1	1.0	1.0	1.0	1.0
Leu	1.1	1.2	1.1	1.1	1.2	1.3
Lys	9.0	8.8	8.7	8.3	11.5	9.7
Arg	2.1	2.4	2.2	2.0	2.7	2.3
Pro*	(2)	(2)	(2)	(2)	(2)	(2)
Total	106	113	118	109	118	115

Fractions corresponding to the prothymosin α peaks (Fig. 2) were pooled and aliquots were hydrolyzed with redistilled 5.7 M HCl at 150°C for 1 hr. Amino acid analyses were carried out as described (8). For calculation of composition, the content of isoleucine was taken as unity, except for spleen, where aspartic acid was taken as 25.

*Not assayed in the procedure employed here. The content of proline is assumed to be the same as that reported (8).

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Correction. In the article "β-Thalassemia in American Blacks: Novel mutations in the 'TATA' box and an acceptor splice site" by Stylianos E. Antonarakis, Stuart H. Irkin [*sic*], Tu-chen Cheng, Alan F. Scott, Julianne P. Sexton, Stephen P. Trusko, Samuel Charache, and Haig H. Kazazian, Jr., which appeared in number 4, February 1984, of *Proc. Natl. Acad. Sci. USA* (**81,** 1154–1158), the following undetected printer's error should be noted. The second author's surname is Orkin, not Irkin.

Correction. In the article "Distribution of prothymosin α in rat tissues" by A. A. Haritos, O. Tsolas, and B. L. Horecker, which appeared in number 5, March 1984, of *Proc. Natl. Acad. Sci. USA* (**81,** 1391–1393), an error occurred in the *Proceedings* editorial office. Ref. 8 should read: Haritos, A. A., Goodall, G. J. & Horecker, B. L. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1008–1011.