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Thymosin β_4: A ubiquitous peptide in rat and mouse tissues

(thymus/spleen/brain/macrophages/HPLC of peptides)

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ABSTRACT **Thymosin β_4, recently isolated from calf thymus, is present in a number of rat and mouse tissues, including spleen, thymus, brain, lung, liver, and heart muscle. High concentrations are found in peritoneal macrophages, suggesting that its occurrence in other tissues may be related to the presence of macrophages or macrophage-like cells in these tissues. The conclusion that "thymosin" β_4 does not originate solely in the thymus gland is supported by the high concentrations found in tissues of athymic (*nu/nu*) mice.**

Thymosin β_4 has recently been characterized (1) as one of the peptides present in calf thymus fraction 5 (2). It contains 43 amino acids with a high proportion of lysyl and glutamyl residues (1). It has also been isolated from fresh-frozen calf thymus by a procedure that minimizes the possibility of proteolytic modification (3). Thymosin β_4 has been reported to enhance the activity of terminal deoxynucleotidyltransferase in lymphocytes in immunosuppressed mice (1) and to induce the release of hypothalamic luteinizing hormone-releasing hormone (4). We now report that this peptide is present in a variety of rat and mouse tissues, including macrophages. Its concentration is elevated in tissues from athymic (*nu/nu*) mice.

MATERIALS AND METHODS

Materials. Altex ODS columns (Ultrasphere; 5 μm, 4.6 mm × 250 mm) were purchased from Beckman. Sep-Pak C_{18} cartridges from Waters Associates were primed before use as recommended by the supplier. Guanidinium chloride and fluorescamine (Fluram) were from Sigma and Hoffmann–LaRoche, respectively. Other chemicals and solvents were chromatography grade.

Methods. *Isolation of tissue samples and preparation of extracts.* Male Sprague–Dawley CD rats weighing 80–100 g or female Charles River CD1 *nu/+* or *nu/nu* mice, 45 days old, were sacrificed by decapitation and tissues were removed and immediately frozen in liquid N_2. Peritoneal exudate macrophages were harvested from rats by introduction of 15 ml of 0.34 M sucrose into the peritoneal cavity. After a brief massage, the solution was withdrawn with a pipette and an aliquot of the cell suspension was taken for estimation of cell number by hemocytometry. The cells were collected by centrifugation for 10 min at 180 × g and the cell pellet was solubilized by addition of guanidinium chloride. The same procedure was used to collect mouse macrophages, except that 5 ml of sucrose solution was injected into each mouse. Usually, 10^7–10^8 macrophages were extracted directly with 10 ml of 6 M guanidinium chloride.

Aliquots of frozen rat tissues (0.01–1.5 g) or pooled tissue from several mice were homogenized in 10 ml of ice-cold 6 M guanidinium chloride using a Potter–Elvejhem Teflon/glass homogenizer at high speed or a Polytron homogenizer (Brinkmann). Each homogenate was added to 10 ml of 0.4 M pyridine/2 M formic acid buffer, pH 2.8, and the mixture was centrifuged for 30 min at 18,000 × g. The floating lipid layer was removed, and the solutions were forced through the primed Sep-Pak cartridges by using a 20-ml Luer-Lok syringe. The cartridges were washed with 20 ml of the 0.2 M pyridine/1 M formic acid buffer and the peptides were eluted with buffer/20% or 40% propanol. The eluates were lyophilized and each residue was dissolved in 0.5 ml of buffer. By HPLC analysis (see below) of diluted aliquots of the original guanidinium chloride extracts and of the lyophilized Sep-Pak eluates, it was established that the recovery of thymosin β_4 by this procedure was ≥80%.

HPLC analysis of peptides. An automatic peptide analyzer constructed in this laboratory was used, following the procedure described by Stein and Moschera (5). Amino acid analyses were carried out on aliquots of peptide hydrolyzed with 5.7 M HCl at 150°C for 1 hr, using a modified Glenco MM-70 analyzer adapted for use of *o*-phthalaldehyde and fluorescence detection (6).

RESULTS

Identification of Thymosin β_4 in Extracts of Rat and Mouse Tissues. A peptide having the retention time of thymosin β_4 was a major component in the extracts of rat thymus, brain, spleen, and peritoneal macrophages (Fig. 1). This peptide was also present in extracts of lung, liver, and kidney (see Table 2). Its identity was confirmed by amino acid analysis (Table 1) and, in the case of the peptide from rat thymus, by digestion with trypsin and HPLC analysis of the tryptic peptides. The peptide from rat liver, which appeared to contain a small amount of impurity, was cleaved with cyanogen bromide, and the fragments corresponding to residues 1–6 and 7–43 were separated by HPLC. The amino acid compositions of these cyanogen bromide peptides were as predicted from the reported sequence of thymosin β_4 (1, 7). The identity of the peptide from rat brain was confirmed by the isolation of tryptic peptides corresponding to residues 1–14, 15 and 16, 17–19, and 39–43.

Thymosin β_4 isolated from mouse thymus, brain, spleen, and macrophages was similarly identified by HPLC retention time, amino acid composition, and isolation of tryptic peptides corresponding to residues 1–11 or 1–14, 15 and 16, and 39–43.

Content of Thymosin β_4 in Rat and Mouse Tissues. The results of analyses carried out with tissues from rats and from heterozygous (*nu/+*) and homozygous athymic (*nu/nu*) mice are shown in Table 2. The quantities of β_4 were calculated from the height of the fluorescamine peaks (see Fig. 1) standardized by analysis of a sample of thymosin β_4 purified from calf thymus

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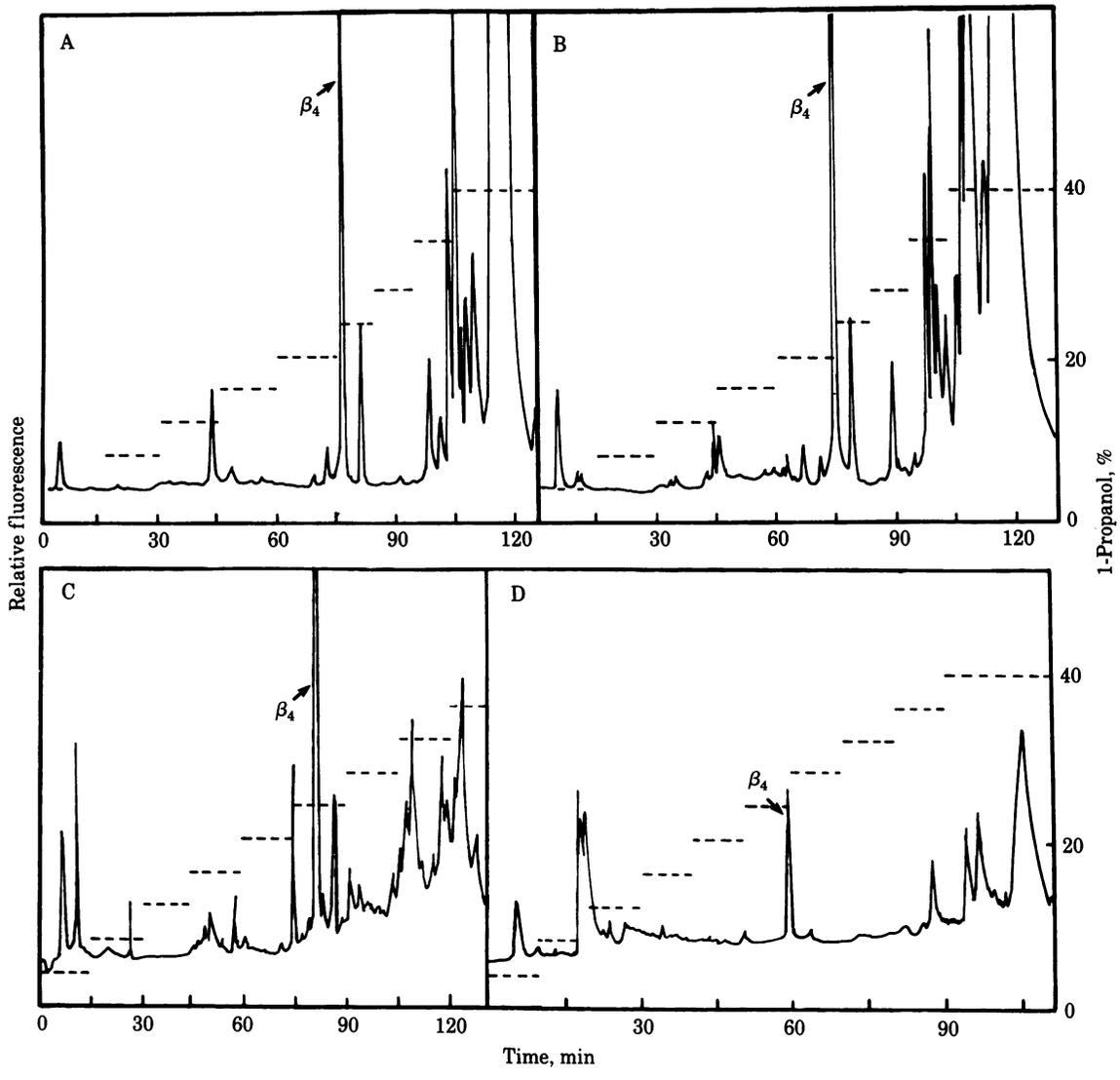

FIG. 1. HPLC analysis of peptides in rat tissues. (*A*) Thymus. (*B*) Brain. (*C*) Spleen. (*D*) Peritoneal macrophages. Frozen tissues were extracted with guanidinium chloride and peptides were concentrated by adsorption onto Sep-Pak cartridges. Elution from the Sep-Pak cartridges was with 20 ml of 0.2 M pyridine/1 M formic acid buffer/40% propanol, except for the peptides from spleen and macrophages, which were eluted with buffer/20% propanol. This accounts for the decreased quantities of larger peptides in the samples analyzed. Automated HPLC analysis was carried out, eluting with 0.2 M pyridine/1 M formic acid, pH 2.8, and increasing concentrations of 1-propanol as indicated (----) at a flow rate of 0.5 ml/min. Quantities of tissues extracted were thymus, 0.15 g; brain, 1.0 g; spleen, 1.8 g (pooled samples from two rats); peritoneal macrophages, 3×10^7 cells (pooled macrophages from two rats).

(3). The results were confirmed by the amino acid analyses shown in Table 1. In the rat, the concentration of thymosin β_4 was high in spleen, thymus, and peritoneal macrophages. Significant quantities were also recovered from brain, liver, and lung, but in bone marrow, blood, adrenal medulla, mast cells, and testes, the peptide was present only in low levels or was undetectable. When splenic lymphocytes were separated into adherent and nonadherent cells by adsorption of the former on plastic Petri plates, the β_4 content was highest in the adherent cell fraction, which is known to contain macrophages. However, spleen cannot be the sole source of β_4, since the levels of this peptide in other tissues were not reduced in splenectomized animals.

The concentration of thymosin β_4 appeared to be higher in mouse than in rat tissues, but this may be partly due to the fact that the mouse tissues were extracted using the Polytron homogenizer and the peptides were absorbed with two Sep-Pak cartridges in series. The β_4 content of tissues obtained from athymic (*nu/nu*) mice was consistently higher than that of tissues from heterozygous (*nu/+*) mice.

Distribution of Thymosin β_4 in Rat Brain. When various regions of rat brain were dissected and analyzed, the thymosin β_4 content was highest in the olfactory bulb (Table 3). This region is particularly rich in macrophages (unpublished observations). Similar concentrations of thymosin β_4 were found in comparable regions from brains of young or mature rats.

DISCUSSION

The HPLC elution patterns of the small peptides derived from the four rat tissues shown in Fig. 1 and the other rat tissues analyzed were remarkably similar, with thymosin β_4 as the major component. There was more variation in the pattern of larger peptides eluted with high concentrations of 1-propanol. Thymosin β_4 was also the major small peptide in the mouse tissues analyzed (data not shown), as well as in calf thymus (3, 7).

Table 1. Amino acid composition of peptides isolated from rat tissues

Residue	β_4*	Thymus ($n = 3$)	Spleen ($n = 2$)	Brain ($n = 2$)	Lung ($n = 1$)	Liver ($n = 1$)	Kidney ($n = 1$)	Macrophages ($n = 3$)
Asp	4	4.4	4.3	4.2	3.8	4.2	5.0	4.0
Thr	3	2.8	2.9	2.9	3.3	2.3	2.9	2.7
Ser	4	3.7	3.7	3.6	2.5	2.9	4.1	3.3
Glu	11	11.2	11.4	10.9	11.0	11.0	11.1	10.5
Gly	1	1.5	1.3	1.4	1.3	1.8	3.3	1.2
Ala	2	2.2	2.1	2.1	2.0	2.5	3.0	2.1
Val	0	0.2	0.1	0.2	0.2	0.6	0.8	0.2
Met	1	0.6	0.6	0.8	1.0	0.8	0.5	0.4
Ile	2	1.8	1.9	1.9	1.7	1.3	1.6	1.9
Leu	2	2.0	2.0	2.0	1.9	1.7	2.0	2.1
Tyr	0	0	0.1	0	0	0.1	0.2	0.1
Phe	1	1.0	1.0	1.0	0.9	0.9	0.9	1.0
Lys	9	8.5	8.7	8.9	7.8	7.0	7.3	8.7
His	0	0	0.1	0	0	0	0.2	0.1
Arg	0	0	0.2	0.2	0.5	0.9	0.8	0.3

For $n \geq 2$, each sample was from a different animal, except for macrophages, which were pooled from two rats for each analysis.
* Predicted from the amino acid sequence (1).

Thymosin β_4 therefore appears to be a ubiquitous peptide in mammalian tissues and identical in all species thus far examined.‡ On the other hand, thymosins β_8 and β_9, two peptides closely homologous to thymosin β_4 (7), have been found only in calf thymus and were not detected in the rat and mouse tissues analyzed. Instead, rat and mouse thymus, brain, and spleen contain a peptide that emerges several minutes later in the HPLC analyses (Fig. 1).

From our results, it may be concluded that thymosin β_4 is not a thymus-specific peptide; its presence in a variety of rat and mouse tissues appears to be related to the presence of macrophages (e.g., in thymus, spleen, and lung) or other phagocytic cells (e.g., Kupfer cells in liver). Its concentration in various regions of the brain also follows the pattern expected from the distribution of macrophages in this tissue. The failure of splenectomy to reduce the levels of thymic and extrathymic thymosin β_4 also precludes the spleen as the sole source of β_4.

Compelling evidence for the nonthymic origin of thymosin β_4 is its presence in tissues of athymic (*nu/nu*) mice; in fact, its concentration appeared to be elevated in *nu/nu* tissues as compared with those from heterozygous *nu/+* mice. In this context, it is of interest that the macrophages of *nu/nu* mice have been suggested (9) as being responsible for the unexpected ability of *nude* mice to resist infections that are lethal to normal littermates (10, 11).

The presence of large amounts of thymosin β_4 in macrophages and tissues rich in macrophage-like phagocytic cells also raises interesting questions regarding its possible role in protein degradation. Another widely distributed peptide, ubiquitin (12), has been shown (13) to be the factor that activates ATP-dependent proteolysis in erythrocytes (14), and a similar function may account for the presence of thymosin β_4 in macrophages. In light of the results reported here, it becomes important to reexamine the role of this peptide in cellular immunity.

‡ Thymosin β_4 has also been isolated from calf spleen, in which its content is ≈0.8 mg/g protein (S. Erickson-Viitanen, unpublished observations).

Table 2. Content of thymosin β_4 in rat and mouse tissues

Tissue	Thymosin β_4		
		Mouse	
	Rat	*nu/+*	*nu/nu*
Thymus	0.33 ± 0.08 ($n = 5$)	1.4	NA
Spleen	0.55 ± 0.18 ($n = 3$)	3.8	5.0
Brain	0.13 ± 0.03 ($n = 3$)	0.47	0.70
Lung	0.10 ± 0.04 ($n = 3$)		
Liver	0.05 ± 0.03 ($n = 3$)	NA	NA
Kidney	0.07 ± 0.03 ($n = 3$)	NA	NA
Macrophages	0.36	0.7–1.56	1.0

Tissue protein content was determined on weighed aliquots dissolved in 1 M NaOH by using the method of Lowry *et al.* (8). Frozen rat tissues were homogenized with a glass/Teflon homogenizer. Frozen organs from eight *nu/+* and nine *nu/nu* mice were pooled, tissues were extracted by using a Polytron homogenizer, and extracts were desalted by using two Sep-Pak cartridges in series, except for macrophages, which were treated as the rat tissues. n values are for analyses with organs taken from individual rats. Intraortic perfusion of the blood system with cold phosphate-buffered saline or splenectomy 6 days before sacrifice did not affect thymosin β_4 levels. Results represent mg of thymosin β_4 per mg of protein, except for macrophages (pooled from two rats or six mice), which are reported as mg of thymosin β_4 per 10^9 cells. NA, Not analyzed.

1. Low, T. L. K., Hu, S.-K. & Goldstein, A. L. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1162–1166.
2. Hooper, J. A., McDaniel, M. C., Thurman, G. B., Cohen, G. H., Schulof, R. S. & Goldstein, A. L. (1975) *Ann. N.Y. Acad. Sci.* **249**, 125–144.
3. Hannappel, E., Davoust, S. & Horecker, B. L. (1982) *Biochem. Biophys. Res. Commun.* **104**, 266–271.

Table 3. Content of thymosin β_4 in regions of rat brain

Region	Thymosin β_4, mg/g of protein	
	Young	Mature
Thalamus	—	0.39
Stem	0.39	0.36
Cortex	0.29	0.52
Cerebellum	0.49	0.41
Olfactory bulb	0.75	0.61

Tissue was collected as described in *Materials and Methods* except that brains were dissected and sections were frozen separately. Extraction was with the Polytron homogenizer and desalting was with two Sep-Pak cartridges in series. Brain areas from six young male rats (80–100 g) or six mature rats (200–220 g) were excised, pooled, and analyzed.

4. Rebar, R. W., Miyake, A., Low, T. L. K. & Goldstein, A. L. (1981) *Science* **214,** 669–671.
5. Stein, S. & Moschera, J. (1981) *Methods Enzymol.* **78,** 435–447.
6. Benson, J. R. & Hare, P. E. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 619–622.
7. Hannappel, E., Davoust, S. & Horecker, B. L. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 1708–1711.
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
9. Johnston, R. B., Jr. (1981) in *Lymphokines*, ed. Pick, E. (Academic, New York), Vol. 3, pp. 33–56.
10. Emmerling, P., Finger, H. & Hof, H. (1977) *Infect. Immun.* **15,** 382–385.
11. Mogensen, S. C. & Andersen, H. K. (1978) *Infect. Immun.* **19,** 792–798.
12. Schlesinger, D. H., Goldstein, G. & Niall, H. D. (1975) *Biochemistry* **14,** 2214–2218.
13. Wilkinson, K. D., Urban, M. K. & Haas, A. L. (1980) *J. Biol. Chem.* **255,** 7529–7532.
14. Ciehanover, A., Hod, Y. & Hershko, A. (1978) *Biochem. Biophys. Res. Commun.* **81,** 1100–1105.