

## Purification and Biological Activity of Thymosin, a Hormone of the Thymus Gland (thymus/rosette-forming cells/thymectomy/lymphoid cells/mouse)

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**ABSTRACT** The purification and chemical properties of thymosin, obtained from bovine thymus tissue, are described. The biological activity of the thymic hormone has been assessed by a newly developed rosette assay, which permits measurement of thymus-dependent lymphoid cells. Thymosin activity is associated with a physico-chemically homogeneous protein of molecular weight 12,600. The hormonal activity is evident in *in vitro* incubation assay, after injection into adult thymectomized mice, and in prolonging survival of neonatally thymectomized mice and the reconstitution of their response to a skin allograft.

The extraction, partial purification, and assay of a thymic factor, which has been termed thymosin, from calf thymic tissue has been described by this laboratory (1). Thymosin-containing fractions lowered the incidence, in neonatally thymectomized mice, of a wasting disease characterized by an atrophy of lymphoid tissue and high mortality (2) and a failure of development of cell-mediated immune responses, including the capacity of host cells to elicit a normal graft-versus-host response (3) and to reject histoincompatible skin grafts (4). In addition, thymosin administration to newborn normal mice accelerated the ontogenesis of cell-mediated immunity, as measured by the capacity of spleen cells from the treated animals to elicit a normal graft-versus-host response (5) and by the development of resistance to progressive tumor growth after inoculation with Moloney sarcoma virus (6). The addition of thymosin preparations *in vitro* to discrete populations of lymphoid stem cells from bone marrow rapidly converted such cells into immunologically competent cells, as measured by a graft-versus-host assay (5) and by the appearance of cells with characteristics of T-cells (7) in the rosette bioassay.

In this preliminary communication, we describe the further purification and characterization of thymosin. We have isolated from thymus glands of calves a carbohydrate- and lipid-free homogeneous protein that, in preliminary assays, has several of the biological activities that we have reported for crude fractions (8, 9). The purification and activity are based upon a modification of a new rosette-forming cell assay described recently (7). A complete paper on the chemistry and additional properties of thymosin will be reported in a separate communication (Guha, A. *et al.*, in preparation).

Abbreviations: T-cells, thymus dependent cells; B-cells, bone-marrow derived cells; sRF cells, spontaneous rosette-forming cells. Present address: \* Division of Biochemistry, Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77550.

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### MATERIALS AND METHODS

**Animals.** CBA/J and A/J male mice, 60 days old, were purchased from Jackson Laboratories, Bar Harbor, Me. CBA/Wh mice of the same age were raised in our own colony. All animals received food and water ad libitum until killed.

**Chemicals and Reagents.** Azathioprine was generously provided by Burroughs-Wellcome as its sodium salt. Sephadex G-150 (40-120  $\mu$ m) was purchased from Pharmacia Chemicals, Inc.; Cellex E (Ecteola; 0.43 meq/g exchange capacity) and Biogel HTP were obtained from Bio-Rad Laboratories. All other chemicals were of analytical or reagent grade and were used without further purification.

**Rosette Assays.** Two types of rosette assays for thymosin have been used. One is an *in vitro* assay in which aliquots of  $3 \times 10^6$  spleen cells were incubated for 90 min in 0.1 ml of Hank's balanced salts solution containing bicarbonate (pH 7.2), with either thymosin or control fractions from other tissues. Each fraction tested was added in 0.15 ml of the medium to tubes, each of which contained various volumes (usually 0.15-0.20 ml) of a solution of the sodium salt of azathioprine (10  $\mu$ g/ml of medium). At the end of the 90 min incubation period,  $1.2 \times 10^7$  sheep erythrocytes (in 0.1 ml of medium) were added to each tube, the mixture was centrifuged for 5 min at  $200 \times g$ , and the pellet was then resuspended slowly by gentle rotary agitation for 5 min. Duplicate aliquots of this mixture were then transferred to a hemocytometer and the number of rosettes present in two fields of 6000 cells each were counted. These counts were compared with those of controls of similarly incubated spleen lymphoid cells exposed only to azathioprine. Previous studies have shown that the concentrations of azathioprine used inhibit T-cell spontaneous rosette-forming cells (sRF cells), but do not inhibit B-cell sRF cells. Additional controls included tubes with thymosin alone, as well as tubes with azathioprine alone. Results are calculated on the basis of the minimal active concentration of thymosin. The minimal inhibitory concentration of azathioprine is defined as the quantity that reduces by more than 50% the number of sRF cells counted in a given field, as compared to untreated controls.

In the *in vivo* rosette assay for thymosin, the fractions to be assayed were injected intravenously into 60-day-old male CBA/J mice that had been thymectomized 1-2 weeks previously. 24 hr after injection, the animals were killed, their spleens were removed, and suspensions of cells were prepared and incubated *in vitro* for 90 min with azathioprine, followed by the subsequent steps described above.

**Neonatal Thymectomy and Skin Allograft Procedures.** New-born CBA/Wh mice were thymectomized within 24 hr of birth. From 1 day after surgery, each animal received intraperitoneal injections of 2.5  $\mu$ g of the fraction to be tested three times a week for the first 2 weeks, then 5.0  $\mu$ g daily by subcutaneous injection. At 6 weeks of age, surviving control and experimental mice were grafted (4) with full-thickness histoincompatible A/J skin, and graft survival was measured over the next 60 days.

## RESULTS

The extraction and fractionation procedure used for the preparation of thymosin is diagrammed in Fig. 1. Fresh calf thymus was obtained from a local abattoir. 1 kg of tissue was cleaned, defatted, and homogenized in 0.15 M NaCl (tissue-saline 1:3) in a Waring Blender. All procedures, except where indicated, were done at 0–5°. The homogenate was centrifuged at 1200  $\times$  g for 15 min. The supernatant fluid was centrifuged at 46,000  $\times$  g for 2 hr and then passed through glass wool to remove particulate material. The clear extract (Fraction 1) was heated in a water bath at 80° for 15 min, then cooled in an ice bath. A large, white voluminous precipitate that formed during the heat step was removed by centrifugation at 20,000  $\times$  g for 15 min. The supernatant solution was then passed through a Millipore filter (0.45- $\mu$ m pore size) (Fraction 2). The filtrate was then added slowly with constant stirring to 10 volumes of cold (–20°) acetone. The precipitate was collected by filtration on a large Buchner funnel on Whatman No. 1 filter paper. The wet precipitate was washed with several volumes of cold acetone. The white powdery precipitate was transferred to a glass dish and dried overnight under reduced pressure at 4°. Extraction with 0.1 M sodium phosphate buffer (pH 7.2) solubilized more than 95% of the powder, which is about 30% protein by analysis (10). The small insoluble residue was removed by centrifugation. The clear supernatant solution (Fraction 3), at a concentration of about 100 mg/ml, was brought to 25% saturation by addition of a solution of saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (previously adjusted to pH 7.2 by the addition of NH<sub>4</sub>OH) with constant stirring. The precipitate was removed by centrifugation at 10,000  $\times$  g for 30 min. To the clear supernatant solution, saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to a concentration of 50% saturation. The precipitate was collected by centrifugation, redissolved in 0.1 M phosphate buffer, pH 7.2, and reprecipitated by addition of an equal volume of saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. The precipitate was collected, dissolved in buffer as above, and passed through a Millipore filter (0.45- $\mu$ m pore size). The solution was dialyzed overnight against two changes of glass-distilled water and lyophilized (Fraction 4). Fraction 4 (200 mg in 5 ml of 0.01 M phosphate buffer, pH 7.2), was subjected to gel filtration on a Sephadex G-150 column (2.5  $\times$  90 cm), equilibrated with the same buffer, and eluted with this buffer.

The active fractions were combined (Fraction 5) and further purified by chromatography on an Ecteola-cellulose column (2  $\times$  40 cm). 60 mg of Fraction 5 in 60 ml of 0.01 M phosphate buffer, pH 7.2, were placed on the column and eluted with a linear NaCl gradient to 1.0 M in this buffer. Thymosin activity was associated primarily with the peak eluting with the salt gradient between 0.15 and 0.2 M NaCl. The active fraction was dialyzed against 0.01 M phosphate buffer, pH 6.5, and then chromatographed on a hydroxylapatite column (1.2  $\times$  10 cm) that had been washed extensively with 0.01 M

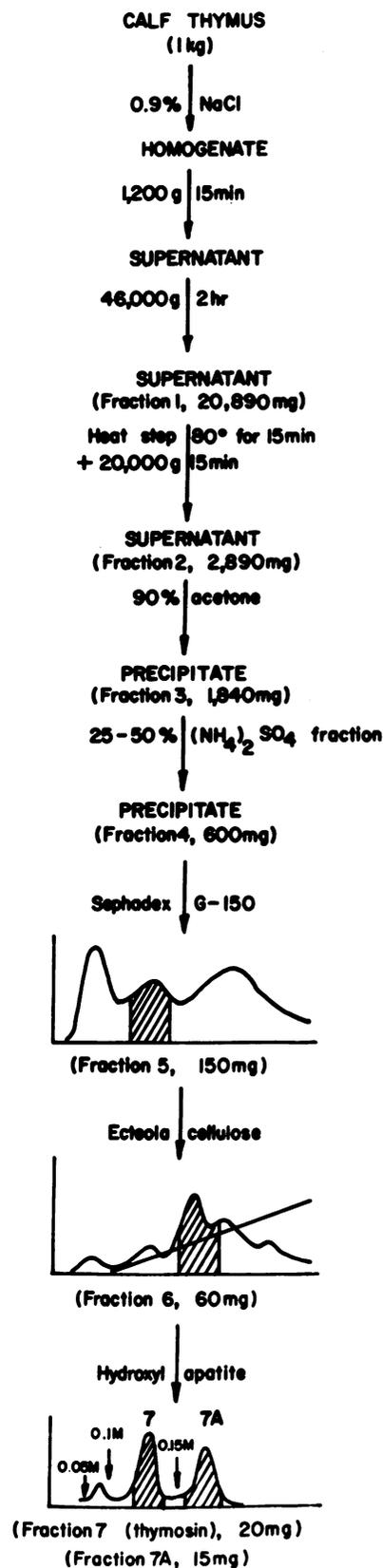


FIG. 1. Flow diagram of extraction and fractionation of thymosin activity from calf thymic tissue. Protein concentrations were estimated by the method of Lowry *et al.* (10). Shaded areas in elution profiles indicate fractions with thymosin activity. In these profiles, the *abscissa* is tube number and the *ordinate* absorption at 280 nm. For details, see text.

TABLE 1. *Effect of purified thymosin fractions administered in vivo on spontaneous rosette-forming spleen cells*

| Experimental groups | Fractions tested* | Azathioprine sensitivity† (μg) |
|---------------------|-------------------|--------------------------------|
| Normal              | —                 | 0.75                           |
| Normal              | 6                 | 0.75                           |
| Thymectomy‡         | —                 | 12.5                           |
| Thymectomy          | 6                 | 3.1                            |
| Thymectomy          | 7                 | 1.5                            |
| Thymectomy          | 7A                | 12.5                           |

\* 20 μg of protein in 0.1 ml of saline injected intravenously 24 hr before death. See Fig. 1 for details of fractionation.

† Amount of azathioprine resulting in more than 50% inhibition of rosette formation.

‡ 60-day-old CBA/Wh mice 14 days after thymectomy.

phosphate buffer, pH 6.5. The adsorbed material was eluted with successive portions of 0.05, 0.1, 0.15, and 0.2 M phosphate buffer, pH 6.5. The second eluted peak of protein contained the bulk of the thymosin activity (Fraction 7). The third peak contained some thymosin activity (Fraction 7A).

Fraction 7 behaved as a single protein on polyacrylamide gel electrophoresis at pH 8.6 and 3.1. Fraction 7A showed at least two slow-moving components, and also a rapidly moving component (Fraction 7). On dialysis of Fraction 7A against 0.1 M phosphate buffer, followed by rechromatography on hydroxylapatite as described above, Fraction 7A was freed of Fraction 7, but still revealed several bands on electrophoresis.

Sedimentation equilibrium studies of Fraction 7 indicate a homogeneous protein of molecular weight of 12,600 ± 200. Amino-acid analyses according to the method of Spackman *et al.* (11) showed the presence of 105 amino-acid residues, from which was calculated a molecular weight of 11,200. No unusual amino acid is present and the protein is free of carbohydrate and lipid.

The purification of thymosin described during fractionation is based upon the increase in activity per mg of protein by the rosette assay of Bach *et al.* (7). This fractionation, which we presently use in our laboratory for the preparation of purified thymosin, includes several modifications from that described in previous publications (1, 8). These modifications have been developed with calf-thymus tissue, but are equally applicable to the isolation of thymosin-containing fractions

TABLE 2. *Effect of purified thymosin fractions added in vitro on spontaneous rosette-forming spleen cells*

| Experimental groups | Fractions tested | Minimal active thymosin concentration (μg)* | Azathioprine sensitivity† (μg) |
|---------------------|------------------|---|--------------------------------|
| Normal              | —                | —   | 0.75                           |
| Thymectomy‡         | —                | —   | 12.5                           |
| Thymectomy          | 6                | 0.25  | 0.75                           |
| Thymectomy          | 7                | 0.12  | 0.75                           |
| Thymectomy          | 7A               | 1.0   | 12.5                           |

\* Per 3 × 10<sup>6</sup> spleen cells in 0.5 ml of Hank's balanced salts solution.

† Amount of azathioprine resulting in more than 50% inhibition of rosette formation.

‡ 60-Day-old CBA/Wh mice 14 days after thymectomy.

from thymic tissue of other animals. Thus, we have demonstrated thymosin activity (Fraction 3) from mouse, rat, rabbit, guinea pig, hog, cow, steer, and human thymus (*ref. 7 and unpublished studies*).

The effect of various thymosin fractions administered *in vivo* on the reconstitution of sRF cells in a spleen population from adult thymectomized mice is shown in Table 1. Previous studies by Bach *et al.* (12) have demonstrated that within 5 days after thymectomy, the number of azathioprine-sensitive sRF cells in a spleen is significantly decreased. The data in Table 1 show that 20 μg of Fraction 7 administered intravenously to adult, thymectomized mice 24 hr before death totally reconstituted the azathioprine sensitivity of spleen sRF cells, to the sensitivity observed with spleen cells from control, unoperated mice. Rechromatographed fraction 7A, as well as other calf tissue fractions, *e.g.*, spleen, liver, and brain, are without significant activity. The effect of addition of thymosin Fractions 6 and 7 *in vitro* on the sensitivity to azathioprine of spontaneous rosette-forming cells in the spleens of adult thymectomized mice is shown in Table 2. Thymosin Fractions 6 and 7 can reconstitute the populations of thymic-dependent sRF cells. Fraction 7A is inactive at concentrations as high as 1 μg per assay vial.

The activity of Fraction 6 *in vivo*, as assessed by the survival of neonatally thymectomized CBA/Wh mice and reconstitution of the capacity to reject skin allografts, is shown by the

TABLE 3. *Effect of thymosin (fraction 6) on survival and reconstitution of allograft response in neonatally thymectomized CBA/Wh mice*

| Daily treatment before grafting | Treatment after grafting | No. of animals | Survival at 8 weeks (%) | No. of intact A/J skin allografts 48 days after grafting* |
|---------------------------------|--------------------------|----------------|-------------------------|---|
| Thymectomy + no treatment       | None                     | 25             | 40                      | 7/7   |
| + saline                        | Saline                   | 30             | 30                      | 5/7   |
| + albumin†                      | Albumin†                 | 20             | 35                      | 5/6   |
| + thymosin†                     | Thymosin†                | 18             | 89                      | 0/16  |
| Unoperated                      | —                        | 16             | 100                     | 0/10  |

\* Number of intact A/J skin grafts per total number of surviving animals.

† 2.5 μg intraperitoneally, three injections per week for 2 weeks; 5.0 μg subcutaneously daily thereafter; *albumin*, bovine serum albumin.

data in Table 3. Similar studies are in progress with Fraction 7. The data in Table 3 show that the majority of thymosin-treated mice (89%), in contrast to saline-treated (30%) or bovine-serum albumin-treated controls (35%), are alive 8 weeks after thymectomy, and have the capacity to reject their skin allografts in a manner comparable to normal mice.

### DISCUSSION

A homogeneous protein preparation with thymosin activity has been prepared with the aid of a new rosette assay developed by Bach *et al.* (7). The purified thymosin preparation does not contain lipid or carbohydrate. It has been established by Bach and Dardenne (13) that the majority of spontaneous rosette-forming cells in spleens of normal, unsensitized mice are thymic-dependent. Evaluation of thymic dependency has been based upon the extreme sensitivity of sRF cells from nonimmunized animals to azathioprine, antilymphocyte serum, and antitheta serum. Spleen cells from neonatally thymectomized mice, from "nude mice" that congenitally lack a thymus, or from adult, thymectomized, irradiated, and bone marrow reconstituted mice are much less sensitive to azathioprine (13). Recently, Bach *et al.* (12) have demonstrated that within 7 days after thymectomy of adult mice, the population of cells that are sensitive to azathioprine, antilymphocyte serum, and antitheta serum is significantly reduced in the spleen.

The studies cited above appear in contrast to the majority of reports that thymus-dependent cells are not markedly affected immediately after adult thymectomy (14). Newer data indicate, however, that subpopulations of T-cells exist that apparently require the presence of the thymus gland. These cell types give rise to azathioprine-sensitive cells, which may be similar to the population of T-cell precursors designated as "T<sub>1</sub>" by Raff and Cantor (15). T<sub>1</sub>-cells are characterized by being present in greater numbers in spleen and thymus than in lymph nodes, blood, or thoracic duct lymph, are relatively insensitive to antilymphocyte serum *in vivo*, and are more sensitive *in vitro* to lysis by antitheta serum and complement (15).

Our observation (7) that thymosin can restore the population of azathioprine-sensitive cells when added *in vitro* to cell suspensions prepared from spleens of adult, thymectomized mice suggests the presence of a thymosin-sensitive thymus-dependent cell. This cell is present in spleen in adequate numbers, and requires only an endocrine influence of the thymus gland to be converted to the more mature thymosin-activated cell. We have reported (7) that this thymosin-sensitive cell is also present in bone marrow, as evidenced by its conversion to a thymosin activated cell (azathioprine sensi-

tive) after incubation *in vitro* with thymosin. We shall discuss elsewhere, in detail, a possible sequence of events leading to the maturation of immunologically competent cells (Goldstein, A. L. & White, A., in preparation).

The availability of thymosin in purified form makes possible a detailed study of its mode of action, and the development of a radioimmunoassay to measure its concentration in biological fluids. This approach should facilitate delineation of the endocrine functions of the thymus gland in the ontogenesis and maintenance of the immune system, and perhaps will provide a better understanding of the role of the thymus in the development of resistance to disease.

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