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ISOLATION, PARTIAL CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF THYMONE B

by

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SUMMARY

A peptide, thymone B, was isolated from bovine thymus by sequences of solvent extractions, gel filtrations, ion exchange chromatography and micromanipulation. Paucity of sample restricted all assays. Thymone B showed essentially single spots by TLC in 3 systems and by electrophoresis in one system. Levels of 100 nanograms (lower?) and 10 micrograms (lower?) of thymone B stimulated incorporation of [^{3}H]-thymidine into DNA, and the synthesis of cGMP, respectively; 100 micrograms did not stimulate cAMP. Trypsin destroyed activity indicating an active peptide. Analysis indicated up to 13 individual amino acids: Asp, Glu, Gly, Ala, Val, Ile, Leu, Ser, Pro, Thr, His, Lys and Arg, and a molecule considerably smaller than that of thymone A.

INTRODUCTION

The function of the thymus gland in the control of immunity is established. The control is mediated presumably by endocrine factors and hormones. There is an increasing consideration that there may not be a single thymic hormone, but rather a group of hormones which control the complex mechanisms of normal immunocompetence. The potential importance of the medical use of such thymic hormones has been explored, particularly by two groups of investigators.

An extract of thymus was purified to yield a mixture, designated fraction 5 (1), which has been important to study the effectiveness of substances in the thymus for the clinical treatment of thymus-dependent immunological diseases (2,3). Fraction 5 has also been reported to cause reversal of certain immunological parameters which are known to decrease in cancer patients (4). When fraction 5 was used in a combined modality to treat carcinoma of the lung by a randomized protocol, both survival time and number increased (5).

Kook, Yakir and Trainin (6), 1975, described their purification and partial chemical characterization of their thymic humoral factor (THF). Preparations of their THF were used by Varsano et al. (7) to treat severe disseminated adenovirus infection, which was reported in 1977 to be successful. A preparation of THF was also used by Zaizov et al. (8) for therapy with immunosuppressed children who had lymphoproliferative neoplasia and generalized varicella.

These exemplary clinical studies with preparations from A. Goldstein et al.

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and Trainin et al. underscore the importance of the chemical isolation and elucidation of thymic factors and hormones.

A summary, 1979, of purified preparations and isolated peptides from the thymus, particularly as they have been tested for cyclic nucleotide effects, has been made by Naylor and A. Goldstein (9). Supplementing this summary, Low and A. Goldstein et al. announced, 1980, (10) that they have isolated two new thymic peptides, designated thymosin β_3 and β_4, which appear to act on stem cells. These two peptides have an M.W. of about 5000-5500.

We have isolated a peptide from the thymus which is apparently different from other peptides which have been described. We designate this peptide as thymone B, and summarize our findings on the basis of the very limited amount which has been available.

EXPERIMENTAL

Biological Assay Methods

A newly devised assay which is based upon the incorporation of [^{3}H]-thymidine into DNA by spleen cells, particularly from neonatally thymectomized C57BL/6 mice, was used to guide the chemical fractionations. The mice were obtained from the Charles River Breeding Labs., Inc., Wilmington, MA. The essential steps of this assay have been described as they were used for the isolation of thymone A (11). After a desired refinement of this assay, the details of the method will be separately published. Kits from the New England Nuclear, Boston, MA, were utilized for the determinations of cAMP and cGMP. The details of the procedures of the radioimmunoassays will be included in a separate account of assays and other biological data.

Chemical Isolation and Partial Characterization

Although there was some flexibility in the experimentation to develop a sequence of purifications, the following sequence exemplifies such steps which were used for the isolation of thymone B: thymus tissue was lyophilized; extraction with methylene chloride was used for defatting; extraction of the defatted residue was made with methanol; the methanol residue was extracted with acetic acid; the extractives from acetic acid were purified over Sephadex G-50; purification over DEAE-Sephadex A-25; purification over CM-Sephadex C-25; purification over Sephadex G-25.

Manipulation of fraction 7 from Sephadex G-25 yielded thymone B. Examination for purity by electrophoresis afforded the following data: Rf = 0.05, relative to Lys; pyridine:AcOH:H_2O (2.6:30:867) pH 3.5; 1000 V, 3 mA, 20 min: cellulose plate (Merck) 5 x 10 cm. Examination for purity by TLC gave the following results:

Rf = 0.53; n-BuOH:Pyridine:AcOH:H_2O (30:30:6:24)
Rf = 0.48; EtOAc:Pyridine:AcOH:H_2O (5:5:1:3)
Rf = 0.61; n-BuOH:AcOH:EtOAc:H_2O (1:1:3:1)

After hydrolysis of 20 µg with hydrochloric acid under the usual conditions, the analysis for amino acids resulted in the data in Table I. Cys, Met, Tyr, Phe and Trp were apparently absent.

An advanced fraction after DEAE-Sephadex A-25 of thymone B (ca. 1 mg) was dissolved in 1 ml of 0.1 M N-ethyl-morpholine acetate buffer, pH 8.2. Digestion was carried out both with 0.2% and 1% of trypsin. The action of trypsin was terminated by addition of a trypsin inhibitor (1:1, w/w).

After 10 and 100 min., at a concentration of 0.2% trypsin, the activity in the assay for the incorporation of [^{3}H]-thymidine was 34,143 cpm ($p<0.02$)

TABLE I. Amino Acid Data

Amino Acid	x 10^{-2} μmoles	Amino Acid	x 10^{-2} μmoles
Asp	0.369	Ala	0.432
Thr	0.237	Val	0.189
Ser	1.017	Ile	0.084
Glu	0.783	Leu	0.118
Pro	0.214	His	0.635
Gly	1.005	Lys	0.160
		Arg	0.229

TABLE II. Incorporation of [^{3}H]-Thymidine into DNA

Substance	Level	cpm±SEM	P
Control	--	13,513±1394	
Fraction of Thymone B	30 μg	39,388±1278	<0.001
	1 μg	19,081±271	<0.02
	0.1 μg	15,323±489	n.s.
Control	--	9,780±1028	
Fraction of Thymone B*	0.1 μg	12,886±320	<0.05

*from Sephadex G-25

and 33,236 cpm ($p<0.05$), respectively, in comparison with the control value of 35,045 cpm before treatment. However, after digestion for 7 hrs, the level of cpm had decreased to 24,237 ($p<0.001$). At a concentration of 1% trypsin, the activity had decreased from an initial value of 35,045 cpm ($p<0.001$) to 30,535 cpm ($p<0.01$) and 21,605 cpm ($p<0.001$) after 10 and 100 min., respectively, and had decreased to 18,946 cpm (p 0.001) after 7 h.

Biological Assays

Incorporation of [^{3}H]-Thymidine into DNA

A fraction from DEAE-Sephadex, which was not pure, but highly purified was shown to have activity to stimulate the incorporation of [^{3}H]-thymidine into DNA, according to the data in Table II.

TABLE III. Stimulation of cGMP Synthesis

Substance	Level μg/ml	Time min.	cGMP fmols/10^6 cells±SEM	P
Control	0	0	6.95±0.89	--
Fraction of Thymone B	100	1	7.77±0.96	n.s.
	"	5	10.66±1.33	<0.001
	"	10	11.45±1.12	<0.001
	"	20	6.01±2.82	n.s.

TABLE IV. Stimulation of cGMP Synthesis

Substance	Level μg/ml	Time min.	fmols/10^6 cells±SEM	P
Control	0	0	6.60±0.84	--
Fraction of Thymone B	100	10	11.40±0.87	<0.001
	10	"	9.29±1.15	<0.001
	1	"	7.51±2.12	n.s.

Stimulation of Synthesis of cGMP

The same fraction from DEAE-Sephadex which was used to obtain the data in Table II was used in tests fro possible stimulation of levels of cyclic nucleotides. The data in Table III show that this fraction of thymone B stimulates the synthesis of cGMP with time as a variable. The data in Table IV show the stimulation by levels of the fraction of thymone B as a variable.

The same fraction of thymone B was also tested for the stimulation of the synthesis of cAMP when the time of incubation and the level of thymone B were variables. After periods of 1, 5, 10, 20 and 30 min, a level of 100 ug/ml did not result in any statistically significant increase or decrease; the control value was 3.39 pmoles of cAMP/10^7 cells. At levels of 0.01, 0.1, 1, 10 and 100 μg during an incubation of 10 min, there was no significant increase or decrease in the levels of cAMP in comparison with the control value of 3.75 pmoles of cAMP/10^7 cells.

RESULTS AND DISCUSSION

Diverse sequences and series of steps were explored during the fractionation of extracts of thymus tissue toward the isolation of one or more active factors or hormones. When an assay for the stimulation of the synthesis of cAMP was used to guide the fractionation, glutathione was unexpectedly isolated; Folkers et al. (12). Pure glutathione, both from isolation and purchase, was found to show activity for the stimulation of cAMP and by a dose-response assay.

When a new assay was used, which is based on the stimulation of the incorporation of [^{3}H]-thymidine into DNA by lymphocytes from spleens from neonatally thymectomized mice, a peptide, designated thymone A, was isolated in essentially pure form; Folkers et al. (11).

During the multi-fractionations which led to the isolation of thymone A, the activity of another stimulatory factor in fractions, which were widely separately from those containing thymone A, was increasingly observed. These fractions ultimately yielded a different compound in essentially pure form which has been designated thymone B.

Thymone B was apparently present in lower concentrations in fractions than was thymone A, and was correspondingly more difficult to isolate in an essentially pure state. Therefore, the paucity of the compound restricted the initial chemical and biological determinations of its properties.

Since thymone B showed essentially single spots by TLC in three solvent systems and also a single spot by electrophoresis in one system, it appeared that thymone B was indeed essentially pure, but possibly not of absolute purity.

Hydrolysis of thymone B with hydrochloric acid and then analysis for the presence of amino acids revealed up to 13 individual amino acids which were Asp, Glu, Gly, Ala, Val, Ile, Leu, Ser, Pro, Thr, His, Lys and Arg. Both by amino acid analysis and behavior over gel filtration columns, the molecular size of thymone B is considerably smaller than that of thymone A.

Treatment of thymone B with trypsin led to inactivation which indicated that the peptide was apparently responsible for the biological activities rather than a non-peptidic impurity.

Thymone B at a level of 100 nanograms (lower levels not tested) effectively stimulated the incorporation of [^{3}H]-thymidine into the DNA of spleen cells which were from neonatally thymectomized mice. This activity of thymone B resembles that of thymone A.

Thymone B at a level of 10 μg (lower levels not tested) stimulated the synthesis of cGMP, but even the level of 100 μg did not stimulate the synthesis of cAMP.

On the basis of the available chemical evidence, thymone B is different from thymosin α_1, the thymopoietins, and the facteur thymique serique, all of which have been isolated, sequenced, and synthesized. Therefore, thymone B is apparently a new peptide in this group of peptides which has been isolated from thymus tissue.

Naylor and A. Goldstein (9) have summarized data from various investigators on the tests of thymic mixtures and peptides for effects on the cyclic nucleotides. These diverse studies on levels of cyclic nucleotides shwo that levels of both cAMP and cGMP are important parameters in research on the mechanisms of action of thymic hormone (14,15,16). To paraphrase, it appears that a valid thymic hormone may be expected to have a direct effect on cyclic nucleotides, i.e., either cAMP or cGMP. If these criteria are valid, it is important to note that the thymosin fraction 5 mixture elevated levels of cGMP, but not of cAMP. Thymosin α_1, isolated from the thymosin fraction 5 mixture, did not elevate either cGMP or cAMP.

In the review (9), thymopoietin was reported to elevate cGMP, but its effect of cAMP was ambiguous. The preparation of the thymic humoral factor (THF) was reported to elevate cAMP, a result which was stated to characterize THF as a hormone. The facteur thymique serique (FTS) was reported not to elevate levels of cAMP. The effect of the human serum thymic factor (SF) to elevate levels of cAMP was ambiguous.

On the basis of all this prior emphasis of the importance of effects of these diverse mixtures and identified peptides from the thymus on levels of cyclid nucleotides, it may be significant that thymone A elevates levels of cAMP but not that of cGMP, and that thymone B elevates levels of cGMP but not that of cAMP.

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