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ISOLATION OF THYMONE A FROM BOVINE THYMUS PARTIAL CHEMICAL AND BIOLOGICAL CHARACTERIZATION

by

Karl Folkers, Naoki Sakura, Teresa Kubiak and Henryk Stepien

Institute for Biomedical Research, The University of Texas at Austin
Austin, Texas 78712

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SUMMARY

A peptide, designated thymone A, was isolated from bovine thymus by a sequence of eight procedures prior to micromanipulation. Trypsin destroyed the activity indicating the absence of a non-peptidic but active component. The peptide showed essentially single spots under three conditions of electrophoresis. Analysis revealed up to 14 individual amino acids: Asp, Glu, Gly, Ala, Val, Ile, Leu, Pro, Ser, Thr, Met, Lys, Arg and His, and a composition of $\geq$ 68-71 amino acids (MW 7291-7677). The MW by behavior over Bio-Gel P-6 was ca. 8000. A level between 10-100 ng of thymone A stimulated incorporation of [^{3}H]-thymidine into DNA; a level of 1 μg (lower?) stimulated the synthesis of cAMP. Thymone A may have functional activity or be an active pro-hormone.

INTRODUCTION

It is generally understood that the thymus gland is essential for immunocompetence. As the age of man increases, normal immune functions decline. Associated with this decline, is the emergence of diseases which profoundly affect many tissues. Many investigators are endeavoring to isolate one or more factors or hormones from the thymus which may have specific functions in the complex mechanisms of immunocompetence.

A. Goldstein (1) standardized a 5-step purification of a thymus extract, and designated the mixture as "fraction 5". This fraction has been used for diverse biological research and also for exploratory clinical studies in patients with immune deficiency diseases. A. Goldstein et al.(2), 1977, reported the isolation and sequence of thymosin α_1 from fraction 5 which is a peptide having 28 amino acids. Low and A. Goldstein et al.(3), 1980, announced the isolation of thymosin β_3 and β_4, which have ca. 50 and 43 amino acids, respectively.

Schlesinger and G. Goldstein (4), in 1975, reported the sequence of thymopoietin I and II. Both peptides have 49 amino acids, and differ in sequence only in positions 1, 2 and 43.

Kook et al., 1975, described amino acid analytical data on a fraction of their thymic humoral factor (THF) as having a composition of about 31 amino acids from 10 individual amino acids; these investigators in 1976(5)described

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another amino acid analysis indicating 27 amino acids and a total of 13 individual amino acids.

Bach et al. (6), 1977, described the sequence of their FTS as a nonapeptide, which was synthesized.

Astaldi et al. reported in 1979 (7) that their serum factor (SF) was apparently pure by observations of one spot in TLC systems and appeared to have a MW of <500 and to consist of possibly four amino acids.

We have made the first isolation of a different peptide in a very small amount which must now be laboriously reisolated. We presently designate this peptide as thymone A and summarize our findings.

EXPERIMENTAL

Biological Assay Methods

The newly devised assay was used to guide chemical fractionation. The assay is based upon the incorporation of [^{3}H]-thymidine into DNA. The prime steps of the assay are: (a) Spleens are removed rom intact or neonatally thymectomized C57BL/6 mice; Charles River Breeding Labs., Inc., Wilmington,MA; (b) Spleen cells were obtained by enzymatic digestion with collagenase (Millipore Corp., Bedford, MA; (c) The spleen cells were exposed to the fractions for assay during incubation; (d) [^{3}H]-thymidine was added to allow incorporation; (e) Trichloroacetic acid was added for precipitation of DNA; (f) Radioactivity as cpm/min was determined with a Beckman Liquid Scintillation Counter. This assay is based upon the following reports.

Houck et al. (9), 1971, utilized the incorporation of [^{3}H]-thymidine by lymphocytes, stimulated by PHA, to study a thermolabile inhibitor of about 30,000-50,000 daltons. Caspary and Hughes (10), 1972, used [^{3}H]-thymidine incorporation for studies of transformations of lymphocytes in cultures. Kiger et al.(11), 1977, studied purification and characterization of a lymphocyte-inhibiting factor from thymus (LIFT) as based on the incorporation of [^{3}H]-thymidine on spontaneous DNA synthesis.

The determinations of cAMP and cGMP utilized kits provided by the New England Nuclear, Boston, MA. The procedures of radioimmunoassay were based upon reports, as follows.

Steiner et al.(12), 1972, reported on a radioimmunoassay for cyclic nucleotides. Frandsen et al. (13), 1976, described a simple ultrasensitive method to assay cAMP and cGMP in tissues. Naylor et al.(14), 1979, examined the changes in cyclic nucleotides in murine lymphocytes after exposure to fraction 5 (thymosin).

The details of these assay methods will be published separately, and particularly after refinement of the assay based upon the stimulation of the incorporation of [^{3}H]-thymidine by spleen cells.

Chemical Isolation and Partial Characterization

The following sequence of steps was used for the isolation of thymone A: lyophilization of thymus tissue; extraction with methylene chloride for defatting; extraction with methanol; extraction of methanol residue with acetic acid; dialysis of extractives with a membrane allowing passage of molecules of molecular weight up to about 8000; purification over Sephadex G-10; purification with DEAE-Sephadex, CM-Sephadex and with Bio-Gel P-6.

Thymone A was isolated (samples of a few hundred micrograms) from fractions from Bio-Gel P-6 in a state of high purity, particularly as revealed by essentially one spot in electrophoresis in three solvent systems, each at a different pH, as follows.

1. 2000 V, 6 mA, 20 min., pyridine-acetic acid-water(1:10:190), pH 3.5
2. 800 V, 22 mA, 50 min., pyridine-acetic acid-water(4:1:45), pH 5.3
3. 2000 V, 6 mA, 15 min., pyridine-acetic acid-water(10:0.4:89.6), pH 6.45

Electrophoresis was performed on cellulose plates (10 x 20 cm) from E. Merck Darmstadt, Germany. The plates were sprayed with chlorine/o-tolidine reagent.

When lysine was used for reference in electrophoresis, the values were:

Rf = 0.37; 800 V; 22 mA; 15 min; pH 5.3; System 2 above
Rf = 0.33; 1000 V; 6 mA; 20 min; pH 3.5; System 1 above
Rf = 0.28; 2000 V; 6 mA; 10 min; pH 6.45; System 3 above

After a standard hydrolysis with hydrochloric acid, an analysis for amino acids gave the following results.

ESTIMATE OF AMINO ACID COMPOSITION

Amino Acid	Analysis x 10^{-2} μmoles	Molar Ratios Based on Leu=3	Molar Ratios Based on His=2	No. Amino Acids
1. Asp	1.00	4.10	4.46	4(?5)
2. Thr	0.909	3.73	4.06	4
3. Ser	1.355	5.56	6.05	6
4. Glu	2.270	9.31	10.13	10(?9)
5. Pro	1.876	7.69	8.38	8
6. Gly	1.381	5.67	6.17	6
7. Ala	1.305	5.35	5.83	6(?5)
8. Val	0.765	3.13	3.42	3(?4)
9. Met	0.181	0.741	0.80	1
10. Ile	0.401	1.09	1.79	2(?1)
11. Leu	0.731	3.0	3.26	3
12. His	0.448	1.84	2.00	2
13. Lys	2.722	11.17	12.15	12(?11)
14. Arg	0.831	3.39	3.71	4(?3)
				ca. 71(?68)

The amino acids Cys, Tyr, Phe, and Trp were apparently absent.

The numbers of 7 of the 14 amino acids were the same whether the numbers were based upon Leu=3 or His=2. The numbers of the other 7 amino acids varied by 1 for each amino acid depending upon whether the calculation was based upon Leu or His. On this basis, the total number of amino acids was estimated at 68-71 with a MW of ca. 7291-7677.

The behavior of the sample of thymone A over a standardized column(bovine serum albumin, LHRH, alanine) of Bio-Gel P-6 indicated that this peptide has a molecular weight higher than 6000.

An enzymatic degradation of 30 μg of thymone A was conducted with 0.1% trypsin by weight for 10 min. There was complete inactivation by the assay based upon the incorporation of tritiated thymidine into DNA.

The paucity of thymone A, and the known difficulties of promptly identifying a successful solvent system and packing for the HPLC of peptides of the molecular size of thymone A has delayed our analysis of thymone A by HPLC.

Biological Assays of Thymone A

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

The data in Table I show that thymone A has activity to stimulate the incorporation of [^{3}H]-thymidine into DNA.

Stimulation of cAMP Synthesis

The data from the assay of thymone A for stimulation of cAMP are in Table II.

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

Substance	Level	cpm±SEM	P
Control	--	6,942±1355	--
Thymone A	5 μg	17,223±621	<0.001
	1 μg	15,634±1069	<0.001
	100 ng	11,519±468	<0.02
	10 ng	6,841±1007	n.s.

TABLE II. Stimulation of cAMP by Thymone A

Substance	Level μg/ml	Incubation min.	cAMP pmoles/10^7 cells	
Control	-	0	3.57±0.26	--
Thymone A	1	1	3.44±0.53	n.s.
	"	5	4.36±0.58	P<0.05
	"	10	7.14±0.78	P<0.001
	"	20	7.04±0.59	P<0.001
	"	30	5.57±1.36	P<0.001

Stimulation of cGMP Synthesis

Thymone A was also assayed for stimulation of cGMP. The test was conducted with a dose level of 1 μg/ml of thymone A. The times of incubation were 1, 5, 10, 20 and 30 min. The control level of cGMP was 7.12±2.61 fmoles/10^6 cells. At the single dosage used for the five time intervals, there was no significant stimulation of cGMP.

RESULTS AND DISCUSSION

The entire process of isolation, as generalized in the Experimental Section, exemplifies a complete sequence which has been used, but this sequence is flexible and subject to improvements. All of the steps were designed on the basis that the sought after factors or hormones of the thymus are probably peptides, although a non-peptidic nature of one or more factors or hormones is not excluded.

At one stage of the purification, an assay for the stimulation of cAMP was used to guide the fraction. However, this assay led to the unexpected isolation of glutathione, as described by Folkers et al.(15). Pure glutathione, both isolated and purchased, showed a dose-response in a cAMP assay. Glutathione was not active in the mixed lymphocyte culture assay. When glutathione was tested in assays using t-rosettes and the graft vs. host assay, positive responses were inconsistently obtained.

On the basis of the known coenzyme roles of glutathione, including a possible transport of amino acids across membranes, it was considered that glutathione might have a role in mechanisms of the complex immune systems.

Another peptide has now been isolated which is designated as thymone A, for convenience. By electrophoretic analysis in three solvent systems and at

three pH's, thymone A behaved essentially as single spots. Since only very small amounts of thymone A were available, electrophoretic analyses appeared to provide the best evidence for high purity.

Amino acid analysis, after acid hydrolysis, yielded up to 14 amino acids: Asp, Glu, Gly, Ala, Val, Ile, Leu, Ser, Thr, Met, Lys, Arg and His. The amino acids Cys, Tyr, Phe and Trp were absent. The molar ratios of the amino acids indicated that thymone A may have approximately 68-71 amino acids. When the isolation of thymone A is repeated, and the purity of the peptide is refined, a precise composition of amino acids is expected.

A composition of 68-71 amino acids (MW 7291-7677) is also reasonable on the basis that thymone A behaved over a standardized column of Bio-Gel P-6 according to a range of molecular weight higher than 6000.

When thymone A was subjected to enzymatic degradation with trypsin, there was complete inactivation after 10 min of reaction. This result minimized the possibility that thymone A contained a non-peptidic component which was responsible for the activity and that the peptide could be inactive.

Thymone A has a high number of Glu(Gln) and Asp(Asn) residues. Aromatic amino acids are minimal, since only two His are indicated. Thy one A might possibly be a pro-hormone, not only on the basis of its molecular size, but particularly on the basis of the very high content of Lys 12(or 11) and Arg 4 (or 3). The presence of so many Lys and Arg residues indicates the possible presence of double, triple or quadruple moieties of Lys and Arg which is a basis of sequence in pro-hormones, which can allow specific cleavage to one or more fragments having hormonal activities. Exemplifying cleavages at double basic units are the known conversion of bovine insulin to insulin and C peptide, the conversion of pro-glucagon to glucagon, and the conversions of β-lipotropin to β-MSH, β-endorphin and the N-fragment (16).

The nonapeptide, designated FTS (facteur thymique serique) has the sequence: <Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asn-Pro-OH(7). It was proposed that <Glu^1 as Gln^1 in a moiety of FTS might be linked to Arg^{49} of thymopoietin in a new 58-amino acid peptide in the thymus (17,18). Cleavage between Arg^{49} and Gln^{50} in the 58-amino acid peptide could liberate thymopoietin and the [H-Gln^1]-FTS which could cyclize to FTS, enzymically or chemically. Alternatively, FTS could be similarly linked to thymone A, because each of the nine amino acids in FTS is present at least once in thymone A. The concept is not incompatible with the finding of FTS in serum and tissues, because of the specificities of cleavages of pro-hormones. Synthetic models of analogous pro-hormones of the luteinizing hormone releasing hormone (LHRH) and the enzymic conversion of such models to LHRH (a <Glu^1-peptide) were reported by Folkers et al. (19).

All of the amino acids in thymosin α_1 are also in thymone A. The presence of the N-terminal [Ac-Ser][1] in thymosin α_1 could indicate that if thymosin α_1 and thymone A were linked together, thymosin α_1 could be N-terminal in a pro-thymosin α_1. The C-terminal-COOH group of thymosin α_1 is compatible with such a cleavage; otherwise acetylation could occur after cleavage.

On the basis of available knowledge, thymone A is different from the other peptides, purified or isolated from the thymus by other investigators.

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