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(19)

(54) THYMOSIN α_1

(71) We, F. HOFFMANN-LA ROCHE & CO. AKTIENGESELLSCHAFT a Swiss Company of 124-184, Grenzacherstrasse, Basle, Switzerland and BOARD OF REGENTS OF THE UNIVERSITY OF TEXAS SYSTEM, Austin, Texas, The United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:-

The importance of the thymus gland in the development and senescence of immunological competence in animals, including man is now generally accepted. Although there is little knowledge of the molecular events by which the thymus gland exerts control over T cell development, it appears that a vital part of the process occurs via a hormonal mechanism. The thymus gland produces a family of polypeptides termed thymosin and perhaps several other thymic hormones and/or factors which play an important role in the maturation, differentiation and function of T cells. Thymosin has been found to induce T cell differentiation and to enhance immunological functions in genetically athymic mice, in adult thymectomized mice, in NZB mice with severe autoimmune reactions, in tumor bearing mice and in mice with casein-induced amyloidosis.

It is known that thymosin fraction 5 is a potent immunopotentiating preparation and can act in lieu of the thymus gland to reconstitute immune functions in thymic deprived and/or immunodeprived individuals. Thymosin fraction 5 is described in the article by Mesper *et al.*, Annals New York Acad. Sci. 249, 125 (1975). Ongoing clinical trials with fraction 5 suggest that thymosin is effective in increasing T cell numbers and normalizing immune function in children with thymic dependent primary immunodeficiency diseases and can increase T cell numbers in immunodepressed cancer patients.

Analytical polyacrylamide gel electrophoresis and iso-electric focusing have demonstrated that fraction 5 consists of 10-15 major components and 20 or more minor components with molecular weights ranging from 1,000 to 15,000.

The present invention relates to the isolation and first complete structural determination of an acidic polypeptide isolated from thymosin fraction 5. This peptide has been termed thymosin α_1 . Thymosin α_1 has been found to be 10 to 1,000 times more active than fraction 5 in several *in vitro* and *in vivo* assay systems designed to measure T cell differentiation and function.

Thymosin α_1 has a molecular weight of 3,108 and a pI in the range of 4.0-4.3 as determined by gel isoelectric focusing at a pH range of 3-5. The compound has the following amino acid sequence:

(N-acetyl)-Ser-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-OH.

According to one aspect of this invention we provide a method of preparing thymosin α_1 having the amino acid sequence:

(N-acetyl)-Ser-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-OH;

from thymosin fraction 5, said method comprising

(a) chromatographing lyophilized thymosin fraction 5 on a column of carboxymethyl-cellulose in a sodium acetate/2-mercaptoethanol buffer of pH 5.0, washing the column, after the fraction 5 has been introduced, first with this buffer and then eluting with a linear gradient of said buffer and said buffer plus 1.0 M NaCl;

(b) fractionating the first protein peak of (a) on a column of a dextran gel (diameter of dry particle = 10 to 300 μ m; volume of dry gel = 4 to 6 ml/g; water regain value = 2.5 ml/g dry gel);

(c) chromatographing the second protein peak from (b) on a 2-diethylaminoethyl-cellulose column equilibrated with tris/2-mercaptoethanol buffer of pH 8.0, eluting with said buffer alone followed by a linear gradient of said buffer and said buffer plus 0.8 M NaCl;

(d) passing the first sixth of the eluted protein peak from (c) through a column of a dextran gel (diameter of dry particle = 40 to 120 μ m; volume of dry gel = 12 to 15 ml/g; water regain value = 7.5 ml/g dry gel) in guanidine-hydrochloride/tris buffer of pH 7.5 and

(e) taking a single narrow cut from the protein peak of (d) and desalting on a column of a dextran gel (diameter of dry particle = 40 to 120 μ m; volume of dry gel = 2 to 3 ml/g; water regain value = 1 ml/g dry gel).

According to a further aspect of this invention we provide thymosin α_1 having the amino acid sequence

(N-acetyl)-Ser-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-OH

and pharmaceutically acceptable acid addition salts and base salts thereof, essentially free of other thymic polypeptides.

Thymosin α_1 was isolated from fraction 5 by a combination of ion-exchange chromatography and gel filtration in the following way. It will be appreciated that the following experimental conditions could be varied by a skilled peptide chemist within the scope of the invention as defined in the appended claims.

Lyophilized thymosin fraction 5 was chromatographed on a column of carboxymethylcellulose in 10 mM sodium acetate buffer, pH 5.0, containing 1.0 mM 2-mercaptoethanol. After introduction of the sample of the column, the column was washed first with the buffer followed by a linear gradient of 2 liters each of starting buffer and the same buffer containing 1.0 M NaCl. The first protein fraction was filtered on a column of a dextran gel (Sephadex G-25) using sterile water. The second protein peak from the dextran gel column was applied on a 2-diethylaminoethyl-cellulose column (DE-32) equilibrated with 50 mM tris-HCl buffer containing 1.0 mM 2-mercaptoethanol (pH 8.0). The column was eluted with the starting buffer followed by a gradient of 1.3 liters each of starting buffer and the same buffer containing 0.8 M NaCl. The first sixth of the protein peak from the DE-32 column was further purified by being passed through a column of a dextran gel (Sephadex G-75) in a buffer containing 6.0 M guanidine hydrochloride and 10 mM tris (pH 7.5). A single narrow cut was made from the protein peak and desalted on a column of a dextran gel (Sephadex G-10) using sterile water. The purified sample so obtained was identified as thymosin α_1 . The yield of thymosin α_1 from fraction 5 was about 0.6%. The preparation was free of carbohydrate and nucleotide. "Sephadex" is a registered Trade Mark.

The structure and complete amino acid sequence of thymosin α_1 was determined using conventional methods such as enzymatic digestion by trypsin, chymotrypsin, thermolysin or subtilisin, separation of the digests by paper electrophoresis and/or chromatography and Edman degradation of the separated peptide fragments.

The following table demonstrates that thymosin α_1 is from 10 to 1,000 times more active than thymosin fraction 5 in an *in vivo* mouse mitogen assay, an *in vitro* lymphokine assay measuring MIF production, and an *in vitro* human E-rosette assay.

Thymosin Activity (μ g) in Various Bioassays

	MIF	E-Rosette	Mitogen*
Thymosin Fraction 5	1-5	1-10	1-10
Thymosin α_1	0.01-0.1	0.001-0.01	0.01-0.1

* *In vivo* 14 daily injections

Thymosin α_1 may be administered to warm blooded mammals by parenteral application either intravenously, subcutaneously or intramuscularly. The compound is a potent immunopotentiating agent with a daily dosage in the range of 1 to 100 μ g/kg of body weight per day for intravenous administration. Obviously the required dosage will vary with the particular condition being treated, the severity of the condition and the duration of the treatment. A suitable dosage form for pharmaceutical use is 1 mg. of lyophilized thymosin α_1 per vial to be reconstituted prior to use by the addition of sterile water or saline.

The invention therefore also includes a method for reconstituting immune functions in thymic deprived or immuno deprived warm blooded non-human mammals which method

comprises administering to said mammals an immunopotentiating effective amount of thymosin α_1 .

Also included within the scope of the present invention are the pharmaceutically acceptable acid addition salts and base salts of thymosin α_1 such as the sodium or potassium salt or salts with strong organic bases such as guanidine. Examples of acid addition salts are the hydrochloride, hydrobromide, sulfate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate and ascorbate.

WHAT WE CLAIM IS:-

1. A method of preparing thymosin α_1 having the amino acid sequence (N-acetyl)-Ser-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-OH from thymosin fraction 5, said method comprising:
 - (a) chromatographing lyophilized thymosin fraction 5 on a column of carboxymethyl cellulose in a sodium acetate/2-mercaptoethanol buffer of pH 5.0, washing the column, after the fraction 5 has been introduced, first with this buffer and then eluting with a linear gradient of said buffer and said buffer plus 1.0 M NaCl;
 - (b) fractionating the first protein peak of (a) on a column of a dextran gel (diameter of particle = 100 to 300 μ m; volume of dry gel = 4 to 6 ml/g; water regain value = 2.5 ml/g dry gel);
 - (c) chromatographing the second protein peak from (b) on a 2-diethylaminoethyl-cellulose column equilibrated with tris/2-mercaptoethanol buffer of pH 8.0, eluting with said buffer alone followed by a linear gradient of said buffer and said buffer plus 0.8 M NaCl;
 - (d) passing the first sixth of the eluted protein peak from (c) through a column of a dextran gel (diameter of dry particle = 40 to 120 μ m; volume of dry gel = 12 to 15 ml/g; water regain value = 7.5 ml/g dry gel) in guanidine hydrochloride/tris buffer of pH 7.5 and
 - (e) taking a single narrow cut from the protein peak of (d) and desalting on a column of a dextran gel (diameter of dry particle = 40 to 120 μ m; volume of dry gel = 2 to 3 ml/g; water regain value = 1 ml/g dry gel).
2. The method of claim 1 wherein the buffer in (a) is 10 mM sodium acetate and 1.0 mM 2-mercaptoethanol.
3. The method of claim 1 or 2 wherein the buffer in (c) is 50 mM tris-HCl and 1.0 mM 2-mercaptoethanol.
4. The method of claim 1, 2 or 3 wherein the buffer in (d) is 6.0 M guanidine hydrochloride and 10 mM tris.
5. The method of any one of claims 1 to 4 wherein (b) and (e) are carried out using sterile water.
6. A method of preparing thymosin α_1 having the amino acid sequence defined in claim 1 as hereinbefore described.
7. A method for reconstituting immune functions in thymic deprived or immunodeprived warm blooded non-human mammals which method comprises administering to said mammals an immunopotentiating effective amount of thymosin α_1 having the amino acid sequence defined in claim 1.
8. The method of claim 7 wherein a daily dosage in the range of from 1 to 100 μ g/kg of body weight per day is administered intravenously.
9. Thymosin α_1 whenever prepared according to a method claimed in any one of claims 1 to 6.
10. Thymosin α_1 having the amino acid sequence
(N-acetyl)-Ser-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-OH
and pharmaceutically acceptable acid addition salts and base salts thereof, essentially free of other thymic polypeptides.

For the Applicants,
CARPMAELS & RANSFORD,
Chartered Patent Agents,
43, Bloomsbury Square,
London, WC1A 2RA.