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Poster Presentation

Rapid Isolation of Thymosin B4 from Human Thymus by Reversed Phase HPLC

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ABSTRACT

THYMOSIN BETA 4 (TB_4) IS A PEPTIDE COMPOSED OF 43 AMINO ACID RESIDUES WITH A MOLECULAR WEIGHT OF 4982 AND A pI OF 5.1. IT WAS ORIGINALLY ISOLATED FROM CALF THYMUS [LOW, ET AL PNAS 78, (1981)].

WE HAVE DEVELOPED A RAPID, EFFICIENT AND REPRODUCIBLE METHOD FOR THE PURIFICATION OF TB_4 FROM HUMAN THYMUS. A NEWLY DEVELOPED METHOD WAS EMPLOYED FOR THE EXTRACTION OF TB_4. THIS EXTRACTION IS SIMPLE AND MINIMIZES THE POSSIBILITY OF PROTEOLYTIC MODIFICATION.

THIS PURIFICATION IS BASED ON THE USE OF HIGH PERFORMANCE REVERSED PHASE (DELTA PAK C_{18}) CHROMATOGRAPHIC COLUMNS. THE HPLC RETENTION TIME, RIA, AND SDS-PAGE HAVE SHOWN THAT PURIFIED TB_4 FROM HUMAN THYMUS IS HOMOGENEOUS AND IS IDENTICAL TO SYNTHETIC TB_4.

SAMPLE PREPARATION FOR HPLC

HUMAN THYMUS (MALE - 16 MONTHS) WAS IMMEDIATELY FROZEN AND STORED AT -90 °C. 2.5 g OF TISSUE WAS HOMOGENIZED IN 7.5 ml OF ICE-COLD 0.5 N PERCHLORIC ACID WITH A TEFLON PESTLE AT 840 rpms WITH 100 UP- AND -DOWN STROKES. THE HOMOGENATE WAS CENTRIFUGED AT 1000g FOR 10 MINUTES. THE pH OF THE SUPERNATANT WAS ADJUSTED TO 7 WITH 1 M POTASSIUM HYDROXIDE ADDED OVER A PERIOD OF 30 MINUTES. THE SOLUTION WAS STIRRED FOR 60 MINUTES AT 4 °C AND CENTRIFUGED AT 20,000g FOR 30 MINUTES. THE SUPERNATANT WAS ALIQUOTED AND STORED AT -20 °C.

THE CONCENTRATION OF PROTEIN IN THE SUPERNATANT WAS ESTIMATED TO BE 0.8 mg/ml BY THE METHOD OF LOWRY (et al-1951) USING BOVINE SERUM ALBUMIN AS THE STANDARD.

CHROMATOGRAPHIC CONDITIONS

STEP 1

COLUMN: 300 X 7.8 mm, DELTA PAK C_{18}, 15 MICRON, 300 ANGSTROM

ELUENT A: 0.02 M AMMONIUM ACETATE, pH 6.8

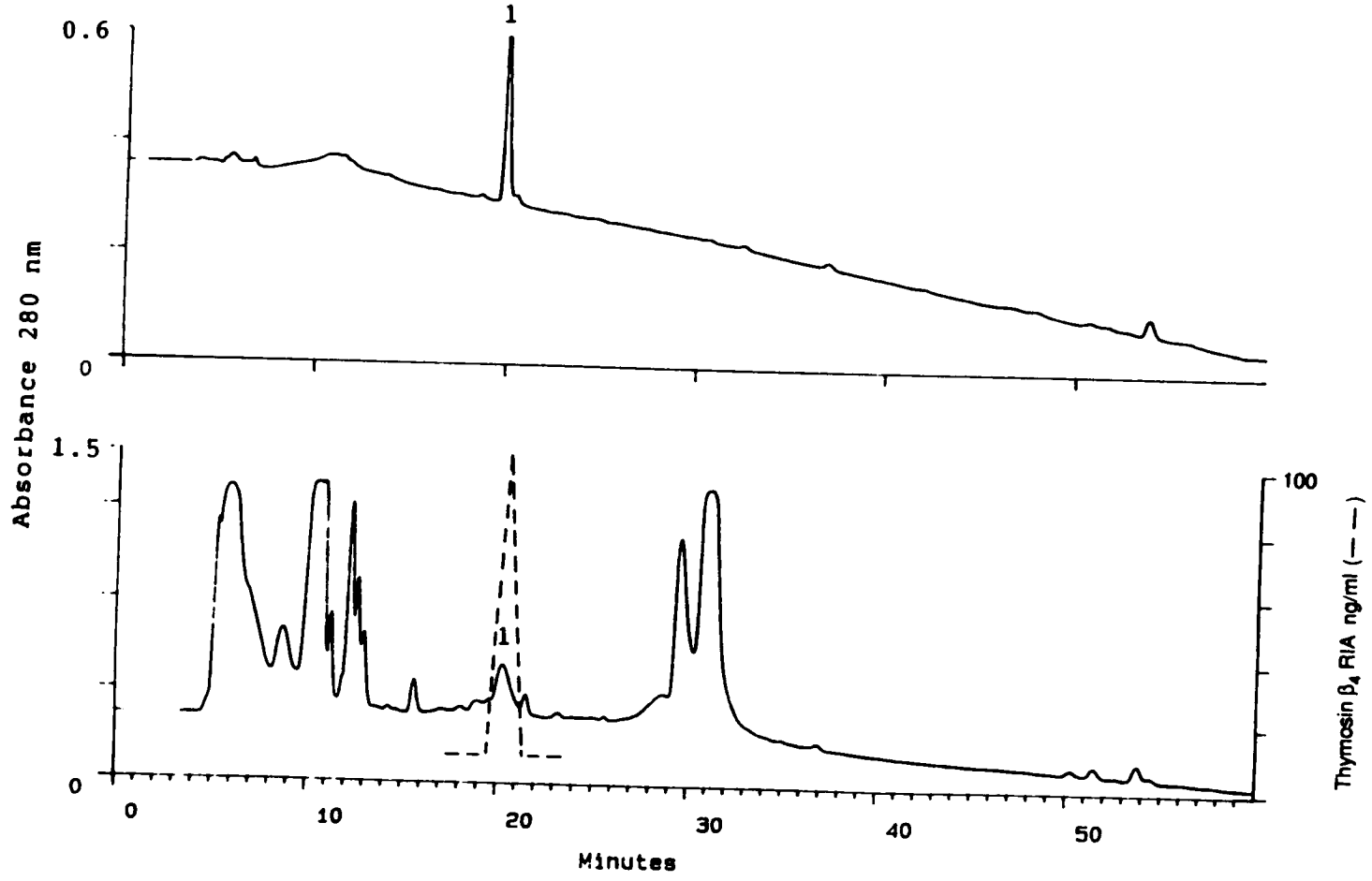
ELUENT B: ACETONITRILE

GRADIENT: 0 - 80% B, 60 MINUTES, LINEAR

FLOW RATE: 2 ML/MIN

DETECTION: 214 NM

RP-HPLC SEPARATION
STEP 1



RP-HPLC SEPARATION OF 20 ug OF SYNTHETIC THYMOSIN BETA 4 (1), TOP PANEL AND 1.8 ml OF HUMAN THYMUS EXTRACT (1.44 mg OF PROTEIN AS DETERMINED BY A LOWRY ASSAY), BOTTOM PANEL. COLLECTED FRACTIONS OF THE THYMUS EXTRACT SEPARATION WERE ASSAYED FOR THYMOSIN BETA 4 USING SOLID PHASE RIA. RESULTS ARE OVERLAID ON THE CHROMATOGRAM.

CHROMATOGRAPHIC CONDITIONS

STEP 2

COLUMN: 150 X 3.9 mm, DELTA PAK C_{18}, 5 MICRON, 300 ANGSTROM

ELUENT A: 0.1% PHOSPHORIC ACID IN WATER

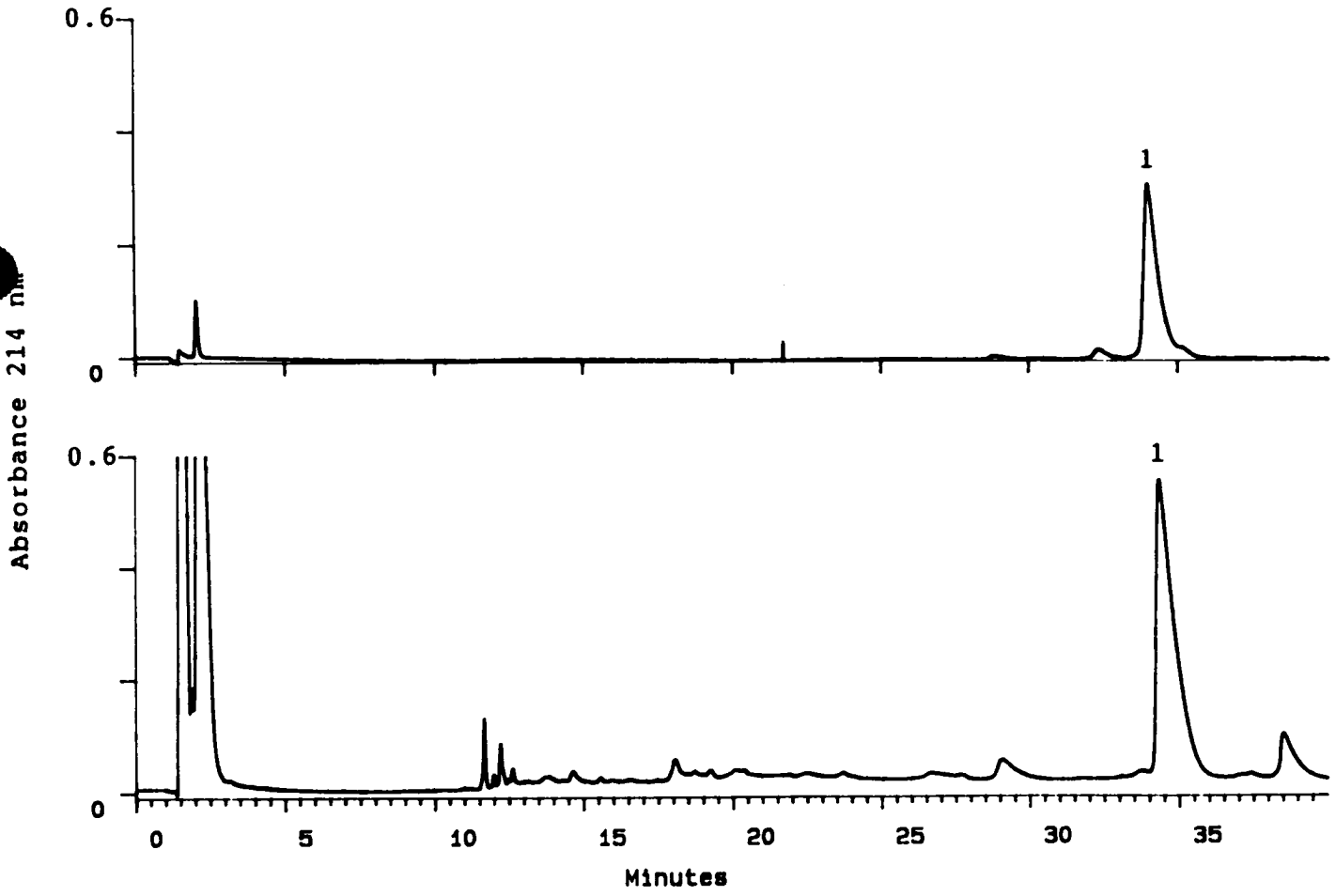
ELUENT B: 0.1% PHOSPHORIC ACID IN ACETONITRILE

GRADIENT: 0 - 14% B, 10 MINUTES, LINEAR, HOLD AT 14% 10 MINUTES,
14 - 18% B, 10 MINUTES, LINEAR, HOLD AT 18% 10 MINUTES

FLOW RATE: 1 ML/MIN

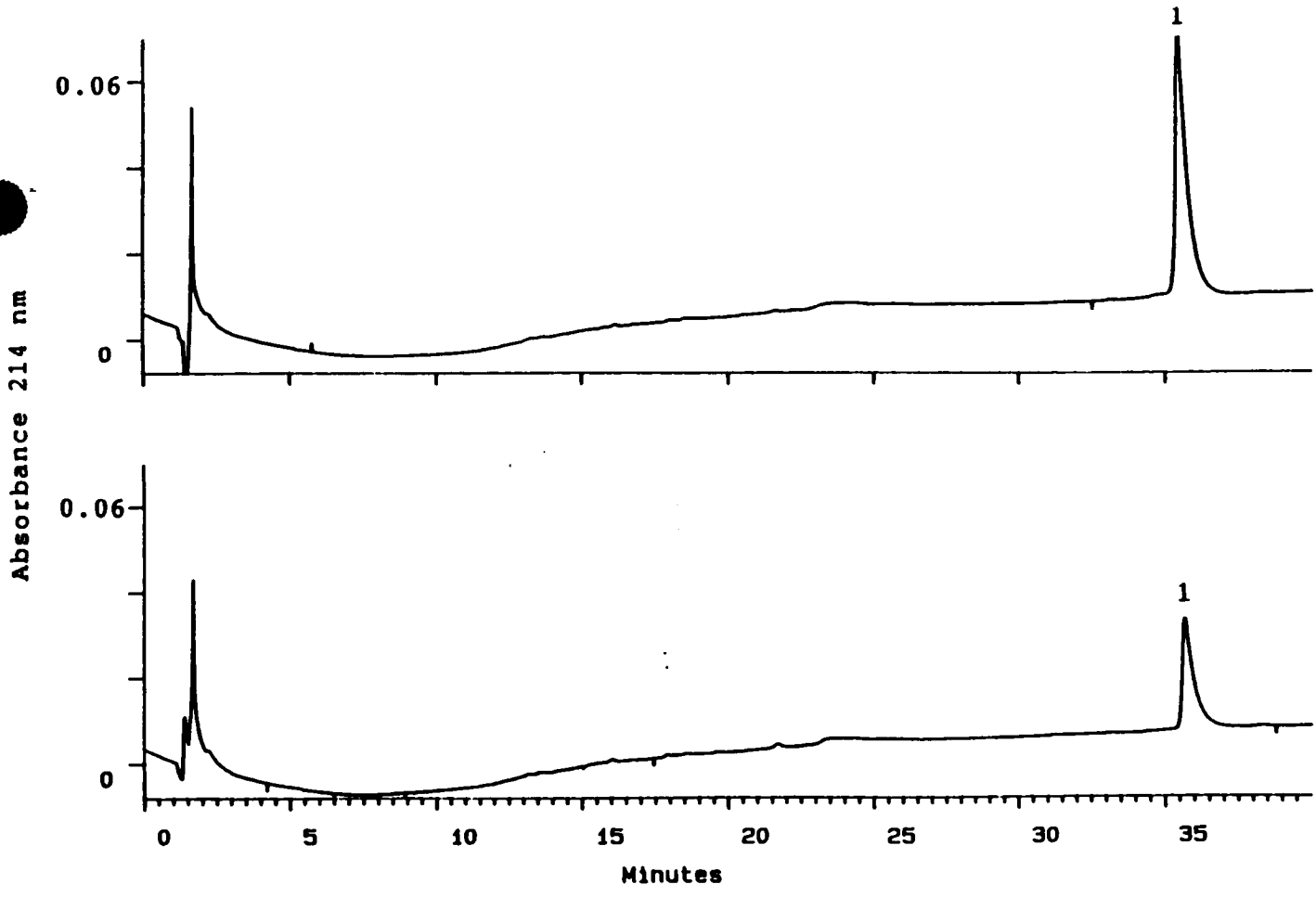
DETECTION: 214 NM

RP-HPLC SEPARATION
STEP 2



RP-HPLC SEPARATION OF 20 ug OF SYNTHETIC THYMOSIN BETA 4 (1), TOP PANEL AND 44 ug OF THYMOSIN BETA 4 FROM THE POOLED FRACTIONS 20 AND 21 FROM THE SEPARATION OF HUMAN THYMUS EXTRACT. THE POOLED FRACTIONS (4 ML) WERE CONCENTRATED TO 300 ul. THE COLLECTED FRACTIONS WERE ASSAYED FOR THYMOSIN BETA 4 USING SOLID PHASE RIA.

RP-HPLC ANALYSES



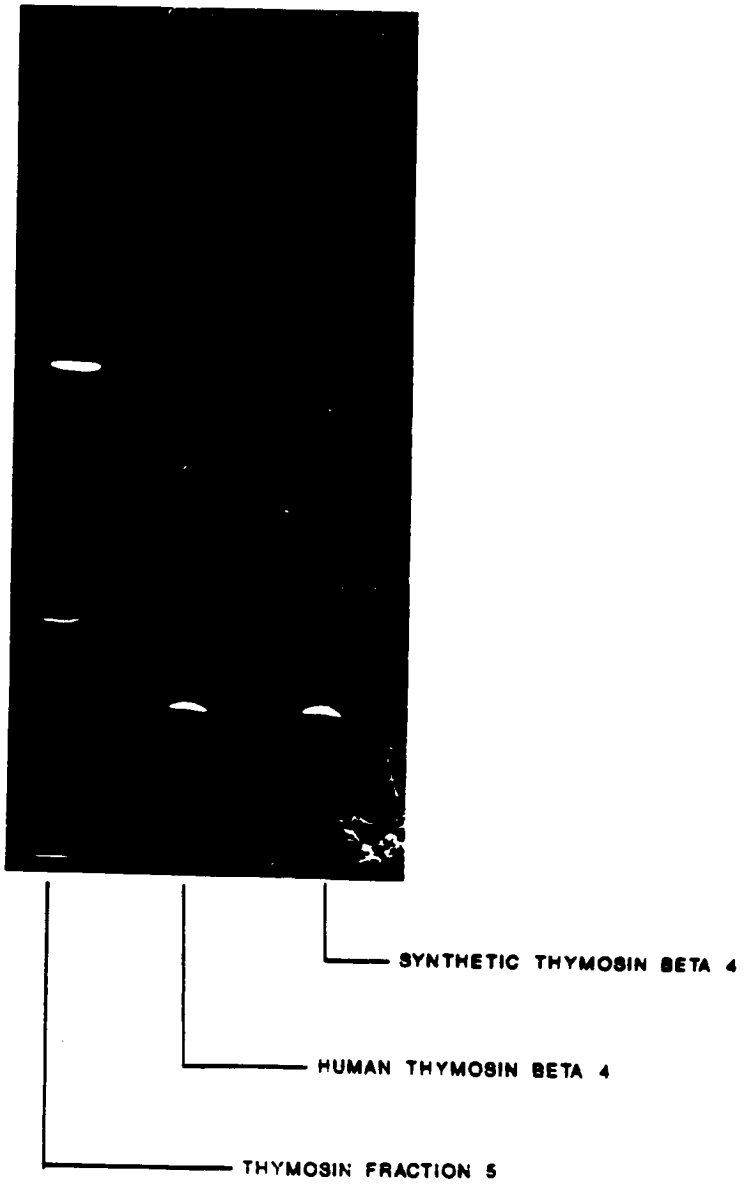
ANALYTICAL RP-HPLC OF 100 ul ALIQUOTS OF FRACTIONS 35 AND 36. 100 ul OF WATER WAS ADDED TO EACH SAMPLE BEFORE ANALYSIS. STEP 2 CHROMATOGRAPHIC CONDITIONS WERE EMPLOYED. BETA 4 (1).

AMINO ACID COMPOSITION* OF NATURAL AND SYNTHETIC THYMOSIN BETA $_4$

AMINO ACID	SYNTHETIC TB_4	NATURAL TB_4	REPORTED SEQUENCE**
ASP	3.9	4.2	4
GLU	10.8	11.1	11
SER	3.5	3.9	4
GLY	1.1	1.4	1
THR	2.8	2.9	3
ALA	2.2	2.4	2
PRO	2.9	3.1	3
MET	0.8	1.2	1
ILE	1.6	2.3	2
LEU	1.6	2.2	2
PHE	0.9	0.7	1
LYS	8.5	9.4	9

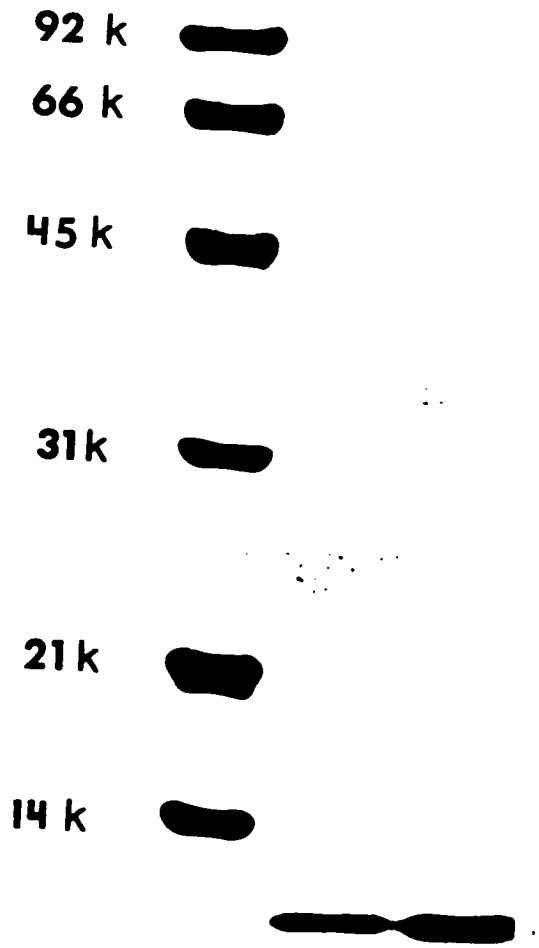
AMINO ACID ANALYSIS WAS PERFORMED WITH A PICO-TAG AMINO ACID ANALYSIS SYSTEM. ABOUT 1-5 ug SAMPLES WERE HYDROLYZED WITH 6 N HCl, CONTAINING 1% PHENOL BY VOLUME AT 110 °C FOR 48 HOURS. THE HYDROLYSATES WERE DRIED AND ANALYZED USING THE STANDARD PICO-TAG PROTOCOL (9).

IEF OF SYNTHETIC AND HUMAN THYMOSIN BETA 4



ISOELECTRIC FOCUSING GEL OF NATURAL AND SYNTHETIC THYMOSIN BETA 4. ABOUT 30-50 ug OF THE SAMPLES WERE RUN ON PHARMACIA/ LKB PAG PLATES (3.5 - 9.5). THE PROTEIN WAS PRECIPITATED WITH 20% TRICHLOROACETIC ACID (TCA) AND 3.5% SULFOSALICYLIC ACID FOR ONE HOUR. THE GELS WERE STAINED WITH 0.1% COOMASSIE BLUE R-250 IN 20% TCA

SDS-PAGE OF SYNTHETIC AND HUMAN THYMOSIN BETA 4



SDS-PAGE OF NATURAL AND SYNTHETIC THYMOSIN BETA 4. ABOUT 1-20 ug OF THE SAMPLES WERE RUN ON A 1.5 mm 12% SDS-POLYACRYLAMIDE GEL ACCORDING TO THE METHOD OF LAEMMELI (8) AND STAINED WITH COOMASSIE BLUE R-250. LANE 1:.PROTEIN STANDARDS, LANE 2 AND 3: SYNTHETIC AND NATURAL THYMOSIN BETA 4, RESPECTIVELY.

CONCLUSION

1. WE WERE ABLE TO PURIFY THYMOSIN BETA 4 FROM HUMAN THYMUS USING A 2-STEP RP-HPLC PROCEDURE. THE PRESENCE OF THYMOSIN BETA 4 WAS FOLLOWED BY RIA AND HPLC RETENTION TIME.

2. SDS-PAGE AND IEF ANALYSIS OF THE HPLC PURIFIED THYMOSIN BETA 4, AFTER STAINING WITH COOMASSIE BLUE, REVEALED SINGLE PEPTIDE BANDS WITH MOLECULAR WEIGHTS BELOW 10 Kd AND pIs OF 5.1.

3. THE HPLC ISOLATED THYMOSIN BETA 4 WAS HOMOGENEOUS AND HAD A SIMILAR HPLC RETENTION TIME AND AMINO ACID COMPOSITION AS THE SYNTHETIC BETA 4.

4. A 98% RECOVERY OF THYMOSIN BETA 4 FROM THE 2-STEP RP-HPLC SEPARATION WAS DETERMINED BY COMPARISON OF PEAK AREAS WITH A THYMOSIN BETA 4 STANDARD. THE AMOUNT OF PROTEIN IN THYMUS EXTRACT WAS DETERMINED BY LOWRY ASSAY. THE AMOUNT OF THYMOSIN BETA 4 ISOLATED IN THIS PROCEDURE WAS DETERMINED BY COMPARISON OF PEAK AREAS WITH THYMOSIN BETA 4 STANDARD. FROM THIS WE ESTIMATE THAT HUMAN THYMUS CONTAINS 3% BETA 4.

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