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Poster Presentation

Rapid Isolation of Thymosin Alpha-1 from Thymosin Fraction 5 by Preparative Reverse Phase HPLC

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ABSTRACT

THYMOSIN ALPHA-1 (Ta_1) IS ONE OF THE BIOLOGICALLY ACTIVE PEPTIDES IN THYMOSIN FRACTION 5 (TF5), A PARTIALLY PURIFIED THYMIC PREPARATION FROM CALF THYMUS. Ta_1 HAS HORMONE-LIKE PROPERTIES AND CAN MODULATE IMMUNE AND NEUROENDOCRINE RESPONSES.

WE HAVE DEVELOPED A RAPID AND REPRODUCIBLE METHOD FOR THE PURIFICATION OF Ta_1 FROM TF5. THE PURIFICATION PROCEDURE IS BASED ON THE USE OF HIGH-PERFORMANCE/SEMI-PREPARATIVE AND ANALYTICAL REVERSED-PHASE (C_{18} DELTA-PAK) CHROMATOGRAPHIC COLUMNS.

THE HPLC RETENTION TIME, RIA, SDS-PAGE, AND AMINO ACID COMPOSITION ANALYSIS HAVE SHOWN THAT NATURAL, PURIFIED Ta_1 IS IDENTICAL TO SYNTHETIC a_1.

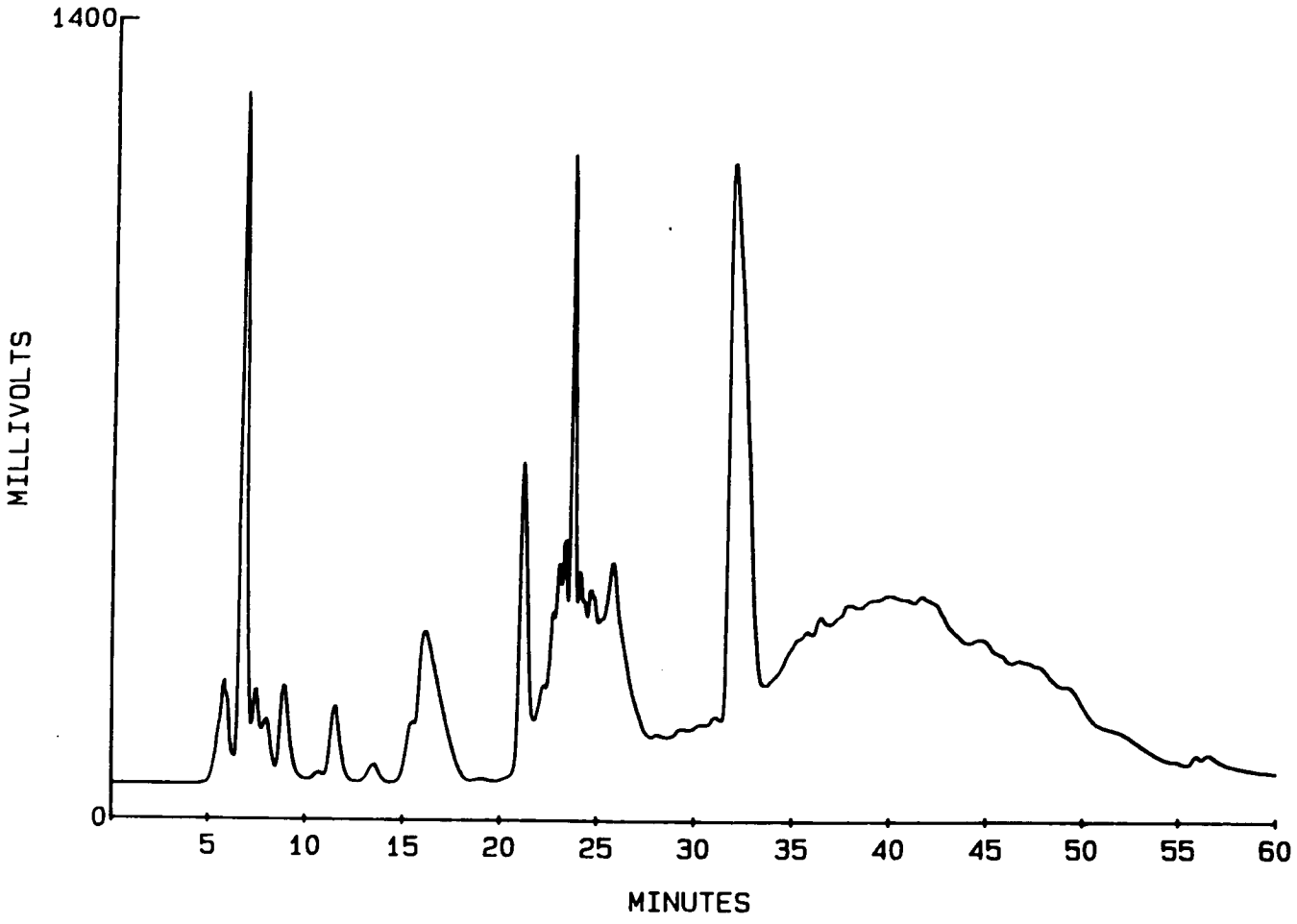
INTRODUCTION

A THYMIC PREPARATION TERMED THYMOSIN FRACTION 5 (TF5) HAS BEEN SHOWN TO BE A POTENT IMMUNO-POTENTIATING AGENT. TF5 CONSISTS OF A FAMILY OF BIOLOGICALLY ACTIVE POLYPEPTIDE COMPONENTS WITH HORMONE-LIKE ACTIVITIES. THYMOSIN A_1 (TA_1) WAS THE FIRST BIOLOGICALLY ACTIVE POLYPEPTIDE TO BE PURIFIED FROM TF5 AND COMPLETELY CHARACTERIZED. IT IS AN ACIDIC PEPTIDE WITH AN ISOELECTRIC POINT OF 4.2, AND A MOLECULAR WEIGHT OF 3108. THIS PEPTIDE IS HIGHLY ACTIVE IN AMPLIFYING T-CELL IMMUNITY AND IS ACTIVE IN MODULATING THE EXPRESSION OF TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE (TdT). INTERCEREBRAL INJECTIONS OF TA_1 IN MICE STIMULATES CORTICOSTERONE PRODUCTION. TA_1 WAS ISOLATED AND PURIFIED FROM TF5 BY ION-EXCHANGE CHROMATOGRAPHY ON CM-CELLULOSE AND DEAE-CELLULOSE, AS WELL AS BY GEL FILTRATION CHROMATOGRAPHY ON SEPHADEX G-75. TA_1 HAS ALSO BEEN ISOLATED BY HPLC TECHNIQUES, USING A u-BONDAPAK C_{18} COLUMN. THE YIELD OF TA_1 FROM TF5 IS ABOUT 0.6%. LOW RECOVERIES HAVE ALWAYS BEEN A PROBLEM IN THE ISOLATION OF TF5 PEPTIDES AND HAVE LIMITED THE AMOUNT OF PURIFIED PEPTIDE AVAILABLE FOR FURTHER CHARACTERIZATION. IN THIS PAPER, WE REPORT A VERY FAST, REPRODUCIBLE, AND EASY, LARGE-SCALE ISOLATION PROCEDURE AS WELL AS AN ANALYTICAL REVERSED-PHASE HPLC (RP-HPLC) PROCEDURE FOR THE PURIFICATION OF THE TA_1 AND OTHER PEPTIDES FROM TF5.

FIGURE 1. REVERSED PHASE HPLC SEPARATION OF 900 UG OF THYMOSIN FRACTION 5 (TF5) ON A 3.9 MM X 30 CM DELTA PAK, 300 A, 15 u, C18 COLUMN. BUFFER A WAS 0.02 M AMMONIUM ACETATE, pH 6.8, AND BUFFER B ACETONITRILE. A 60 MINUTE LINEAR GRADIENT FROM 0-80% B WAS RUN AT A FLOW RATE OF 0.5 ML/MIN. DETECTION WAS AT 280 NM, 1.4 AUFS.

RP-HPLC OF THYMOSIN FRACTION 5

ANALYTICAL SEPARATION



RP-HPLC OF THYMOSIN FRACTION 5

PREPARATIVE SEPARATION

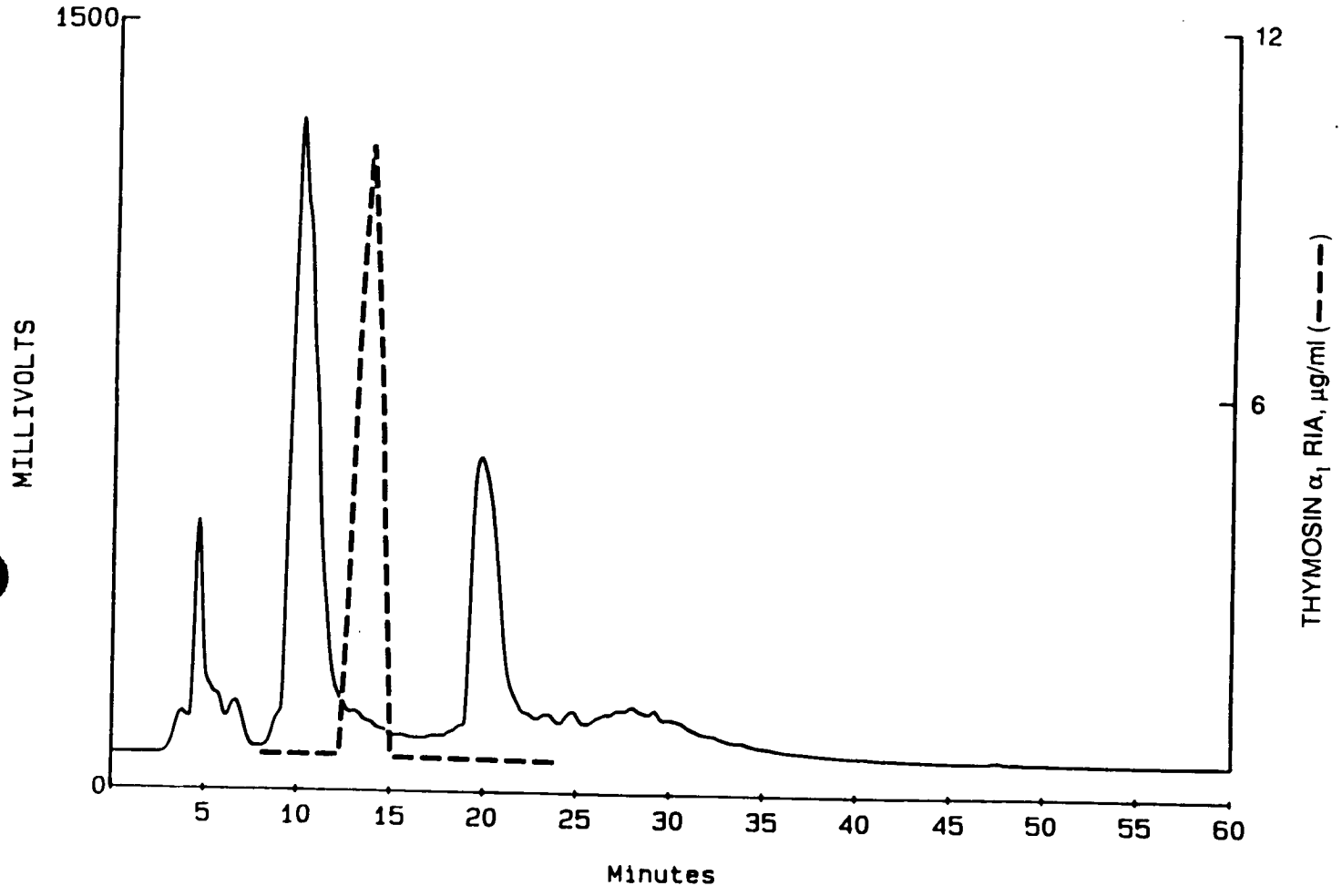


FIGURE 2. REVERSED PHASE HPLC SEPARATION OF 1.5 G OF THYMOSIN FRACTION 5 (TF5) ON A 50 MM X 30 CM DELTA PAK, 300 A, 15 u, C18 COLUMN. BUFFER A WAS 0.02 M AMMONIUM ACETATE, pH 6.8, AND BUFFER B ACETONITRILE. A 60 MINUTE LINEAR GRADIENT FROM 0-80% B WAS RUN AT A FLOW RATE OF 80 ML/MIN. DETECTION WAS AT 280 NM AT 1.5 AUFS. COLLECTED FRACTIONS WERE ASSAYED FOR THYMOSIN ALPHA 1 USING SOLID-PHASE RIA. RESULTS ARE OVERLAID ON THE CHROMATOGRAM.

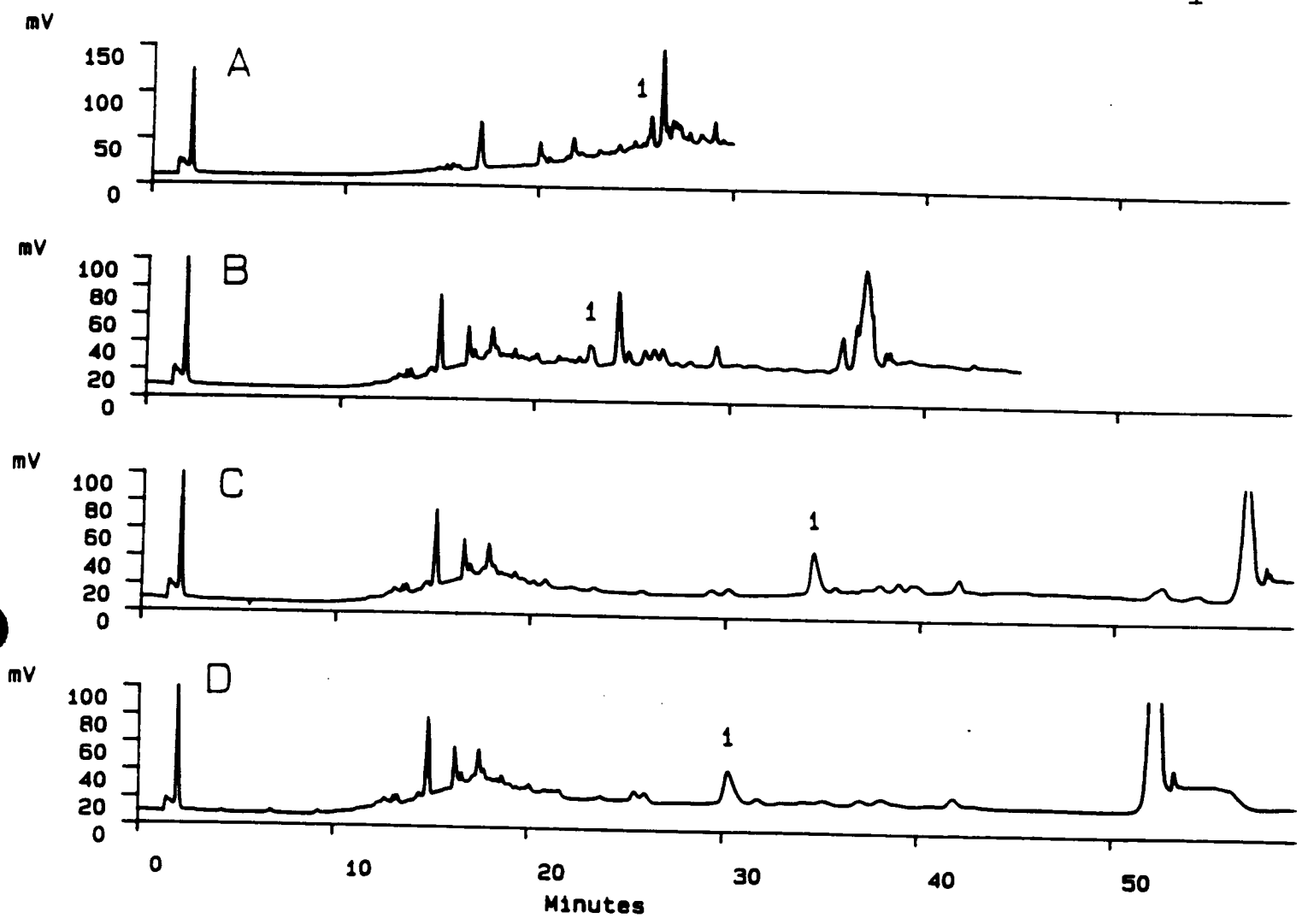


FIGURE 3. OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS FOR THE ISOLATION OF THYMOSIN ALPHA 1 FROM FRACTION 14 OF THE 1.5 G SEPARATION OF THYMOSIN FRACTION 5 ON A 3.9 MM X 15 CM DELTA PAK C18, 300 A, 5 u COLUMN. ELUENT A WAS 0.1% PHOSPHORIC ACID IN WATER AND ELUENT B ACETONITRILE WITH 0.1% PHOSPHORIC ACID. THE GRADIENT CONDITIONS FOR EACH SEPARATION WERE THE FOLLOWING: PANEL A, 0-30% B, 30 MIN. PANEL B, 0-15% B, 10 MIN, 15-23% B 20 MIN, HOLD 10 MIN, 50% B AT 40.1 MIN. PANEL C, 0-15% B, 10 MIN, HOLD 10 MIN, 15-20% B, 20 MIN, HOLD 10 MIN, 50% B AT 50.1 MIN. PANEL D, 0-15% B, 10 MIN, HOLD 5 MIN, 15-17% B, 20 MIN, HOLD 10MIN, 50% B AT 45.1 MIN. ALL THE SEPARATIONS WERE RUN AT A FLOW RATE OF 1 ML/MIN. THYMOSIN ALPHA 1 (1) WAS DETECTED AT 214 NM, PANEL A AT A FULL SCALE ABSORBANCE OF 0.15 AND PANELS B, C, D AT 0.1 AUFS.

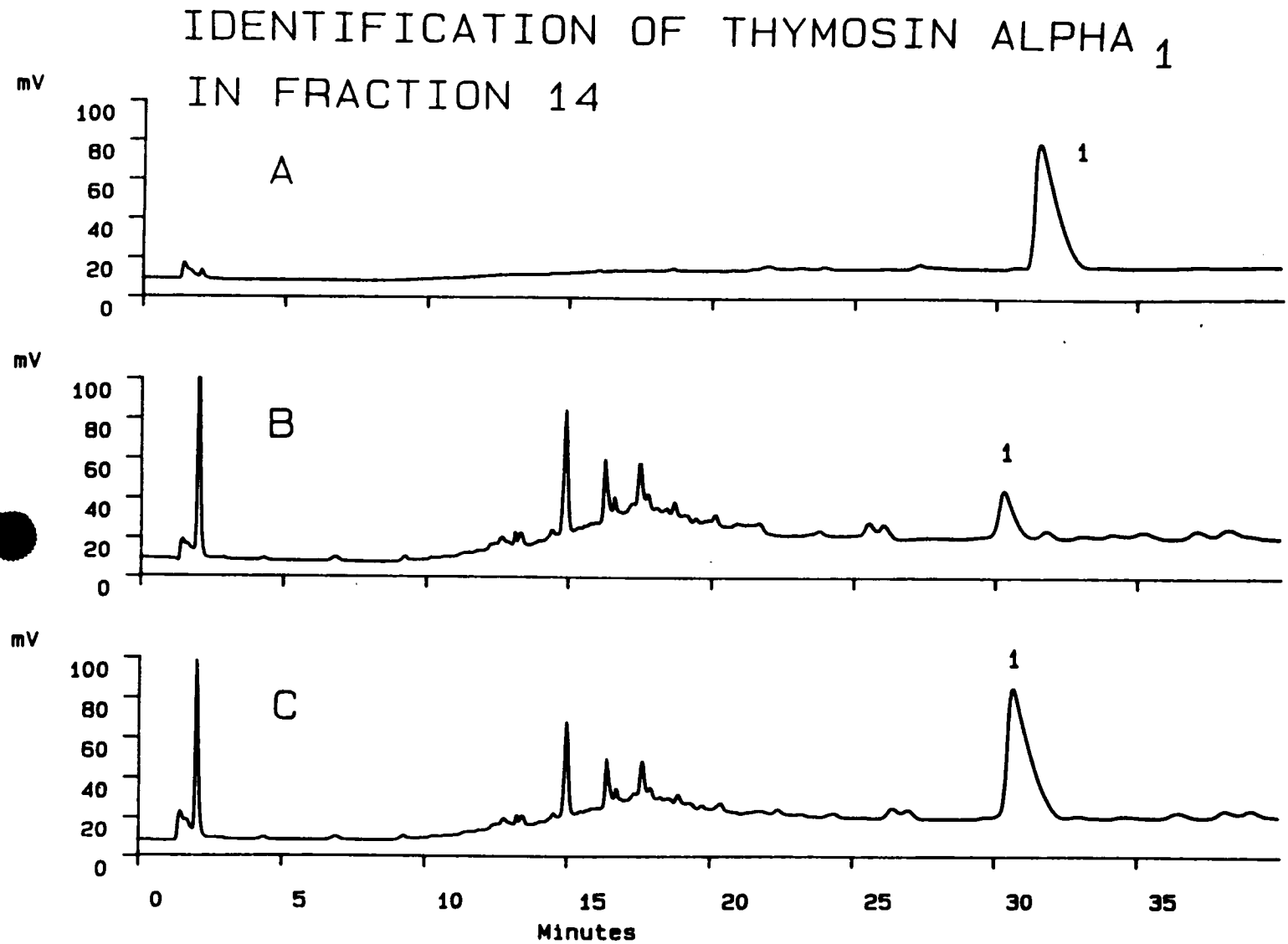


FIGURE 4. IDENTIFICATION OF THYMOSIN ALPHA 1 (1) IN FRACTION 14 FROM THE 1.5 G SEPARATION OF THYMOSIN FRACTION 5 BY COMPARISON WITH SYNTHETIC ALPHA 1 (1) BY REVERSED PHASE HPLC. CHROMATOGRAPHIC CONDITIONS ARE DESCRIBED IN FIGURE 3, PANEL D. PANEL A SHOWS THE SEPARATION OF 11 UG OF SYNTHETIC ALPHA 1. PANEL B SHOWS 80 UG OF FRACTION 14 AND PANEL C 80 UG OF FRACTION 14 SPIKED WITH 9 UG OF SYNTHETIC ALPHA 1. DETECTION WAS AT 214 NM, 0.1 AUFS.

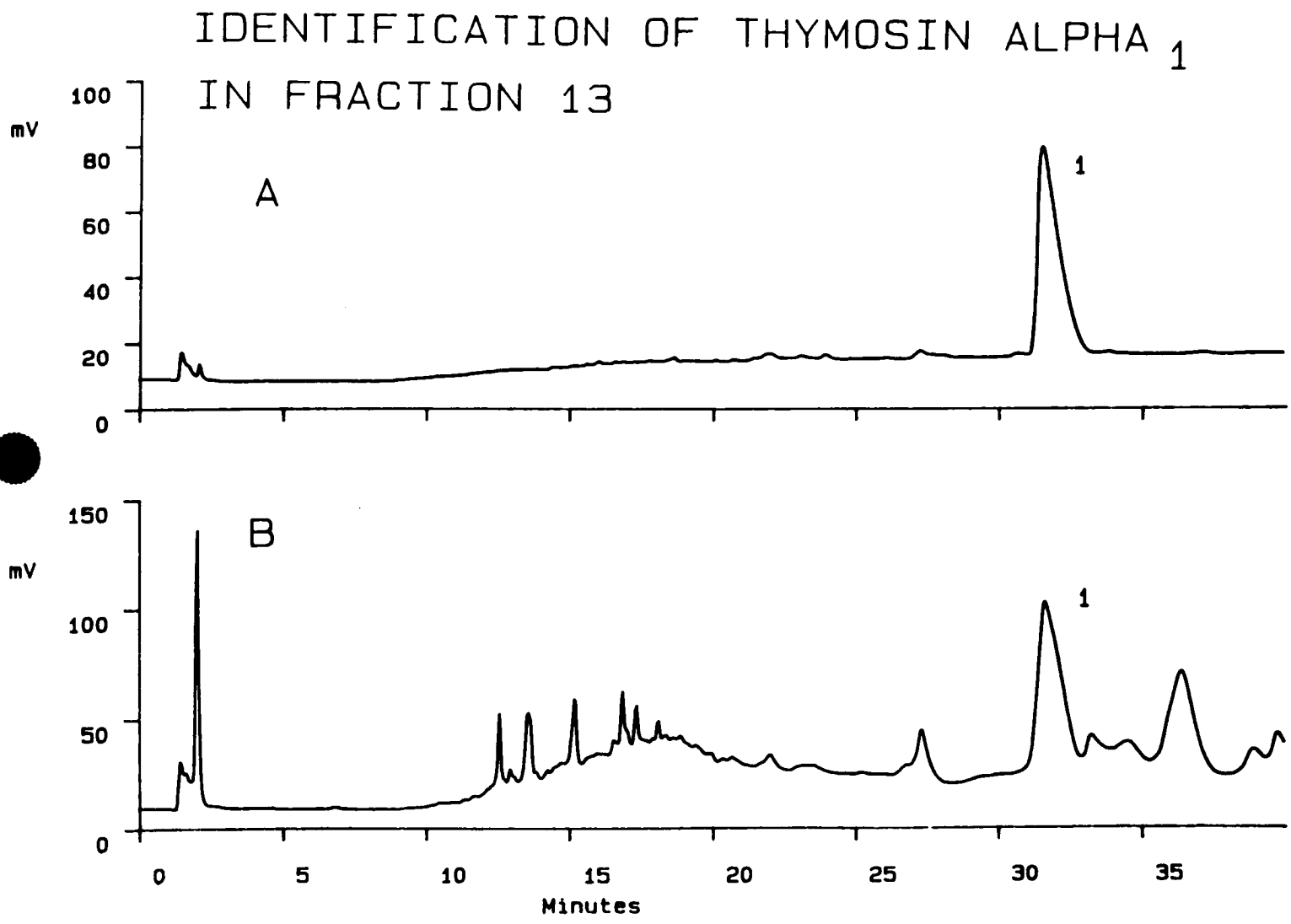


FIGURE 5. IDENTIFICATION OF THYMOSIN ALPHA 1 (1) IN FRACTION 13 FROM THE 1.5 G SEPARATION OF THYMOSIN FRACTION 5 BY COMPARISON WITH SYNTHETIC ALPHA 1 BY REVERSED PHASE HPLC. CHROMATOGRAPHIC CONDITIONS ARE DESCRIBED IN FIGURE 3, PANEL D. PANEL A SHOWS 11 UG OF SYNTHETIC ALPHA 1 AND PANEL B 108 UG OF FRACTION 13. DETECTION WAS AT 214 NM, 0.1 AUFS (PANEL A) AND 0.15 AUFS (PANEL B).

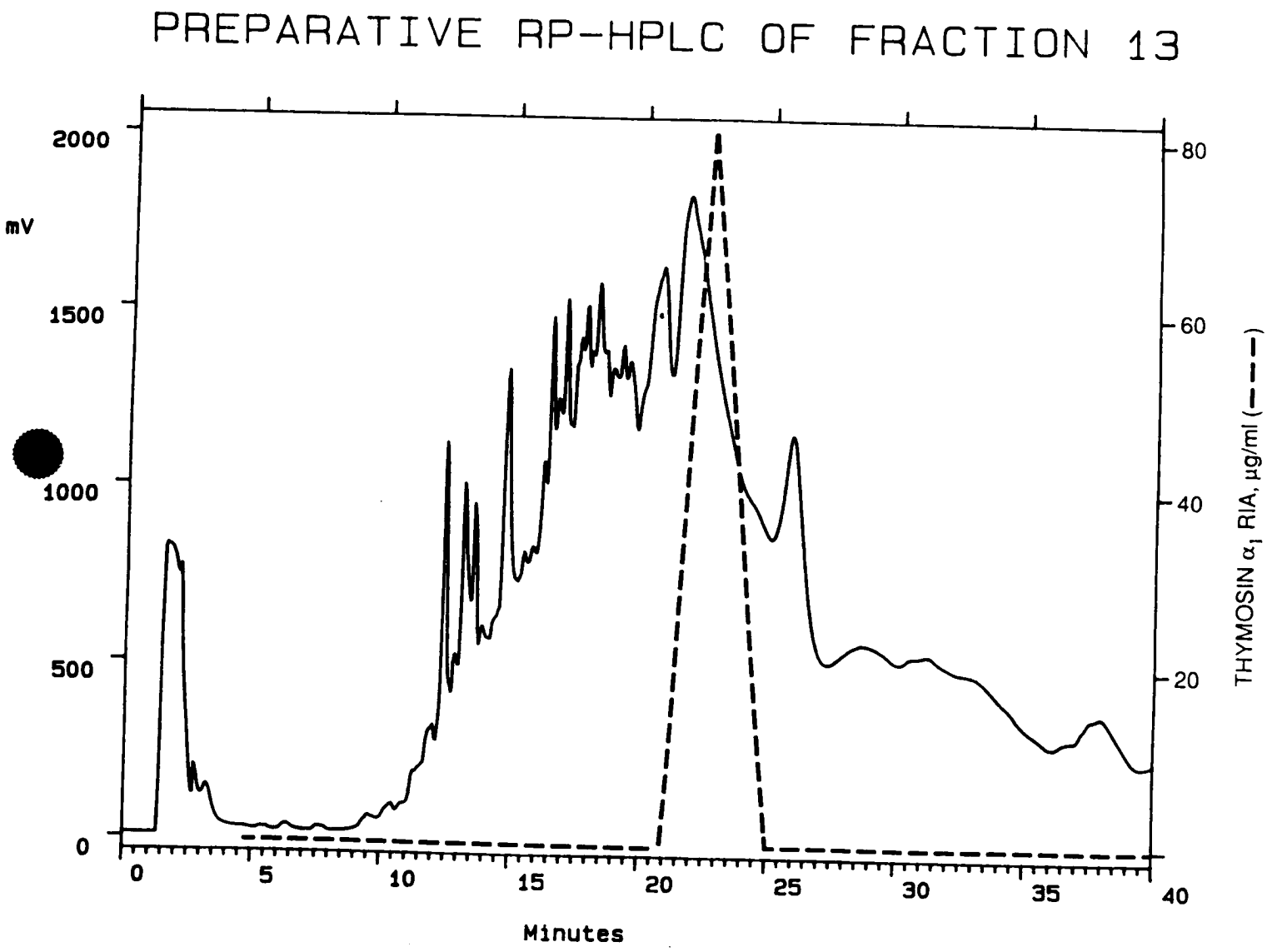


FIGURE 6. REVERSED PHASE HPLC SEPARATION OF 4.3 MG OF FRACTION 13 FROM THE 1.5 G SEPARATION OF THYMOSIN FRACTION 5. CHROMATOGRAPHIC CONDITIONS WERE DESCRIBED IN FIGURE 3, PANEL D. DETECTION WAS AT 214 NM, 2.0 AUFS. COLLECTED FRACTIONS WERE ASSAYED FOR THYMOSIN ALPHA 1 USING SOLID PHASE RIA. RESULTS ARE OVERLAID ON THE CHROMATOGRAM

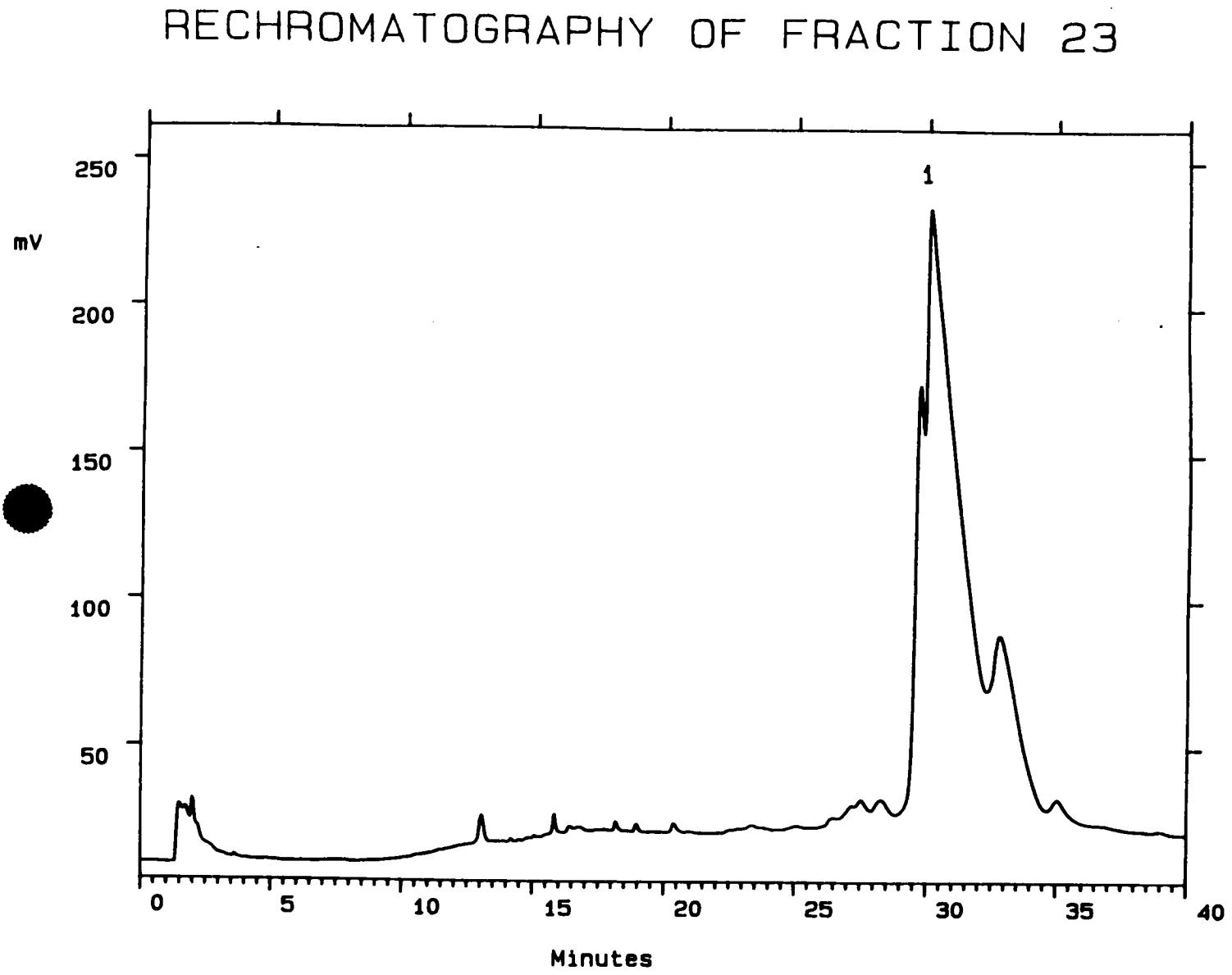


FIGURE 7. RECHROMATOGRAPHY OF 300 UL OF FRACTION 23 FROM THE PREPARATIVE SEPARATION OF FRACTION 13. THE SAMPLE WAS DILUTED WITH 300 UL OF WATER AND INJECTED ON TO THE 3.9 MM X 15 CM DELTA PAK C18 COLUMN. THE CHROMATOGRAPHIC CONDITIONS ARE DESCRIBED IN FIGURE 3, PANEL D. ONE HALF MINUTE FRACTIONS WERE COLLECTED ACROSS THE MAJOR PEAK OF THYMOSIN ALPHA 1 (1). DETECTION WAS AT 214 NM, 0.25 AUFS.

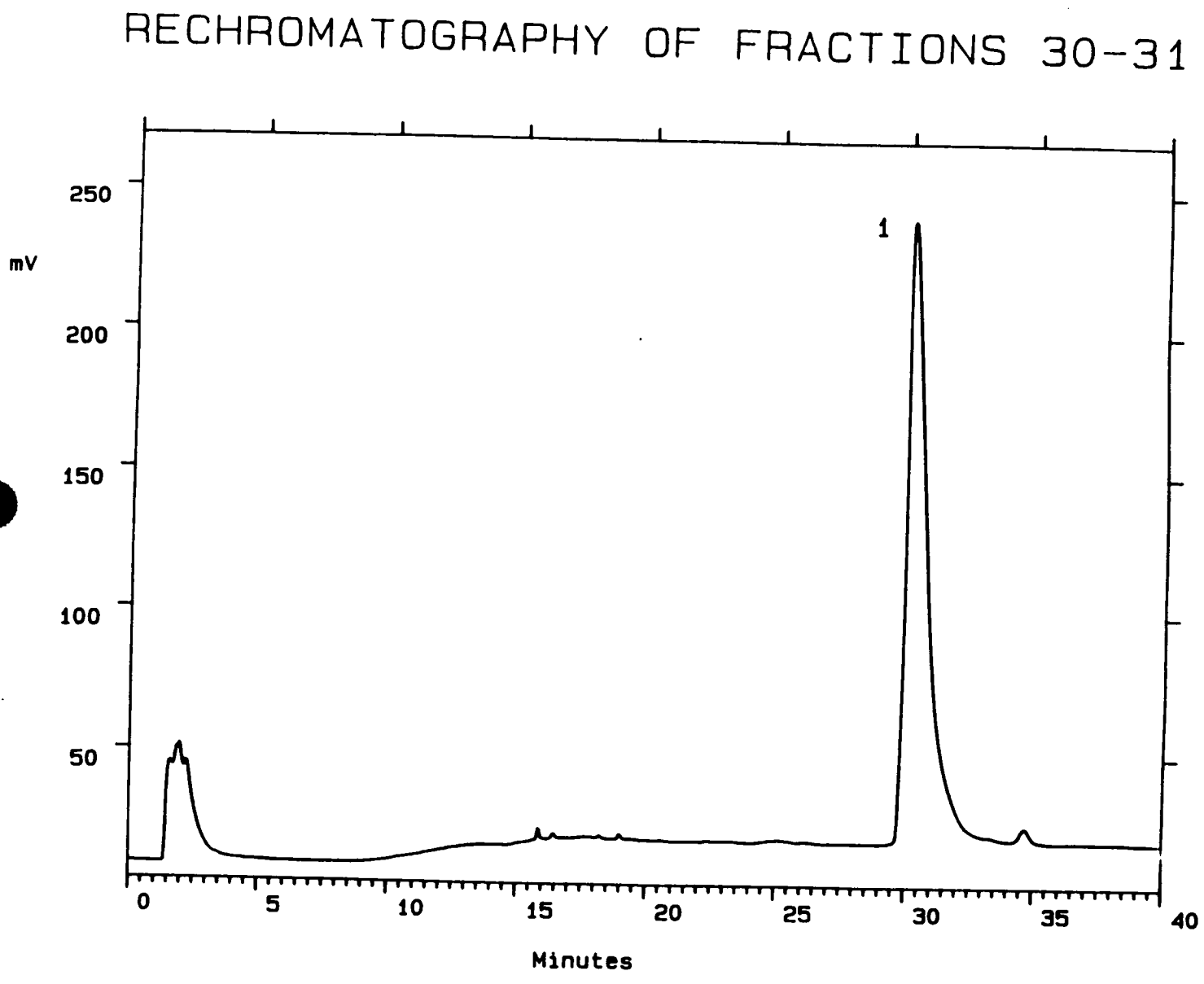


FIGURE 8. RECHROMATOGRAPHY OF FRACTIONS 30-31.5 FROM THE SEPARATION OF FRACTION 23. THE POOLED SAMPLES WERE CONCENTRATED TO 800 UL TO REMOVE THE ACETONITRILE. CHROMATOGRAPHIC CONDITIONS WERE AS DESCRIBED IN FIGURE 3, PANEL D. THE THYMOSIN ALPHA 1 (1) WAS DETECTED AT 214 MN, 0.25 AUFS.

Recovery of Ta_1 from F13

Fraction	Amount recovered in ug
22	96
23	200
24	125
25	78
26	41
Total	540

TABLE 1: RECOVERY OF Ta_1 FROM F13. F13, 4.3 MG FROM THE PREPARATIVE SEPARATION (FIG. 1) WAS FURTHER FRACTIONATED USING THE CONDITIONS DESCRIBED IN FIG. 2, PANEL D. ALL THE FRACTIONS 22-26 CONTAINING IMMUNOREACTIVE Ta_1 WERE RECHROMATOGRAPHED, AND THE AMOUNT OF Ta_1 WAS DETERMINED BY COMPARISON OF PEAK AREA WITH SYNTHETIC a_1 .

Amino Acid Composition[a] of Natural and Synthetic Thymosin a_1

Amino Acid	Synthetic a_1	Natural Ta_1	From Reported Sequence[b]
Asp	3.6	4.15	4
Glu	5.9	6.4	6
Ser	2.7	3.1	3
Thr	2.6	2.5	3
Ala	2.9	3.4	3
Val	2.6	3.0	3
Ile	1.0	1.0	1
Leu	1.3	1.1	1
Lys	4.0	4.4	4

a. The data are presented as assumed numbers of residues per molecule.

b. Number of residues obtained from the reported sequence

TABLE 2: AMINO ACID ANALYSIS WAS PERFORMED WITH A PICO-TAG AMINO ACID ANALYSIS SYSTEM. ABOUT 10 UG SAMPLES OF SYNTHETIC AND NATURAL a_1 WERE HYDROLYZED WITH 6N HCL. CONTAINING 1% PHENOL BY VOLUME AT 110°C FOR 48 HOURS. THE HYDROLYSATES WERE DRIED AND USED FOR AMINO ACID ANALYSIS BY THE PICO-TAG STANDARD PROCEDURE.

CONCLUSION

1. WE WERE ABLE TO PURIFY Ta_1 FROM TF5 IN A TWO STEP RP-HPLC PROCEDURE. THE PRESENCE OF Ta_1 WAS FOLLOWED BY RIA AND HPLC RETENTION TIME OF THE SYNTHETIC a_1.

2. SDS-PAGE ANALYSIS OF THE NATURAL AND HPLC PURIFIED SYNTHETIC a_1, AFTER STAINING WITH COOMASSIE BLUE R-250, REVEALED A SINGLE PEPTIDE BAND WITH A MOLECULAR WEIGHT BELOW 10Kd.

3. THE FINAL PREPARATION OF Ta_1 IS HOMOGENOUS, AND HAS A SIMILAR HPLC RETENTION TIME AND AMINO ACID COMPOSITION AS THE SYNTHETIC a_1.

GENERAL REFERENCES

1. LOW, T.L.K., THRUMAN, G.B., McADDO, M., McCLURE, J.E., ROSSIO, J.L., NAYLOR, P.H., AND GOLDSTEIN, A.L., 1979, J. BIOL. CHEM., 254, 981.

2. LOW, T.L.K., AND GOLDSTEIN, A.L., 1979, J. BIOL. CHEM., 254, 987.