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Poster Presentation

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ISOLATION OF THYMOSIN B_4 FROM THYMOSIN FRACTION 5 BY RP-HPLC

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ABSTRACT

THYMOSIN B_4 (TB_4) IS ONE OF THE BIOLOGICALLY ACTIVE PEPTIDES IN THYMOSIN FRACTION 5 (TF5), A PARTIALLY PURIFIED THYMIC PREPARATION FROM CALF THYMUS. TB_4 HAS HORMONE-LIKE PROPERTIES AND CAN MODULATE IMMUNE AND NEUROENDOCRINE RESPONSES.

WE HAVE DEVELOPED A RAPID TWO-STEP METHOD FOR THE PURIFICATION OF TB_4 FROM TF5. THIS PURIFICATION IS BASED ON THE USE OF HIGH PERFORMANCE SEMI-PREPARATIVE AND ANALYTICAL REVERSED-PHASE (C_{18} DELTA PAK) CHROMATOGRAPHIC COLUMNS. AMINO ACID COMPOSITIONAL ANALYSIS AND POLYACRYLAMIDE GEL ELECTROPHORESIS HAVE SHOWN THAT THE PURIFIED TB_4 IS IDENTICAL TO SYNTHETIC TB_4.

INTRODUCTION

A PARTIALLY PURIFIED THYMOSIN PREPARATION FROM CALF THYMUS TERMED THYMOSIN FRACTION 5 (TF5) HAS BEEN STUDIED EXTENSIVELY FOR BIOLOGICAL ACTIVITY. AS WELL AS IN VIVO AND IN VITRO IMMUNE RESPONSES. TF5 CONSISTS OF A FAMILY OF BIOLOGICALLY ACTIVE POLYPEPTIDE COMPONENTS WITH HORMONE-LIKE ACTIVITIES. THYMOSIN B4 (TB4) IS ONE OF THE SEVERAL PEPTIDES THAT IS PRESENT IN TF5 AND PARTICIPATES IN THE PROCESS OF REGULATION, DIFFERENTIATION AND FUNCTION OF THYMUS-DEPENDENT THYMOCYTES. TB4 IS COMPOSED OF 43 AMINO ACID RESIDUES AND HAS A MOLECULAR WEIGHT OF 4982 AND A PI OF 5.1. THE AMINO-TERMINUS OF THE PEPTIDE IS BLOCKED BY AN ACETYL GROUP. TB4 HAS BEEN PURIFIED FROM TF5 BY CONVENTIONAL COLUMN TECHNIQUES FOR LARGE-SCALE PURIFICATION USING ION-EXCHANGE CHROMATOGRAPHY ON A CARBOXYLMETHYL-CELLULOSE COLUMN FOLLOWED BY GEL FILTRATION ON SEPHADEX G-50 IN 6 M GUANIDINIUM-HCl. THE DESALTED TB4 ON SEPHADEX G-10 HAD A YIELD OF 0.45%. TB4 HAS ALSO BEEN ISOLATED BY HPLC TECHNIQUES WITH A VERY LOW RECOVERY, 2-3%, USING A uBONDAPAK C18 COLUMN. 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 AND COMPARISON OF THE DIFFERENT FORMS OF THE BIOLOGICALLY ACTIVE PEPTIDES. IN THE PRESENT STUDY WE REPORT A VERY FAST, REPRODUCIBLE AND EFFICENT LARGE SCALE PROCEDURE AS WELL AS AN ANALYTICAL RP-HPLC PROCEDURE FOR THE PURIFICATION OF TB4 FROM TF5.

RP-HPLC OF THYMOSIN FRACTION 5

ANALYTICAL SEPARATION

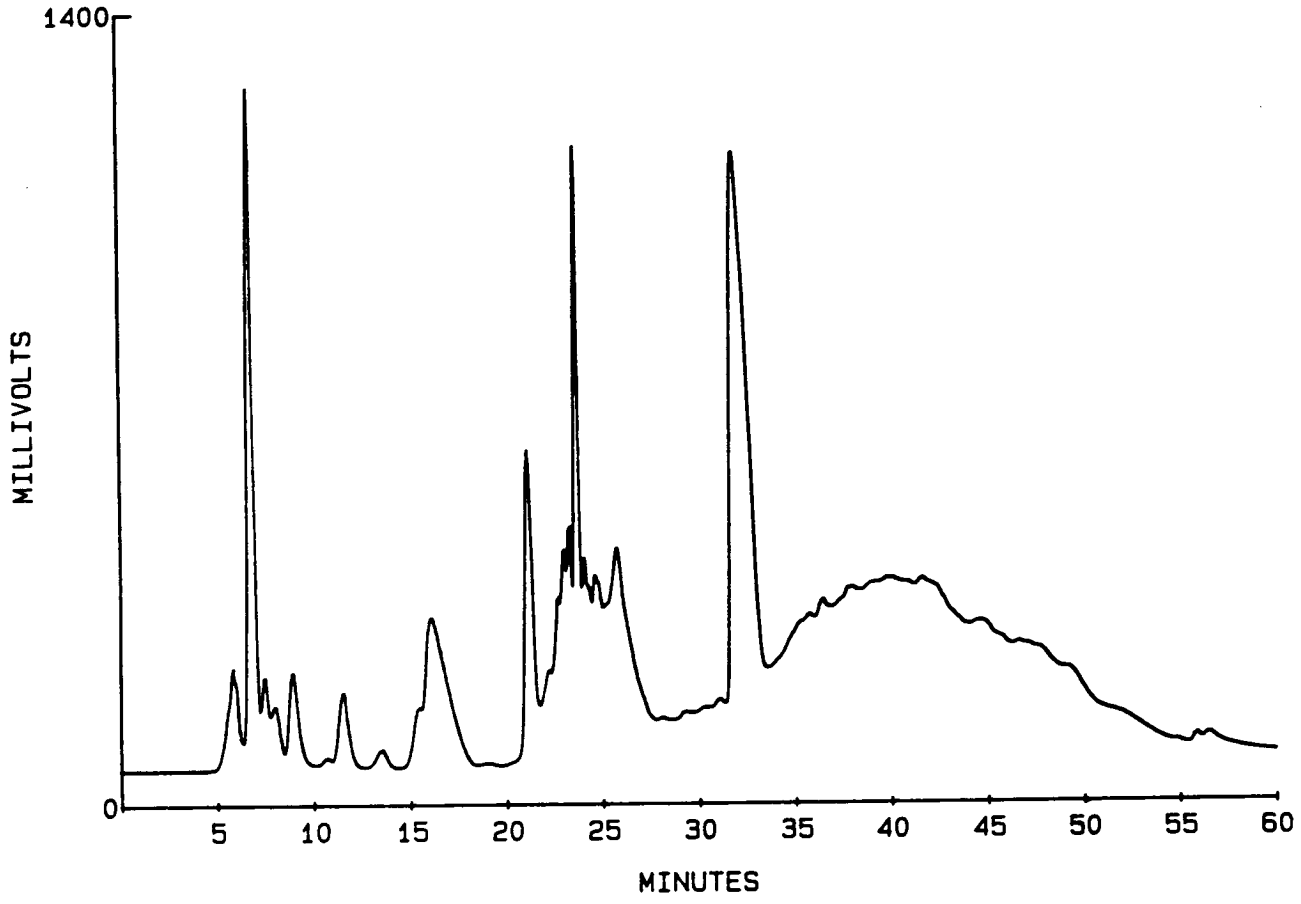

FIGURE 1. REVERSED PHASE HPLC SEPARATION OF 900 UG OF THYMOSIN FRACTION 5 (TF5) ON A 3.9 MM X 30 CM DELTA PAK, 300 A, 15 u, C18 COLUMN. BUFFER A WAS 0.02 M AMMONIUM ACETATE, pH 6.8, AND BUFFER B ACETONITRILE. A 60 MINUTE LINEAR GRADIENT FROM 0-80% B WAS RUN AT A FLOW RATE OF 0.5 ML/MIN. DETECTION WAS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

RP-HPLC OF THYMOSIN FRACTION 5

PREPARATIVE SEPARATION

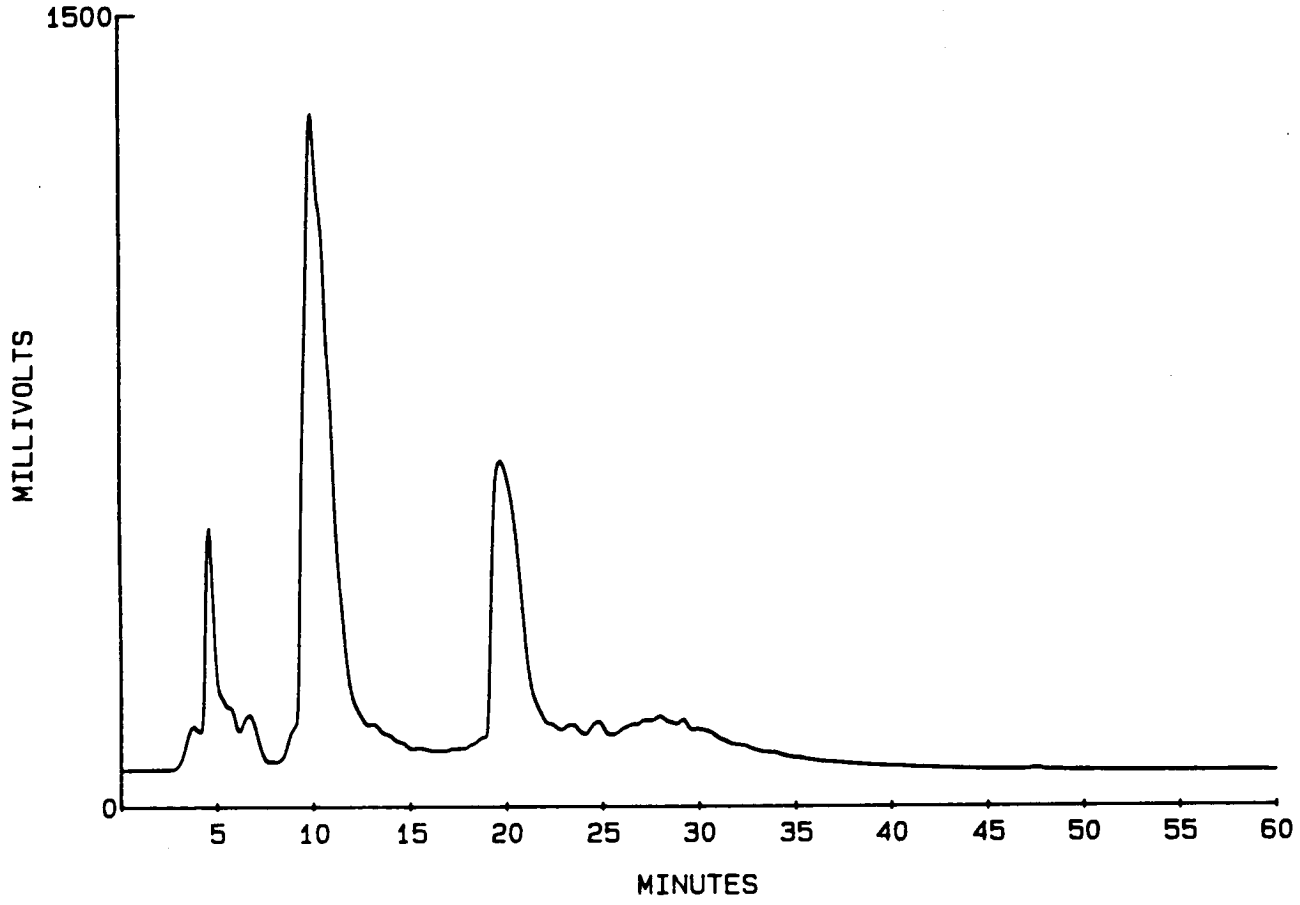

FIGURE 2. REVERSED PHASE HPLC SEPARATION OF 1.5 G OF THYMOSIN FRACTION 5 (TF5) ON A 50 MM X 30 CM DELTA PAK, 300 A, 15 u, C18 COLUMN. BUFFER A WAS 0.02 M AMMONIUM ACETATE, pH 6.8, AND BUFFER B ACETONITRILE. A 60 MINUTE LINEAR GRADIENT FROM 0-80% B WAS RUN AT A FLOW RATE OF 80 ML/MIN. DETECTION WAS AT 280 NM AT 1.5 AUFS. COLLECTED FRACTIONS WERE ASSAYED FOR THYMOSIN B4 USING SOLID-PHASE RIA. RESULTS ARE OVERLAID ON THE CHROMATOGRAM.

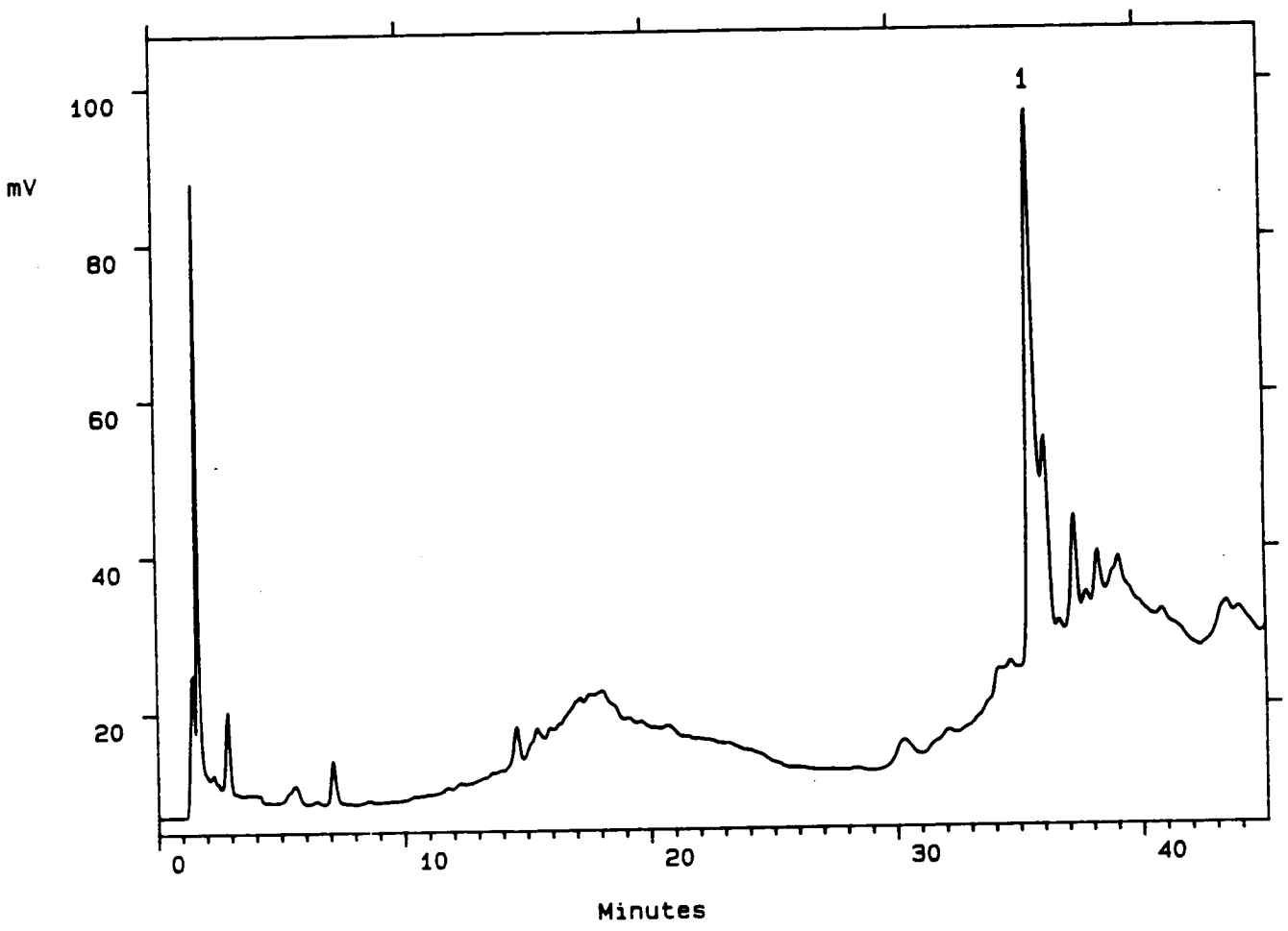

FIGURE 3. REVERSED PHASE HPLC SEPARATION OF 130 UG OF FRACTION 20 FROM THE 1.5 G SEPARATION OF THYMOSIN FRACTION 5 ON A 3.9 MM X 15 CM DELTA PAK, 300 A, 5 u, C18 COLUMN. BUFFER A WAS 0.1% PHOSPHORIC ACID AND BUFFER B ACETONITRILE WITH 0.1% PHOSPHORIC ACID. SEPARATION OF THYMOSIN B4 WAS ACHIEVED USING A 10 MINUTE LINEAR GRADIENT FROM 0-14% B . FOLLOWED BY A 10 MINUTE HOLD AT 14% B. A 10 MINUTE LINEAR GRADIENT TO 18% B ELUTED THE THYMOSIN B4 (1). THE FLOW RATE WAS 1 ML/MIN. DETECTION WAS AT 214 NM, 0.1 AUFS.

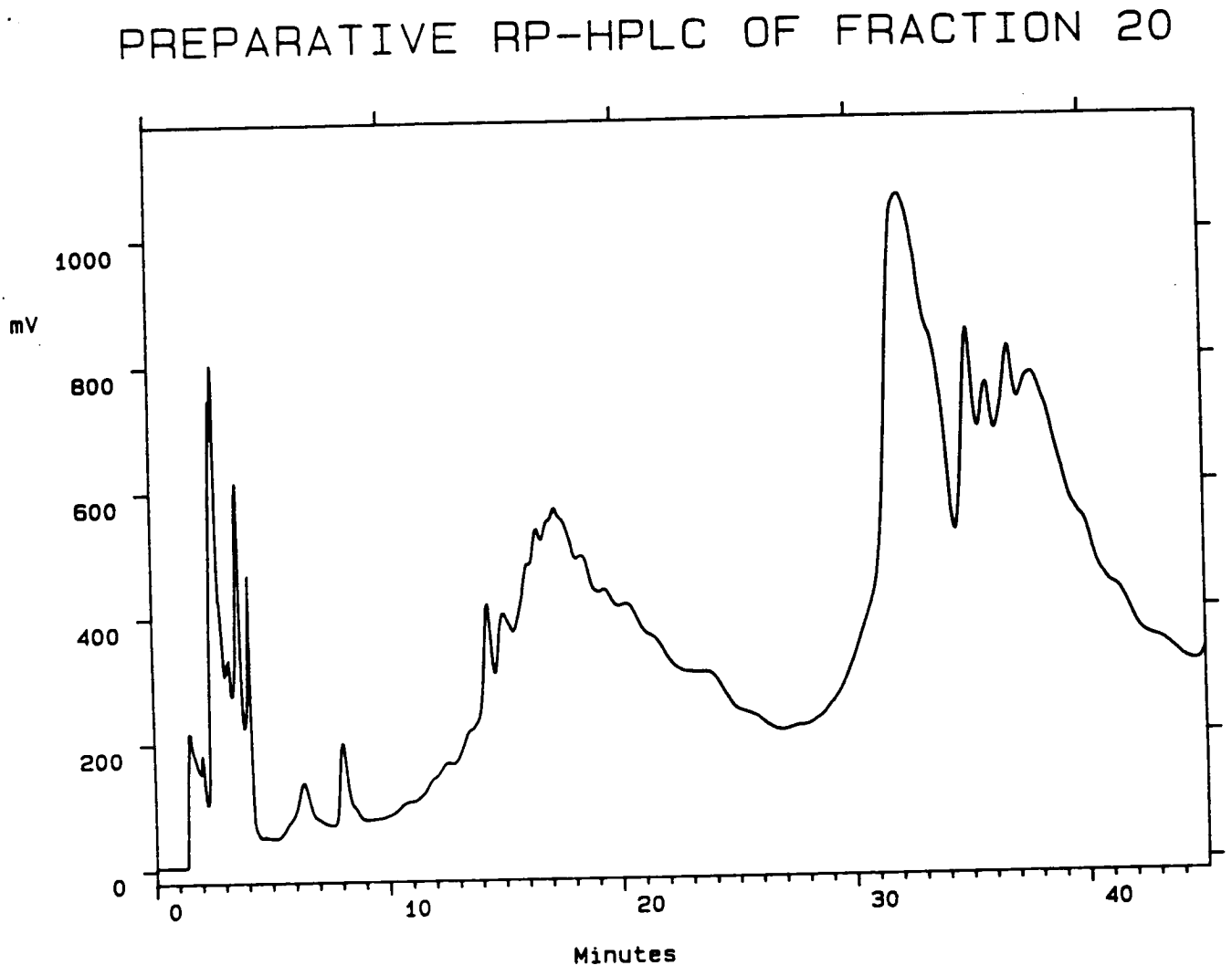

FIGURE 4. REVERSED PHASE HPLC SEPARATION OF 5.6 MG OF FRACTION 20 FROM THE 1.5 G SEPARATION OF TF5 ON A 3.9 MM X 15 CM DELTA PAK, 300 A, 5u, C18 COLUMN. BUFFER A WAS 0.1% PHOSPHORIC ACID AND BUFFER B ACETONITRILE WITH 0.1% PHOSPHORIC ACID. SEPARATION WAS ACHIEVED USING A 10 MINUTE LINEAR GRADIENT FROM 0-14% B, FOLLOWED BY A 10 MINUTE HOLD AT 14% B AND A 10 MINUTE LINEAR GRADIENT TO 18% B. THE FLOW RATE WAS 1 ML/MIN. DETECTION WAS AT 214 NM, 1 AUFS. COLLECTED FRACTIONS WERE ASSAYED FOR THYMOSIN B4 USING SOLID PHASE RIA. RESULTS ARE OVERLAID ON THE CHROMATOGRAM.

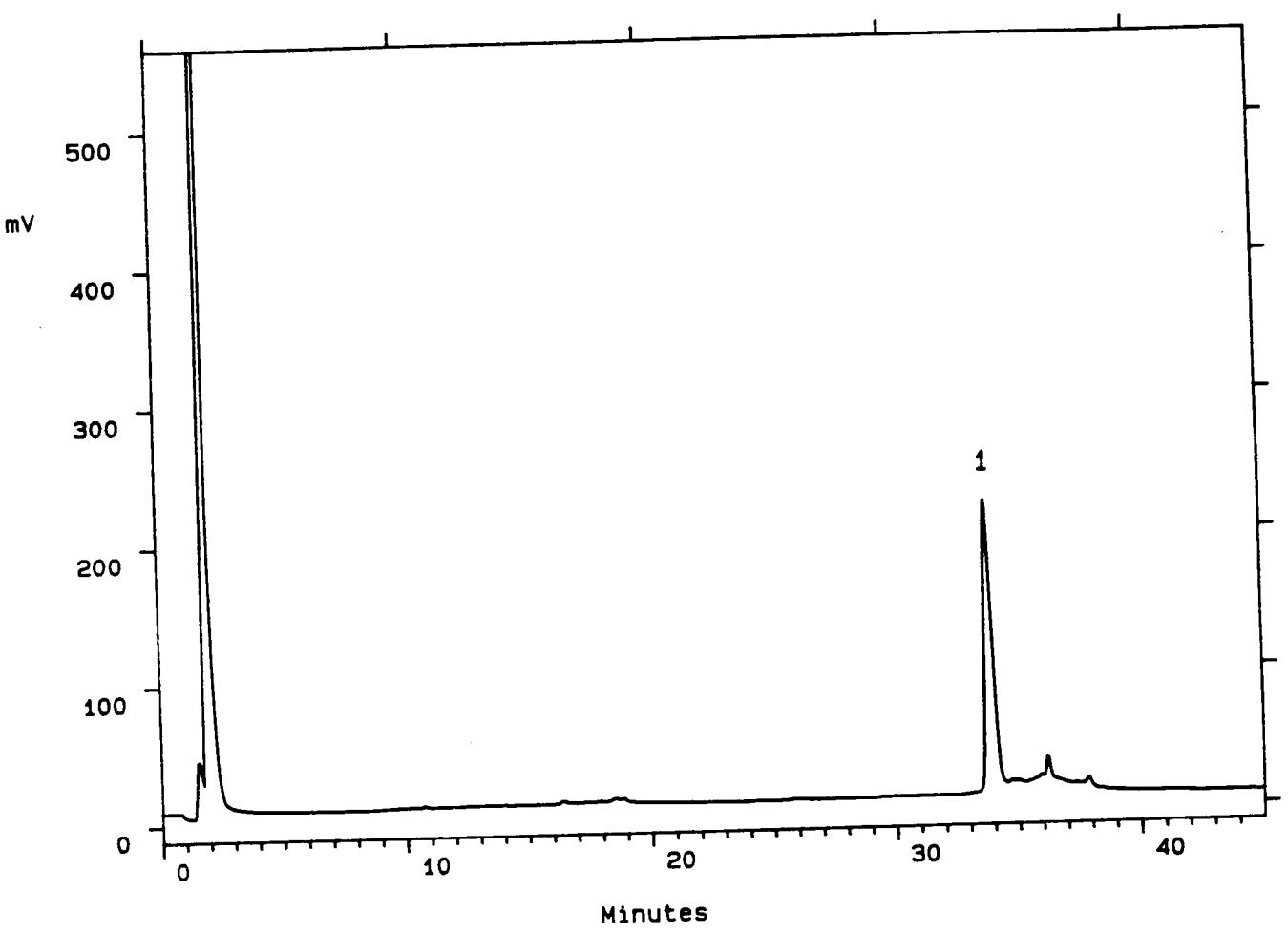

FIGURE 5. RECHROMATOGRAPHY OF 100 UL OF FRACTION 33 FROM THE PREPARATIVE SEPARATION OF FRACTION 20. THE SAMPLE WAS DILUTED WITH 100 UL OF WATER AND INJECTED ON TO THE 3.9 MM X 15 CM DELTA PAK, 300 A, 5 u, C18 COLUMN. BUFFER A WAS 0.1% PHOSPHORIC ACID AND BUFFER B ACETONITRILE WITH 0.1% PHOSPHORIC ACID. A 10 MINUTE LINEAR GRADIENT FROM 0-14% B, FOLLOWED BY A 10 MINUTE HOLD AT 14% B AND A 10 MINUTE LINEAR GRADIENT TO 18% B ELUTED THE THYMOSIN B4 (1). THE FLOW RATE WAS 1 ML/MIN. DETECTION WAS AT 214 NM, 0.5 AUFS.

RECHROMATOGRAPHY OF FRACTIONS 33 & 33.5

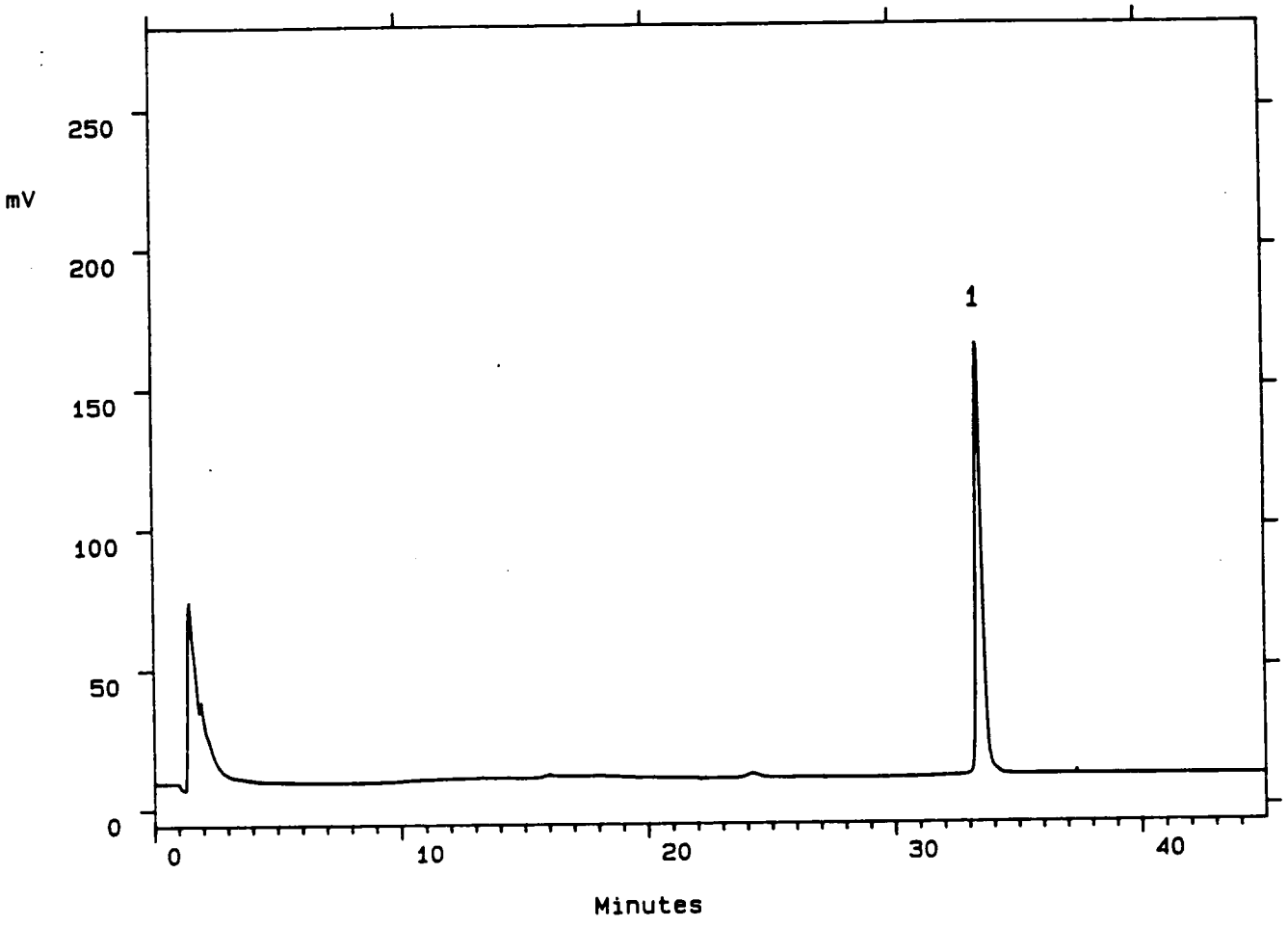

FIGURE 6. RECHROMATOGRAPHY OF FRACTIONS 33 & 33.5 FROM THE SEPARATION OF FRACTION 33. THE SAMPLE WAS CONCENTRATED TO 250 UL, DILUTED WITH WATER AND INJECTED ON TO THE 3.9 MM X 15 CM DELTA PAK, 300 A, 5 u, C18 COLUMN. BUFFER A WAS 0.1% PHOSPHORIC ACID AND BUFFER B ACETONITRILE WITH 0.1% PHOSPHORIC ACID. A 10 MINUTE LINEAR GRADIENT FROM 0-14% B, FOLLOWED BY A 10 MINUTE HOLD AT 14% B AND A 10 MINUTE LINEAR GRADIENT TO 18% B ELUTED THE THYMOSIN B4 (1). THE FLOW RATE WAS 1 ML/MIN. DETECTION WAS AT 214 NM, 0.5 AUFS.

CRUDE SYNTHETIC B4 VS ISOLATED B4

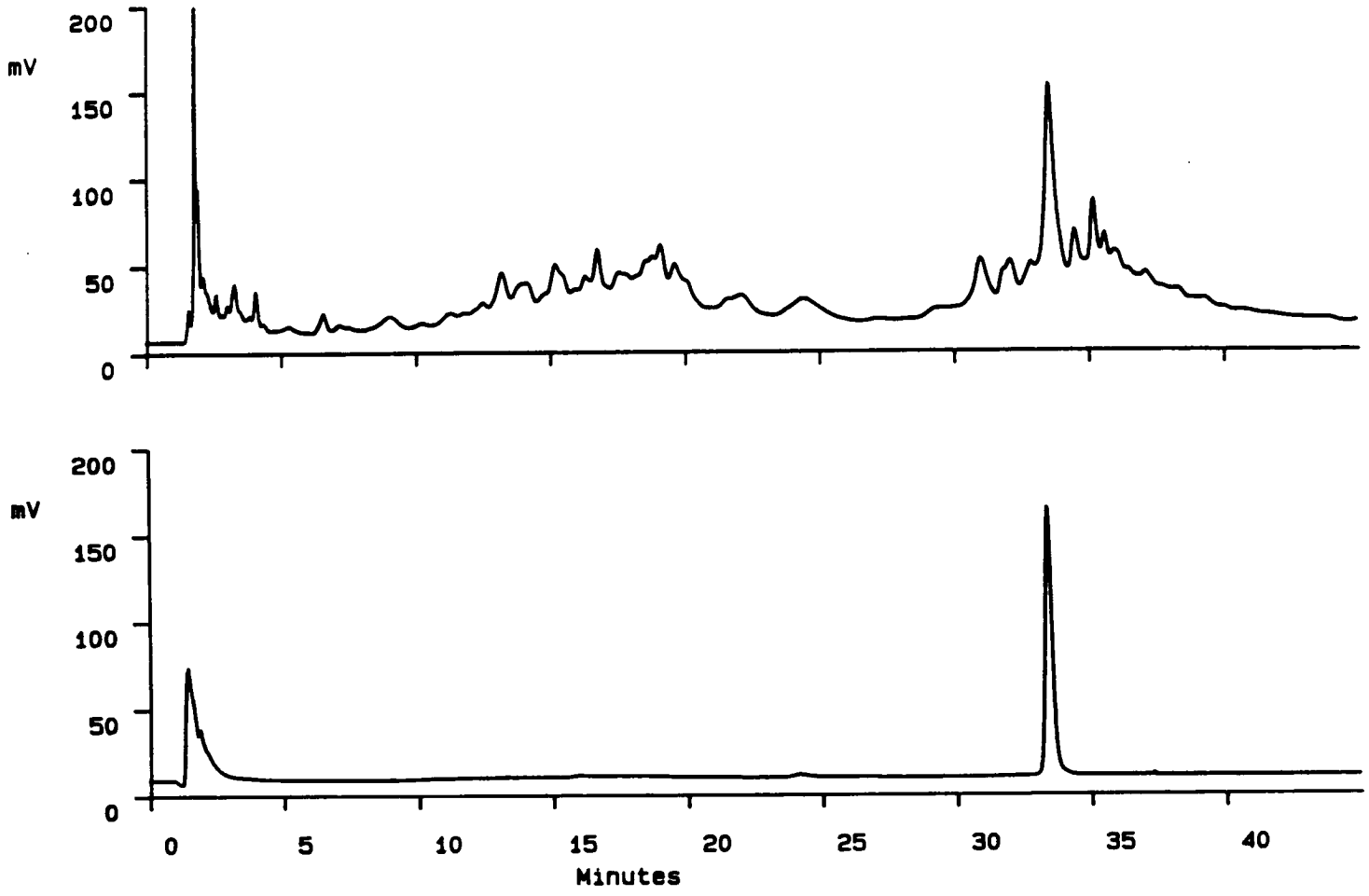

FIGURE 7. COMPARISON OF 50 UG OF CRUDE SYNTHETIC B4 AND B4 ISOLATED BY THE TWO STEP RP-HPLC PROCEDURE ON THE 3.9 MM X 30 CM DELTA PAK, 300 A, 5u COLUMN USING THE GRADIENT DESCRIBED PREVIOUSLY (FIGURE 6). THE SYNTHETIC B4 WAS IDENTIFIED BY SOLID PHASE RIA AND THE RESULTS ARE OVERLAID ON THE CHROMATOGRAM. RED IS SYNTHETIC B4, BLUE IS ISOLATED B 4 AND BLACK IS CPM FROM THE RIA.

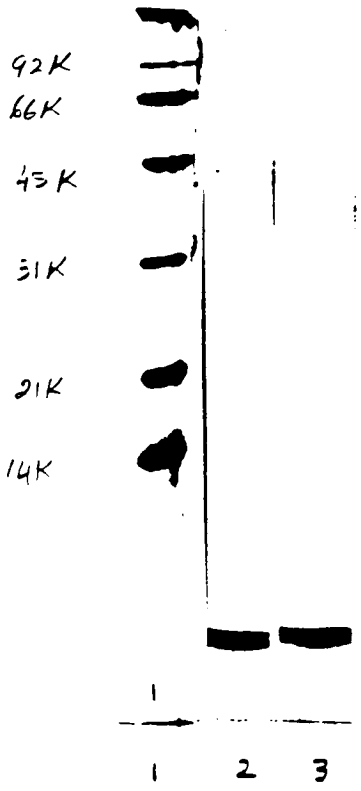

FIGURE 8. SDS-PAGE OF SYNTHETIC AND ISOLATED THYMOSIN B4 . ABOUT 10-20 UG OF THYMOSIN B4 SAMPLES WERE ELECTROPHORESED IN A 1.5 MM 12% SDS-POLYACRYLAMIDE GEL ACCORDING TO THE METHOD OF LAEMMLI (1970) AND STAINED USING A BIO-RAD SILVER STAIN KIT. LANE 1: MOLECULAR WEIGHT MARKERS FROM BIO-RAD; LANES 2 AND 3 SYNTHETIC AND NATURAL THYMOSIN B4 RESPECTIVELY.

AMINO ACID COMPOSITION[a] OF NATURAL AND SYNTHETIC THYMOSIN B4

AMINO ACID	SYNTHETIC B4	NATURAL B4	REPORTED SEQUENCE[b]
Asp	3.9	3.8	4
Glu	10.8	10.6	11
Ser	3.5	3.8	4
Gly	1.1	1.2	1
Thr	2.8	3.2	3
Ala	2.2	1.8	2
Pro	2.9	2.9	3
Met	0.8	0.7	1
Ile	1.6	1.9	2
Leu	1.6	2.2	2
Phe	0.9	0.8	1
Lys	8.5	8.6	9

a. The data are presented as assumed numbers of residues per molecule. b. Number of residues obtained from the reported sequence (Low and Goldstein, 1981, Proc. Natl. Acad. Sci., 78, 1162).

TABLE 1. AMINO ACID ANALYSIS - THIS WAS PERFORMED USING THE PICO TAG AMINO ACID ANALYSIS SYSTEM OF WATERS CHROMATOGRAPHY DIVISION, MILLIPORE. ABOUT 10 UG OF SYNTHETIC AND ISOLATED TB4 SAMPLES WERE HYDROLYZED WITH 6 N HCl CONTAINING 1% PHENOL BY VOLUME AT 110 ^{o}C FOR 48 HRS. THE HYDROLYSATES WERE DRIED AND USED FOR AMINO ACID ANALYSIS BY THE PICO TAG STANDARD PROCEDURE [Bidlingmeyer, et al. (1984) J. Chromatogr. 336, 94-104].

CONCLUSION

1. WE WERE ABLE TO PURIFY TB4 FROM TF5 USING A TWO-STEP RP-HPLC TECHNIQUE WITH AN 80% RECOVERY FOR EACH STEP. THIS PURIFICATION WAS BASED ON THE RIA AND HPLC RETENTION TIME OF THE SYNTHETIC B4. THE FINAL TB4 PREPARATION IS HOMOGENEOUS AND THERE IS A 30-FOLD INCREASE IN ITS RECOVERY COMPARED TO PREVIOUSLY REPORTED PURIFICATION PROCEDURES FOR TB4 FROM TF5 (1-2).

2. SDS-PAGE OF THE ISOLATED AND SYNTHETIC TB4, AFTER SILVER STAINING, REVEALED A SINGLE PEPTIDE BAND WITH A MOLECULAR WEIGHT BELOW 10 KD.

3. THE NATURAL TB4 HAD A SIMILAR HPLC RETENTION TIME AND AMINO ACID COMPOSITION AS THE SYNTHETIC B4.

GENERAL REFERENCES

1. Low, T. L. K., and Goldstein, A. L. (1982) J. Biol. Chem. 257, 1000.

2. Low, T. L. K., and Mercer, R. C. (1984) J. Chromatogr. 301, 221.

3. Low, T. L. K., Hu, S. K., and Goldstein, A. L. (1981) Proc. Natl. Acad. Sci., 78, 1162.