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ISOLATION OF HUMAN MET-ENKEPHALIN AND TWO GROUPS OF PUTATIVE PRECURSORS (2K-PRO-MET-ENKEPHALIN) FROM AN ADRENAL MEDULLARY TUMOUR

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SUMMARY: Human met-enkephalin from an adrenal medullary tumour has been purified and characterised. Two groups of putative human met-enkephalin precursors (MW ∿ 2000) were also isolated and were only opiate active after trypsinisation and yielded met-enkephalin on further treatment with carboxypeptidase B. Amino acid analyses of these peptides show they are not related to β-LPH nor any other reported putative enkephalin precursors. Their amino acid compositions suggest they are structurally closely related and may represent two groups of heterologous peptides.

Although the met-enkephalin sequence is contained in β-LPH and β-endorphin there is increasing evidence that met-enkephalin is derived from precursors distinct from β-LPH and β-endorphin[1]. Putative leu-enkephalin precursors include α-neo-endorphin from pig hypothalami[2] and dynorphin[3] from porcine pituitaries. High molecular weight met-enkephalin related peptides have been reported from bovine striatum[4] and bovine adrenal medulla[1,5]. A series of met-enkephalin related peptides have been isolated and include an adrenal heptapeptide met-enkephalin (arg^6 phe^7)^6, an adrenal tryptic peptide met-enkephalin (lys^6)^6 and met-o-enkephalin (arg^6) from porcine hypothalami[7]. All these reported identified putative leu- and met-enkephalin precursors have been opiate active.

We recently reported high concentrations of met-enkephalin immunoreactivity in the human adrenal medulla[8], and a gradient of met-enkephalin was measured in the human adrenal vein suggesting active release[8]. This and preliminary reports[9-10] led us to investigate met-enkephalin-like peptides in a human adrenal medullary tumour and we describe here the purification and sequence analysis of human met-enkephalin and the isolation, amino acid composition and partial characterisation of two groups of biologically

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inactive putative human met-enkephalin precursors. Met-enkephalin has previously only been isolated and identified from porcine and bovine brains.

MATERIALS AND METHODS

A catecholamine secreting adrenal medullary tumour (40gm) was collected fresh at surgery and homogenised in 200ml 0.1M HCl heated to 80°C for 10 minutes. After addition of 10gm ammonium sulphate, 2.4ml formic acid, 2.4ml trifluoroacetic acid (TFA) the mixture was centrifuged at 10,000g for 1 hour at 4°C. The resulting supernatant was extracted on coarse ODS silica on 5 x 1cm columns[8]. After elution in 80% methanol/1% TFA, drying under nitrogen at 50°C and reconstitution in 1% formic acid the extract was chromatographed on a BioGel P4 column (Fig. 1). A normal human adrenal medulla removed from a patient with widespread carcinoma of the breast was similarly extracted and chromatographed.

The radioimmunoassay employed for met-enkephalin[8,11] does not detect leu-enkephalin. The C-terminal directed leu-enkephalin antibody was raised against leu-enkephalin coupled by its N-terminus by glutaraldehyde to bovine thyroglobulin andthe peptide was radioiodinated and the assay performed as described for met-enkephalin but omitting the oxidation step. Met-enkephalin cross reacts 5% in this assay. Radioimmunoassays for α-endorphin[12], N-β-LPH[13], C-β-LPH (β-endorphin)[14] and ACTH[15] have previously been described.

All purified peptides (∿ 2μg) were analysed on a Jeol JLC 6AH automatic amino acid analyser after acid hydrolysis in 200μl 6N HCl with crystal of phenol in evacuated pyrex tubes at 115°C for 20 hours.

Sequence analysis of 20μg purified human met-enkephalin was performed by subtractive Edman[16] to the third step and carboxypeptidase C (Rohm and Haas, Darmstadt, Germany) digestion of 5μg human met-enkephalin was by 1μg enzyme in 50μl citrate buffer pH 5 left for 2 hours at room temperature.

Putative precursor peaks A and B from HPLC (Fig. 3) were pooled separately after further chromatography on analytical HPLC (Fig. 4 for A). For pooled peak A the predominant N-terminal amino acids were determined by dansylation[18] and the C-terminal amino-acids by carboxypeptidase digests for 20 hours. The content of tryptophan in pooled peak A was assessed by total aminopeptidase digest of 2μg peptide treated with aminopeptidase for 20 hours at room temperature. Tryptic digestion of pooled precursor peaks A and B were performed with 1μg trypsin (TPCK treated, Worthington Biochemical Corporation, Freehold, New Jersey, USA) in 50μl 50mM $NaHCO_3$ pH 8 incubated at 37°C for 2 hours.

All carboxypeptidase B (CPB) digests were performed with 1μg CPB in 50μl 50mM $NaHCO_3$ pH 8 at 37°C for 2 hours.

The carbohydrate content of pooled peak A was studied by Concanavalin A Sepharose[18] andinvestigating if there was a change in elution profile of optical density and immunoreactivity after digestion with endoglycosidase H and endoglycosidase D in the presence of neuraminidase, β-galactosidase and β-N-acetyl hexosaminidase[19] (Miles Laboratories Ltd., Stoke Poges, UK).

Opiate activity was determined using the mouse vas deferens bioassay[20].

RESULTS AND DISCUSSION

The tumour extract content in pmole/mg wet weight of tissue was 50 for met-enkephalin, (some 30 fold higher than the normal adrenal medulla[8]), 0.66 for leu-enkephalin, 0.02 for β-LPH-N, 0.01 for β-LPH-C, 0.003 for ACTH and for α-endorphin <0.001fmole/mg. The leu-enkephalin immunoreactivity could be accounted for by cross reaction with met-enkephalin.

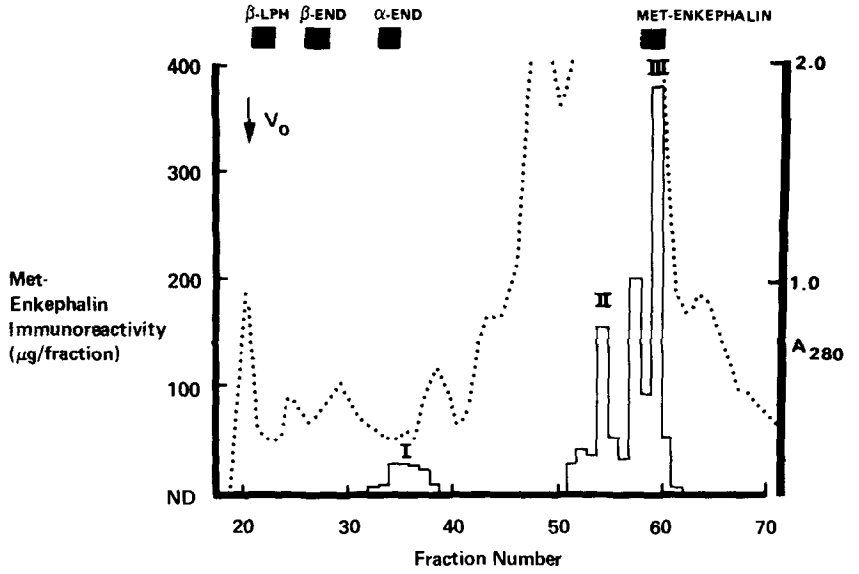

Fig. 1 BioGel P4 chromatography of extracted adrenal medullary tumour (40gm) on a 90 x 2cm column. Hourly 3ml fractions were collected and 10μl samples of each fraction were assayed for met-enkephalin. The arrows mark the elution positions of human β-LPH and β-endorphin, and synthetic α-endorphin and met-enkephalin. The dotted line is the optical density trace read at 280nM.

BioGel P4 chromatography showed several met-enkephalin immunoreactive peaks (Fig. 1). The major peak (peak III) eluted in the position of synthetic met-enkephalin. Both peaks II and III were opiate active prior to trypsinisation. There was an earlier immunoreactive peak I in the elution position of α-endorphin which had neither α- nor β-endorphin immunoreactivity and was opiate active only after trypsinisation.

The BioGel P4 chromatogram of the normal human adrenal medulla showed a similar profile to Fig. 1.

The main met-enkephalin immunoreactive peak (peak III, Fig. 1) was further purified on analytical HPLC (Fig. 2). There was a peak of optical density (∿ 200μg) eluting in the position of synthetic met-enkephalin which was fully immunoreactive and its biological activity was naloxone reversible. Acid hydrolysis yielded the amino acids Gly 2.0, Met 1.0, Tyr 1.0, Phe 1.1. Subtractive Edman degradation (Table 1) and carboxypeptidase C digests (which yielded methionine and a small amount of phenylalanine) confirmed the sequence of human met-enkephalin.

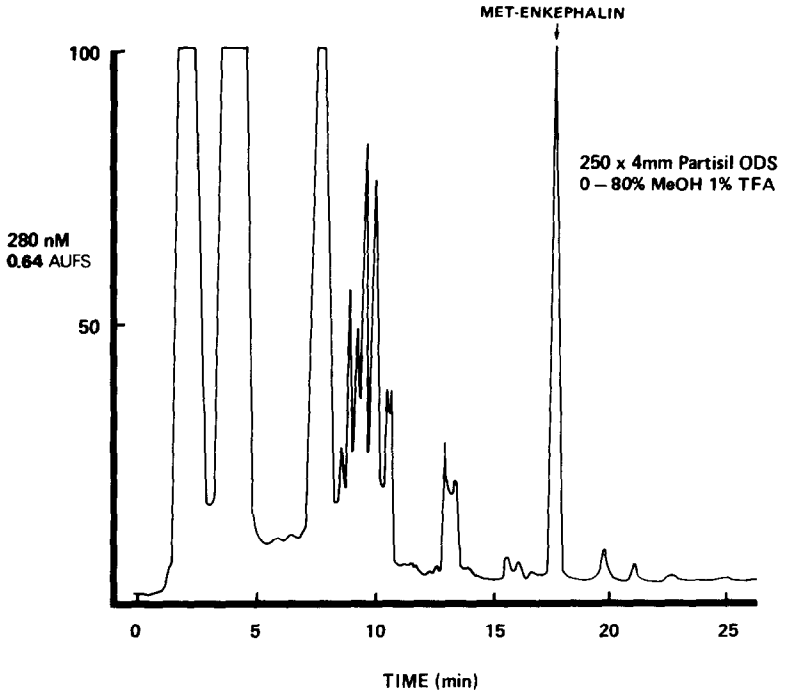

Fig. 2 HPLC (250 x 4mm Partisil ODS, 0.64 AUFS, 280nM) of 1.5ml of peak III (BioGel P4) with gradient of 0-80% methanol/1% TFA eluted at 2ml/min and 0.4ml fractions collected. The arrow marks the elution position of synthetic met-enkephalin.

Peak II BioGel P4 (Fig. 1) on analytical HPLC (profile not shown) yielded two immunoreactive peptides, one which was characterised as met-o-enkephalin and the other Tyr_1 Gly_2 Met_1 Phe_1 Arg_1.

Peak I on BioGel P4 (Fig. 1) was submitted initially to preparative HPLC separation (Fig. 3). Two broad immunoreactive peaks (A and B) were seen, the first (A) corresponding to a larger peak of optical density. To characterise these peaks further the fractions of each peak were dried, reconstituted and rechromatographed separately by analytical HPLC and Fig. 4 shows the elution

TABLE 1

Amino Acids Subtractive Edman Degradation of Purified Human Met-Enkephalin

	Step 1	Step 2	Step 3
Gly	2.2	1.3	0.5
Met	1.0	1.1	1.0
Phe	1.1	0.9	0.9
Tyr	0.1	0.1	0.1

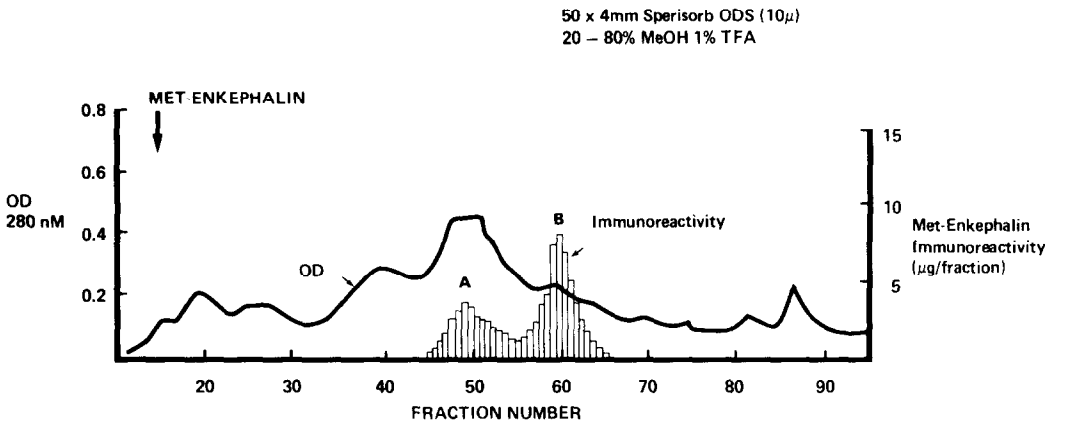

Fig. 3 Preparative HPLC (50 x 4mm Spherisorb ODS, 10μ) of peak I (BioGel P4 volume 21ml) with gradient of 20-80% methanol/1% TFA eluted at 2ml/min, 0.4ml fractions collected. Optical density was monitored at 280nM and aliquots of all fractions were assayed for met-enkephalin. The arrow marks the elution position of synthetic met-enkephalin.

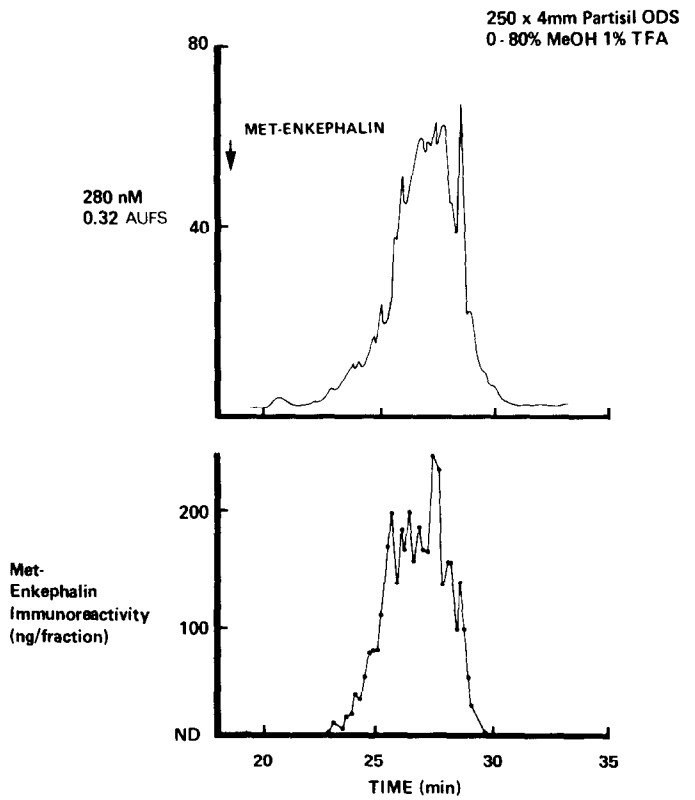

Fig. 4 Analytical HPLC (250 x 4mm Partisil ODS column, 0.32 AUFS, OD 280nM) of reconstituted peak A (Fig. 3) with gradient of 0-80% methanol/1% TFA eluted at 2ml/min, 0.4ml fractions collected. All fractions were assayed for met-enkephalin.

profile of peak A. This shows a collection of peaks of optical density with closely coinciding met-enkephalin immunoreactivity. A very similar chromatographic profile was seen with the second eluting peptide peak B. The yields from the tumour of peptide A and B were 30μg and 10μg respectively. The amino acid composition for the two peak tubes on analytical HPLC of peptide B is shown (Table 2). Analysis of three fractions across peak A showed a series of remarkably closely related peptides (Table 2). The amino acid compositions of A and B were similar, both containing the constituent amino acids of met-enkephalin. However these analyses did not correlate with any portion of β-LPH or other reported enkephalin precursors[2,3,5,6]. Peak A differed from peak B in that it contained tryptophan and only contaminating amounts of valine. For peptide A dansylation yielded N-terminal glutamic acid and carboxypeptidase C digests yielded predominantly leucine with small amounts of alanine and glycine. Total aminopeptidase digests of peak A confirmed the presence of tryptophan which would explain its high optical density to immunoreactivity ratio seen in Fig. 3.

TABLE 2

Amino Acid Compositions of 2K-Pro-Met-Enkephalin A and B

	A*			B
	1	2	3	
Asx	2.9	2.2	2.3	1.7
The	0.5	0.3	0.3	0.4
Ser	1.8	1.4	1.6	1.4
Glx	5.7	5.3	6.0	3.2
Pro	0.6	1.1	0.8	0.8
Gly	2.9	2.4	3.0	2.2
Ala	2.7	2.1	2.6	1.5
Val	0.9	0.4	0.7	1.1
Met	1.3	1.1	1.5	0.5
Ileu	0.3	0.5	0.4	0.5
Leu	2.3	2.0	1.8	1.4
Tyr	1.4	1.2	1.2	1.1
Phe	1.0	1.0	1.0	1.0
Hist	0.5	0.3	0.3	0.3
Lys	2.2	1.8	1.9	1.4
Trp	+	+	+	-
Arg	1.5	1.3	1.3	1.1

*Peptide A - analysis of fractions from HPLC (Fig. 4) at time 1. 26 minutes, 2. 28 minutes, 3. 29 minutes.

The closely correlating immunoreactivity and optical density trace and the similar amino acid compositions across the HPLC profile of peptide A would suggest that the profile seen was not due to contaminating peptides but that peptide A was a group of heterologous but closely related peptides. Peptide A did not adhere to Concanavalin A nor was its immunoreactive nor optical density profile on HPLC altered after treatment by endoglycosidases suggesting that its heterogeneity was not due to glycosylation.

The opiate activity generated by trypsinisation of peptide A and B was comparable to that of met-enkephalin and was antagonised by naloxone.

When treated with trypsin peptide A yielded several peptides separating on HPLC which were immunoreactive in our radioimmunoassay. However the main tryptic products were not met-enkephalin but Tyr_1 Gly_2 Phe_1 Met_1 Lys_1 and Tyr_1 Gly_2 Phe_1 Met_1 Arg_1. Two other tryptic peptides without met-enkephalin

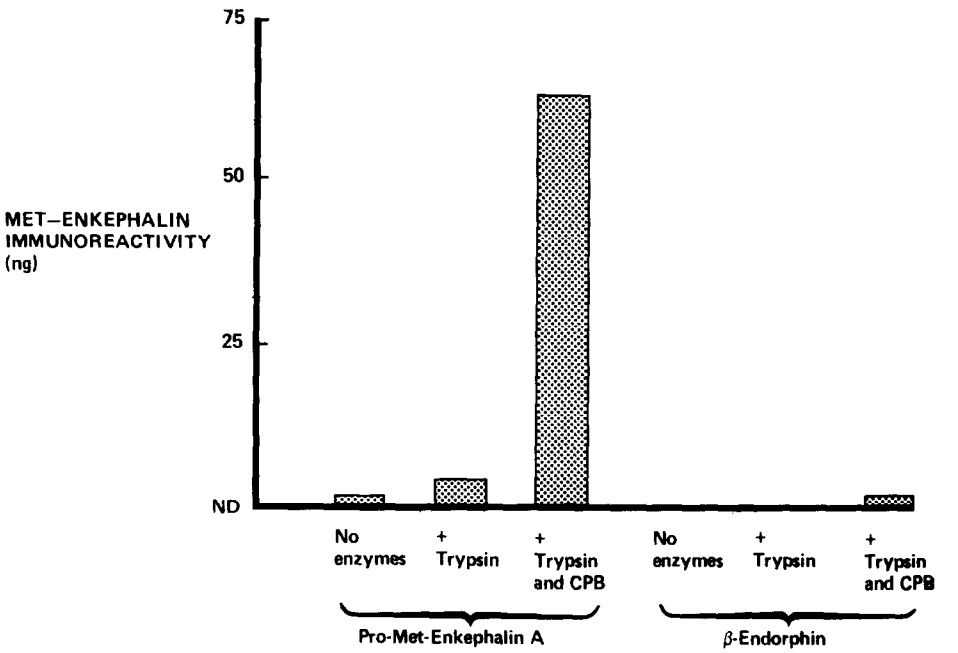

Fig. 5 0.1nmole of peptides A and human β-endorphin were assayed for met-enkephalin before and after treatment at 37°C with a) trypsin alone for 4 hours, b) carboxypeptidase B alone for 4 hours and c) trypsin for 2 hours followed by carboxypeptidase B for 2 hours. Results expressed as a mean of experiments performed in duplicate. Similar treatment of β-LPH revealed the same result as for β-endorphin.

immunoreactivity were isolated including a tryptophanyl tripeptide Trp_1 Ser_1 Lys_1 and a peptide Asx_1 Ser_2 Glx_2 Gly_2 Leu_2 Phe_1 Pro_1 Arg_1. The yield of tryptic fragments however were not sufficient for formal sequence analysis. Tryptic digestion of pooled peptide B did not yield peptides with any clear amino acid analysis after HPLC.

Both peptides A and B were only 3% immunoreactive in our C-terminal directed met-enkephalin assay and inactive in the opiate bioassay suggesting both the C-terminal immunoreactive sites and N-terminal opioid sites may be masked by N- and C-terminal extensions. Fig. 5 shows that after trypsin and carboxypeptidase B treatment there was a 30 fold increase in immunoreactivity of A which on HPLC gave a peak in the position of authentic met-enkephalin. These results contrast with that for β-LPH and β-endorphin which after similar treatment did not yield free met-enkephalin.

In this study human met-enkephalin precursors have been isolated from an adrenal medullary tumour. The elution profile on HPLC of the putative precursors A and B and their variable but closely related amino acid compositions would suggest that they represent two groups of heterologous peptides which we have called human 2K-pro-met-enkephalin A and B.

We would suggest that these observations could be explained by the occurrence of the enkephalin sequence at regular intervals in a large precursor. Lack of evolutionary conservation outside the basic residues flanking the repeating met-enkephalin sequence could lead to these two sets of heterologous peptides. This would serve as an economical way of manufacturing a small pentapeptide from a polypeptide of sufficient size to be translated in the ribosomal protein synthetic system. This heterogeneity may help to explain the different immediate met-enkephalin precursors reported by other workers and ourselves[5,6,7,21].

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