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ANTISERA TO SYNTHETIC PEPTIDE RECOGNIZE HIGH MOLECULAR WEIGHT ENKEPHALIN-CONTAINING PROTEINS

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Antisera to a synthetic peptide corresponding to the 95-117 sequence of proenkephalin were used to develop a sensitive radioimmunoassay. Gel-filtration of acid extracts of bovine adrenal medulla and purified chromaffin granules revealed that the antisera recognized high molecular weight material (M_r approximately 5,000-30,000). The material in peak I (M_r20,000-30,000) and peak II (M_r 10,000-20,000) was further purified by immunoaffinity chromatography. Sequential digestion of each of these fractions with trypsin and carboxypeptidase B generated immunoreactive Met-enkephalin. This study demonstrates that antisera against a synthetic peptide cross-react with high molecular weight enkephalin-containing precursors, validating the use of these antisera in studies of enkephalin biosynthesis.

The primary structure of bovine adrenal preproenkephalin has been determined by sequencing the cDNA of this prohormone (1,2). The sequence of the precursor contains four copies of Met-enkephalin and one copy of each of Leu-enkephalin, Met-enkephalin-Arg^6-Gly^7-Leu^8 and Met-enkephalin-Arg^6-Phe^7. While the steps involved in processing have not been defined fully, a large number of EC peptides have been isolated and sequenced (3).

One of the difficulties encountered in studies of enkephalin biosynthesis is the failure of most antisera to specific enkephalin peptides to recognize high molecular weight intermediates in the processing of enkephalins. In an attempt to overcome this problem we have raised antisera against synthetic peptides corresponding to portions of proenkephalin. An antiserum against a

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Abbreviations. Proenkephalin(95-117) Val-Glu-Glu-Glu-Ala-Asn-Gly-Gly-Glu-Val-Leu-Gly-Lys-Arg-Tyr-Gly-Gly-Phe-Met-Lys-Lys-Asp-Ala; EC, enkephalin-containing; PMSF, phenylmethanesulfonyl fluoride; HPLC, high performance liquid chromatography; PBS, phosphate buffered saline; TFA, trifluoroacetic acid.

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synthetic peptide corresponding to proenkephalin 95-117[4] enabled the detection of high molecular weight forms of EC proteins in bovine adrenal medullary tissue by radioimmunoassay.

MATERIALS AND METHODS

Bovine thyroglobulin, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, PMSF, diphenyl carbamyl chloride-treated trypsin and DFP-treated carboxypeptidase B were purchased from the Sigma Chemical Company, St. Louis, U.S.A. Acetonitrile (HPLC grade) was from Waters Associates, Amherst, MA, U.S.A. Sephadex G-100 and Sepharose 4B were obtained from Pharmacia Fine Chemicals, AB, Uppsala, Sweden. All other reagents were of the highest grade available.

Synthetic peptides Proenkephalin(95-117) was synthesized essentially according to procedures described previously (4) and purified to homogeneity by HPLC. The sequence of this was confirmed by peptide mapping and amino acid analysis. Met-enkephalin and Met-enkephalin-lys^6 were obtained from Sigma and Peninsula laboratories, respectively.

Preparation of antisera Synthetic proenkephalin(95-117), 11.5 mg, was conjugated to bovine thyroglobulin (50 mg) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (2.3 mg) in water (2 ml). The mixture was incubated at room temperature overnight, then stored at 4°C (5). New Zealand white rabbits were immunized with conjugate equivalent to 200 µg of peptide in complete Freund's adjuvant by multiple subcutaneous injections. Immunizations were repeated at two weekly intervals for six weeks, followed by 100 µg of conjugate at monthly intervals. Antisera suitable for radioimmunoassay were obtained after two months.

Radioimmunoassay Proenkephalin(95-117) was iodinated using chloramine T (6) and purified using a C_{18}-silica cartridge (7).

Radioimmunoassay buffer consisting of 50 mM phosphate/5 mM EDTA/0.015% phenol red/0.02% sodium azide, pH 7.5 containing 0.3% bovine serum albumin and 0.1% Triton X-100 was used for all dilutions. The incubation mixture consisted of 100 µl reference standard or unknown, 100 µl ^{125}I-proenkephalin (95-117), 5,000 c.p.m. and 100 µl of antiserum (Rabbit 2, 1;1000 dilution). After incubation at 4°C for 1-2 days, antibody-bound material was precipitated using 20% polyethylene glycol in PBS containing 0.1% Triton X-100.

Antiserum to Met-enkephalin (RB-4) was a generous gift from Dr. S.L. Sabol, National Institutes of Health. This is primarily a carboxyterminal directed antibody (8). Methionine-enkephalin was iodinated using lactoperoxidase (9). Otherwise conditions for the radioimmunoassay were as described for proenkephalin(95-117).

The concentration of all standards was determined by amino acid analysis.

Extraction of adrenal tissue Bovine adrenal glands were collected from local slaughter houses and the medulla carefully dissected. Extracts of adrenal medulla were prepared by homogenizing the tissue with 5 volumes of 1 M acetic acid/20mM HCl /0.1% 2-mercaptoethanol followed by centrifugation at 14,000 r.p.m. for 45 min at 4°C in a SS-34 rotor.

Chromaffin granules were prepared according to the procedure of Bartlett and Smith (10). The final step involved centrifugation through a cushion of 1.8 M sucrose to reduce contamination with lysosomes (11).

Gel-filtration Acid extracts of adrenal medullary tissue were chromatographed on a column (1.6 x 88 cm) of Sephadex G-100 run in 1.0 M acetic acid/20 mM HCl /0.1% 2-mercaptoethanol. Fractions (1.96 ml) were

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[4] The numbering of proenkephalin assumes that preproenkephalin contains a 24 residue signal peptide (2).

collected and aliquots diluted 200-300 fold in radioimmunoassay buffer prior to determination of proenkephalin(95-117) immunoreactivity.

HPLC The HPLC system used in these studies has been described previously (12) Low molecular weight material obtained after fractionating the acid extract of purified chromaffin granules on Sephadex G-100 (Peak III) was pumped onto a Radial-Pak μ-Bondapak C_{18} column equilibrated with 80% solvent A/20% solvent B. After 5 min, a 60 min linear gradient was pumped to the column at 1.5 ml/min to give a final concentration of 30% solvent A/70% solvent B. Solvent A was 0.1% TFA and solvent B consisted of 80% acetonitrile/0.1% TFA. Fractions were freeze-dried and subject to radioimmunoassay.

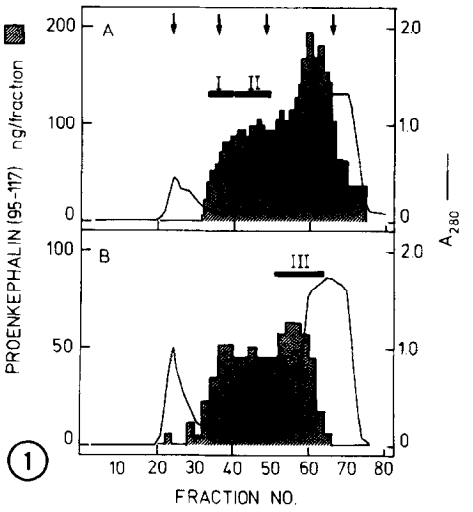
Affinity chromatography Proenkephalin(95-117), 14 mg was coupled to 1 g of CNBr-activated Sepharose 4B (13). Ethanolamine was used to block residual reactive groups. To purify antibodies specific for proenkephalin (95-117) the gamma-globulin fraction from 10 ml of antiserum (Rabbit 1) was obtained by ammonium sulfate precipitation followed by dialysis against PBS. After 2-fold dilution with PBS/2 mM PMSF this material was applied to a column containing the immobilized peptide. Specific antibodies were eluted as described by Sabol *et al.* (14). The affinity-purified antibodies (29% recovery of original binding capacity) were dialysed against 50 mM Tris-HCl, pH 7.5, concentrated using polyethylene glycol (M_r 20,000) and further dialysed against 0.1 M sodium phosphate, pH 7.5 before coupling to CNBr-activated Sepharose 4B (1g). After an overnight incubation unreacted sites were blocked with ethanolamine.

For affinity purification of fractions containing proenkephalin(95-117) immunoreactivity, peak I and peak II material was redissolved in PBS/1 mM PMSF and applied to a column containing affinity purified anti proenkephalin (95-117) antibodies coupled to Sepharose 4B. This was washed with PBS (10 ml), PBS/ 1.0 M NaCl (10 ml), and PBS (10 ml). Immunoreactive material was eluted using 0.5 M acetic acid (10 ml). In order to remove residual salts the eluate was applied to a C_{18} Sep-Pak (Waters Associates) equilibrated with 0.1% TFA. The cartridge was eluted with 80% acetonitrile / 0.1% TFA. Bovine serum albumin (200 μg) and 0.1% 2-mercaptoethanol were added prior to freeze-drying.

Affinity-purified material was redissolved in 50 mM Tris-HCl, pH 8.5 (0.5 ml). and divided into two equal aliquots. One of these was sequentially digested with trypsin followed by carboxypeptidase B (15). The Met-enkephalin and proenkephalin(95-117) immunoreactivity of each aliquot was determined by radioimmunoassay.

RESULTS

Antisera to a synthetic peptide corresponding to the 95-117 sequence of proenkephalin were used to develop a sensitive radioimmunoassay. At a final dilution of 1:3000, the antiserum (Rabbit 2) bound approximately 40 % of the tracer. At this dilution of antiserum the IC_{50} for the peptide was approximately 10 fmol. Preliminary work showed that acid extracts of both bovine adrenal medulla and purified chromaffin granules contained material which cross-reacted in the radioimmunoassay. Initial characterization of the immunoreactivity in these extracts was carried out by gel-filtration on Sephadex G-100 (Fig. 1 A,B) This showed the antisera to recognize material of high molecular weight (M_r approximately 5,000-



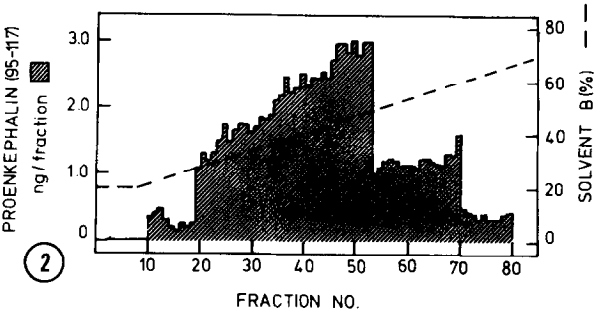


Fig. 1 Gel-filtration profile of proenkephalin(95-117) immunoreactivity in adrenal medulla. Acid extracts of adrenal medulla, 0.6 g (A) and purified chromaffin granules from 20 g medulla (B) were applied to a column (1.6 x 88 cm) of Sephadex G-100 in 1.0 M acetic acid/ 20 mM HCl/ 0.1% 2-mercaptoethanol. Aliquots of column fractions were subject to radioimmunoassay. Further analysis of peaks I, II and III were carried out as described in the text. The arrows indicate the elution positions of blue dextran, ovalbumin, ribonuclease and salt, respectively.

Fig. 2 HPLC profile of peak III immunoreactive material. Peak III from the acid extract of bovine adrenal medullary chromaffin granules (Fig. 1B) was recovered by freeze-drying. This was redissolved in 80% solvent A/20% solvent B (5 ml) and pumped onto a Radial-Pak μ-Bondapak C_{18} column (Waters Associates). After 5 minutes, a 60 minute gradient was pumped onto the column at 1.5 ml/min to give a final concentration of 30% solvent A/ 70% solvent B. Solvent A was 0.1% TFA and solvent B consisted of 80% acetonitrile/0.1% TFA. Fractions (45 seconds) were collected and aliquots taken for the proenkephalin(95-117) radioimmunoassay.

30,000). A similar distribution of immunoreactivity was seen in extracts of both medulla and purified chromaffin granules. Immunoreactive material in these extracts was pooled as indicated and designated peaks I, II and III. Analysis of peak III immunoreactivity by HPLC revealed multiple peaks of immunoreactivity. (Fig. 2).

Immunoreactive material present in peaks I and II was further purified on a column containing affinity purified antibodies to proenkephalin(95-117) coupled to Sepharose 4B. Serial dilutions of immunoaffinity purified material from peaks I and II produced displacement curves which were parallel to that obtained with the synthetic peptide used to raise the antisera (Fig. 3). Sequential digestion of immunoaffinity purified peak I and peak II material with trypsin and carboxypeptidase B generated Met-enkephalin immunoreactivity

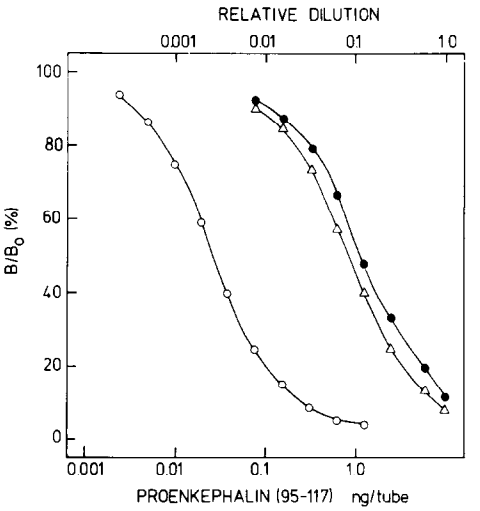


Fig. 3 Displacement curves of synthetic proenkephalin and affinity-purified peak I and peak II material. Material corresponding to peaks I and II from the acid extract of adrenal medulla (Fig. 1A) were purified by adsorption to and elution from column containing affinity-purified antibodies to proenkephalin(95-117) coupled to Sepharose 4B. Dilutions of affinity purified peak I (Δ-Δ) and peak II (●-●) material were compared in the radioimmunoassay with synthetic proenkephalin(95-117) (0-0).

(Table 1). This shows that the antisera against this synthetic peptide cross-react with high molecular weight EC proteins in adrenal medulla.

DISCUSSION

Recent work has shown that synthetic peptides are capable of eliciting antibodies that react with native proteins (16). Interestingly, some of these antibodies cannot be generated in response to the native protein. In the present study antibodies to a synthetic peptide corresponding to the 95-117

TABLE 1 SEQUENTIAL DIGESTION OF AFFINITY PURIFIED PEAK I AND PEAK II MATERIAL* WITH TRYPSIN AND CARBOXYPEPTIDASE B.

	MET-ENKEPHALIN (pmol)	
	I	II
Untreated	1.4	3.2
Trypsin and Carboxypeptidase B	23.0	18.6

*The yields of peak I and peak II material following affinity chromatography were 68 ng and 60 ng, respectively.

sequence of proenkephalin enabled the detection by radioimmunoassay of EC proteins in the molecular weight range of 5,000-30,000 present in bovine adrenal medulla. Of the large number of EC peptides which have been isolated from adrenal medulla, those with molecular weights of 18,200 12,600 and peptide F (M_r, 3600) contain residues corresponding to the 95-117 sequence of proenkephalin (3). Affinity purified peak I and peak II material gave parallel displacement curves to proenkephalin(95-117) in the radioimmunoassay. It was not possible to determine the number of copies of Met-enkephalin contained per mole of affinity purified peak I and peak II material as the relative potency of high molecular weight material has not yet been determined. Peak III material would be expected to contain peptide F. The multiple peaks of immunoreactivity resolved by HPLC indicate that a number of peptides present in chromaffin granules contain determinants present within residues 95-117 of proenkephalin. A similar distribution of immunoreactivity was observed following HPLC of peak III material from extracts of adrenal medulla (data not shown). Some of these peptides may be cleaved from peptide F in a manner similar to that described for the production of the non-opioid peptide corresponding to the 168-180 sequence of proenkephalin (17). A radioimmunoassay to peptide F has been used previously to characterize intermediates of proenkephalin in bovine hypothalamus and adrenal medulla (18) although it is not clear that the specificity is the same as that described in this study. During the course of this work, an antiserum generated against Met-enkephalin-Arg^6-Phe^7 was shown to recognize N-terminally extended forms of Met-enkephalin with variable affinity (14). Larger proteins derived from the N-terminus of proenkephalin have also been detected using an antiserum with determinants with the N-terminal 1-72 residues of proenkephalin (19).

In this study we have shown that an antiserum against a synthetic peptide recognizes high molecular weight proenkephalin-like material in bovine adrenal medulla. This antiserum also reacts with high molecular weight forms of proenkephalin in rat brain (Birch and Christie, unpublished).

This specificity will be of value in studies concerned with characterization of high molecular weight intermediates in the processing of proenkephalin as well as studies of enkephalin biosynthesis *in vitro*. Application of these antisera are validated by the present work.

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