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Identification of Methionine Enkephalin in the Bovine Cornea by Fast Atom Bombardment–Mass Spectrometry

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Native methionine enkephalin and methionine enkephalin generated proteolytically from a larger peptide were both identified and quantified from bovine cornea by negative ion fast atom bombardment–mass spectrometry. The corneal peptides were purified initially by reversed-phase high performance liquid chromatography and identified tentatively by radioreceptor assay and/or radioimmunoassay.

Key words: cornea; enkephalin; fast atom bombardment; mass spectrometry; methionine enkephalin; radioimmunoassay; radioreceptor assay.

1. Introduction

The relatively low concentration of neuropeptides within the nervous system has prompted a considerable amount of interest in developing sensitive yet accurate techniques by which to identify these compounds. The biochemical methods of radioreceptor assay (RRA) and radioimmunoassay (RIA), although yielding high sensitivity, do not possess a commensurately high level of accuracy because they deal with a response based on the secondary structure of the ligand being examined. However, maximum molecular specificity can be obtained readily by utilizing mass spectrometric methods to characterize and quantify neuropeptides: in this case, methionine enkephalin (ME) purified from bovine cornea.

ME, a pentapeptide (YGGFM), is very amenable for identification utilizing the desorption technique of fast atom bombardment–mass spectrometry (FAB–MS) (Barber et al., 1981). ME has been identified in many neuronal tissues and was characterized immunologically in the cornea for the first time in this laboratory. (Tinsley et al., 1988). Unambiguous identification, however, will be provided here by negative ion FAB–MS of corneal ME and proteolytically generated ME from a larger ME-containing peptide isolated from bovine cornea. A similar FAB–MS technique has been utilized in this laboratory to identify ME in human teeth (Tanzer et al., 1988).

2. Materials and Methods

Purification and Characterization of Corneal ME

The tissue acquisition, protein precipitation, Sep-Pak chromatography, gradient reversed-phase high performance liquid chromatography (RP-HPLC), isocratic RP-HPLC, and RIA procedures utilized with the bovine cornea were reported previously (Tinsley et al., 1988). The etorphine RRA for opioid-like receptor-activity followed a procedure also developed in this laboratory (Desiderio et al., 1988). The overall scheme is shown in Fig. 1.

Trypsinolysis of Fractions 42–43

A modified version of a trypsinolysis procedure (May et al., 1982) was used. A volume (100–300 μl of immobilized TPCK-treated trypsin (14 U ml^{-1}; Pierce, Rockford, IL) was rinsed three times with 4 ml of 50 mM Tris-HCl at pH 7·4 to remove the packaging glycerol. Fraction 42–43 from the gradient RP-HPLC of the bovine cornea, reconstituted in 2–3 ml of Tris-HCl, was added to the Tris-HCl-washed trypsin beads, stirred for 20 min at ambient temperature, and centrifuged. The supernatant was pipetted from the trypsin beads, and added to 100 μl of soluble carboxypeptidase B (175 U mg protein^{-1}; Sigma, St Louis, MO) for 30 min at ambient temperature. To filter the residue introduced by the trypsin beads and to eliminate the carboxypeptidase B, the supernatant was acidified to pH 3–6 by adding 3–10 μl of concentrated trifluoroacetic acid, followed by elution through a Sep-Pak cartridge.

FAB–MS

FAB–MS was performed on a VG (Manchester, U.K.) 7070E-HF double-focusing forward geometry (the electrostatic analyzer, E, precedes the magnetic sector, B) mass spectrometer equipped with a standard VG FAB ion source and an Ion Tech (Middlesex, U.K.) B11NF saddle-field fast atom gun. A FAB stainless-

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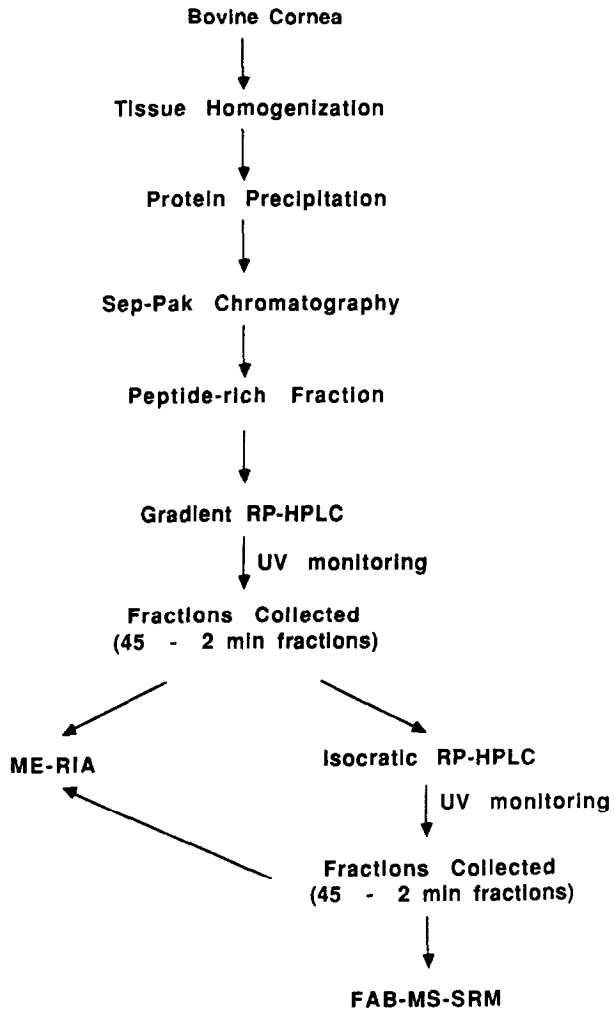

FIG. 1. Schematic representation of the methodology used to purify and to characterize corneal methionine enkephalin.

steel probe tip, with an appropriate angle of incidence, was fitted onto the end of a direct insertion probe. VG 11-2-50 M+ software was utilized for data acquisition.

Samples and standards were reconstituted in water–methanol (1:1, v/v) and applied to the FAB probe tip, which contained approximately 0·4 μl of the FAB matrix glycerol. To the matrix was added glutathione (1 nmol) and 20% NH_4OH, to enhance the detection sensitivity for ME (Tolun, Dass and Desiderio, 1987). The solvents were evaporated from the probe tip with an infrared lamp prior to insertion of the probe into the mass spectrometer. FAB ionization was affected by the impact of high energy (7 keV) xenon atoms. The FAB-generated sample ions were accelerated to a potential of 6 kV.

The B/E linked-field scan of the synthetic ME $[M-H]^-$ anion was used to obtain a spectrum containing amino acid sequence-determining ions. The unique and most abundant ion was the loss of the tyrosine side chain (107 Da) from the $[M-H]^-$ anion, to form a fragment ion of 465 Da (Dass and Desiderio, 1987). This FAB–B/E MS/MS method was utilized for unambiguous detection of ME, and was achieved by focusing the mass spectrometer on the $[M-H]^-$ anion of ME at 572 Da, followed by monitoring the amount of ion current corresponding to the 572–465 Da metastable transition by B/E linked-field selected reaction monitoring (SRM). An external calibration curve of the 572–465 Da transition (loss of 107 Da) was constructed with a series of RP-HPLC blanks spiked correspondingly with three dilutions (100, 150 and 200 ng) of synthetic ME. The calibration curve ($y = 25{\cdot}8x + 171$; $r = 0{\cdot}9680$) obtained from duplicate measurements was plotted as the ion current measured in digital-to-analog converter (DAC) units versus the amount of ME.

3. Results

The RRA results from bovine cornea (Fig. 2) indicate that ME (fractions 10–11), if present in the cornea, is below the detection limit of the RRA. The two areas of greatest receptor activity suggest the possible presence of a β-endorphin (β-End)-like peptide (fractions 37–39) and at least one opioid-like peptide in fractions 42–43. Trypsinolysis of fractions 42–43, followed by gradient RP-HPLC, generates early eluting components with receptor activity (data not shown), thus demonstrating that at least one peptide that eluted in fraction 42–43 is trypsin-sensitive and is probably an opioid-like peptide.

ME–RIA was used to improve assay sensitivity. The RIA results (Fig. 3) indicate not only the presence of ME-like immunoreactivity corresponding to the retention time of synthetic ME, but also immunoreactivity in fractions 42–43. The ME immunoreactive fractions in the gradient region corresponding to the retention time of synthetic ME were combined and purified further by isocratic RP-HPLC. After isocratic elution, a small aliquot from fractions corresponding to the retention time of synthetic ME demonstrated that more than 25 ng of ME-like immunoreactivity was available for MS analysis. The sample, representing more than 400 bovine corneas, was prepared for FAB–MS.

The possibility that the immunoreactivity and receptor activity in fractions 42–43 were at least partially due to a ME precursor was examined by attempting to generate ME by trypsinolysis of fractions 42–43, followed by carboxypeptidase B treatment to remove a carboxy-terminus basic amino acid residue (R or K). Therefore, fractions 42–43 were combined and treated with trypsin followed by carboxypeptidase B. The resultant mixture was eluted through a Sep-Pak, and the elute was subjected to gradient RP-HPLC. Immunoreactivity at the ME retention time was demonstrated after gradient elution, and the remainder of this sample was used for FAB–MS.

Negative ion FAB–MS–SRM, in which the transition corresponding to the loss of 107 Da from the $[M-H]^-$ anion (572 Da) to form the 465 Da ion was monitored, demonstrated a very low level of ion current (308 ± 25

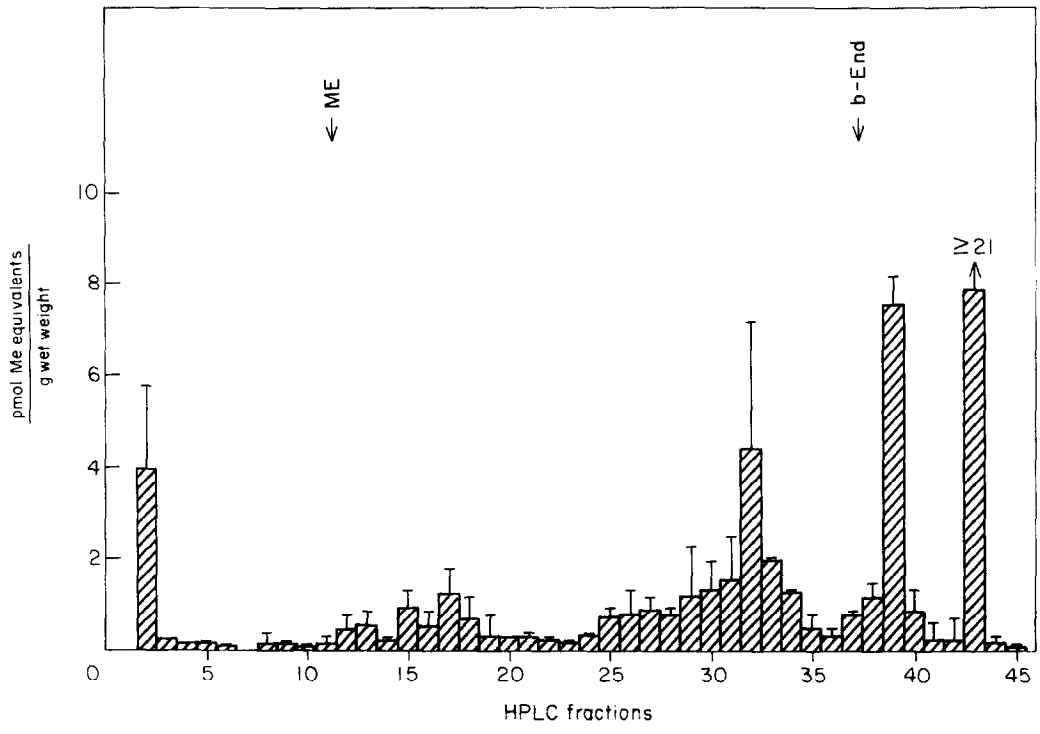

FIG. 2. [^{3}H]etorpine RRA profile of bovine whole cornea after acidified acetone extraction and gradient RP-HPLC. Fractions 1–45 from 45 fractions (2 min per fraction). Two separate RRAs were performed. The measurements were averaged; the lines represent the standard deviation of these two experiments. The down arrow (↓) denotes the retention time for that synthetic neuropeptide, as calibrated in a separate chromatogram and detected by UV. The up arrow (↑) signifies that the quantity indicated exceeded the upper detection limit of the RRA calibration curve.

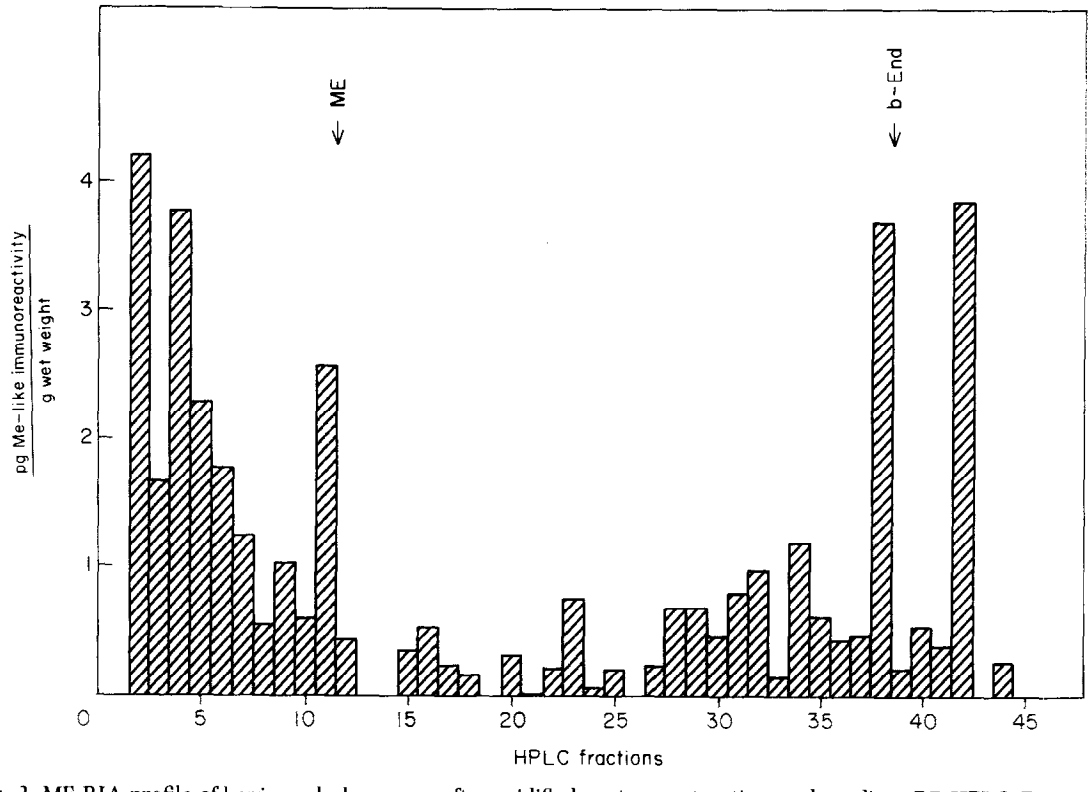

FIG. 3. ME-RIA profile of bovine whole cornea after acidified acetone extraction and gradient RP-HPLC. Fractions 1–45 from 45 fractions (2 min per fraction). The down arrow (↓) denotes the retention time for that synthetic neuropeptide, as calibrated in a separate chromatogram and detected by UV.

DAC units; $n = 3$) in the RP-HPLC blank. The RP-HPLC blanks were the same volume of isocratic eluant that was present in the corneal MS sample. The corneal sample was analyzed by monitoring the same SRM transition, and those data demonstrated a significant level (3650 ± 350 DAC units; $n = 3$) of ion current. Using the external calibration curve, the RP-HPLC blank yielded approximately 2 ng of ME, whereas the average of the three corneal samples yielded a value of 143 ng of ME (141 ng after correcting for the blank) present in the corneal sample.

FAB–MS–SRM of the combined sample corresponding to the ME-like immunoreactivity generated from enzyme treatment of fractions 42–43 yielded a value of 90 ng (2268 ± 110 DAC units; $n = 2$) of ME (87 ng after correcting for the blank). The equivalent RP-HPLC blanks measured 3 ng (335 ± 40 DAC units; $n = 2$) of ME. Thus, at least a portion of the immunoreactivity and receptor activity measured in fractions 42–43 of the gradient RP-HPLC was due to a ME-containing peptide(s).

4. Discussion

For unambiguous proof of a peptide's amino acid sequence, three or more MS sectors must be coupled to yield the technique of MS/MS or tandem MS (McLafferty, 1983). Whereas a two-sector E,B instrument, such as the VG 7070E-HF used in this research, cannot perform three- or four-sector MS/MS, the forward geometry of the VG 7070E-HF can be made to be effectively equivalent to tandem MS by utilizing the B/E linked-field scan mode (Jennings and Mason, 1983). During a B/E linked-field scan, the fixed accelerating potential is uncoupled from the electric sector, and the magnetic sector and the electric sector are scanned simultaneously, maintaining a constant B/E ratio. The B/E linked-field scan collects product ions from a mass-selected precursor ion, generally the $[M+H]+$ cation or the $[M-H]^-$ anion. A metastable reaction is a fragmentation that occurs, either unimolecularly (spontaneously) or collisionally-induced, while the ion traverses through a field-free region within the detection time-frame of the mass analyzer.

FAB–MS–SRM is the technique used in this research to obtain a high level of detection sensitivity and molecular specificity for analyzing endogenous ME. In SRM, instead of monitoring a complete B/E spectrum (a process that consumes precious sample that could be used more effectively by monitoring only one or a few unique sequence-determining ions), the ion current of one select product ion is monitored, where that fragment ion is generated by the fragmentation of a mass-selected precursor ion.

Thus, by using the highly sequence specific technique of B/E FAB–MS–SRM, this study has demonstrated unequivocally the presence of ME and a ME-containing peptide in bovine corneal tissue.

Acknowledgments

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