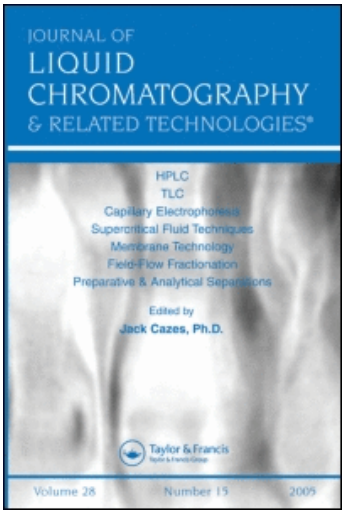

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Hubert E. May[a]; Francis S. Tanzer[b]; Genevieve H. Fridland[a]; Claire Wakelyn[a]; D. M. Desiderio[ac]
[a] Charles B. Stout Neuroscience Mass Spectrometry Laboratory, Memphis, Tennessee [b] Department of Oral Diagnosis, School of Dentistry, Memphis, Tennessee [c] Department of Neurology, School of Medicine, University of Tennessee, Memphis, Tennessee

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND PROTEOLYTIC ENZYME CHARACTERIZATION OF PEPTIDES IN TOOTH PULP EXTRACTS

Hubert E. May[1*], Francis S. Tanzer[2], Genevieve H. Fridland[1], Claire Wakelyn[1,3], and D.M. Desiderio[1]

[1]Charles B. Stout Neuroscience Mass Spectrometry Laboratory, [2]Department of Oral Diagnosis-School of Dentistry, and [3]Department of Neurology-School of Medicine, University of Tennessee Center for Health Sciences, 800 Madison Avenue, Memphis, Tennessee 38163

ABSTRACT

Metabolic profiles are obtained for peptides contained in tooth pulp extracts. To determine which high performance liquid chromatographic peaks are due to peptides, a series of proteolytic enzymes (chymotrypsin, trypsin, and carboxypeptidase A) are utilized. Results from treatment of extracts with immobilized enzymes demonstrate that virtually all peaks in this reverse phase system are due to peptides. This current study is a necessary component in a larger research program focusing on quantification of enkephalin- and endorphin-related peptides in biologic extracts including brain and tooth pulp tissue.

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*On sabbatical leave from Department of Biochemistry, Oral Roberts University, Tulsa, Oklahoma.

INTRODUCTION

The objective of this paper is to describe methodology developed in our laboratory to obtain in a fast and facile manner metabolic profiles of peptides in tooth pulp extracts for the first time by means of proteolytic enzymolysis followed by gradient elution reverse phase high performance liquid chromatography (RP-HPLC). Individual HPLC peaks can then be subjected in a separate study to peptide quantification or amino acid sequence determination.

Following discovery and structural elucidation by mass spectrometry (MS) of the first hypothalamic releasing factor TRF (1), other releasing factors were elucidated (2). Several endogenous neuropeptides were discovered recently which interact with the morphine receptor including beta-endorphin (3), leu-enkephalin (LE) (4), met-enkephalin (ME), dermorphin (5), dynorphin (6), and kyotorphin (7). Furthermore, metabolism of these peptides (8) and the constellation of larger molecular weight precursors are being studied (9). Radioimmunoassay (RIA) is utilized for quantification of these neuropeptides (10-12), but, as many workers are discovering, molecular specificity of antibodies for quantification of only one specific peptide in a biologic

matrix or extract is not sufficiently high for unambiguous measurement or metabolic studies (13, 14).

We are studying molecular mechanisms involved in nociception (pain) (15, 16), and especially processes involving the trigeminal/fifth cranial nerve system. Tooth pulp is naturally suited as a model for study of molecular factors involved in pain because pain is considered to be the only output emanating from tooth pulp tissue independent of stimulus (cold, heat, mechanical, etc.)

RP-HPLC plays a pivotal role in studies of neuropeptides extracted from tooth pulp (15, 16), CSF, and brain tissue (17-20) and offers advantages of high resolution, speed, and sensitivity towards peptides. RP-HPLC, coupled with the unique molecular specificity offered by MS, is utilized for quantification of enkephalins in canine brain tissue extracts including spinal cord (18), hypothalamus (19), thalamus (15), and caudate nucleus (20). Both field desorption (FD) (15-20) and fast atom bombardment (FAB) (21, 22) MS are used for study of neuropeptides. FD is useful for quantitative and FAB for qualitative (amino acid sequence) analysis. Furthermore, collision activation and linked scanning MS analysis of FD- or FAB-produced molecular ions, generally $(M+H)^+$ ions,

provides amino acid sequence data of peptides (23, 24). A more limited metabolic profile of the peptide fraction has been obtained for brain tissue with isocratic RP-HPLC (15-20). Gradient HPLC is useful for qualitative analysis of peptides in a tissue extract while isocratic HPLC is suited for quantitative analysis of individual peptides and analytic purification of (previous gradient-analyzed) chemical and enzymolysis product mixtures.

The purpose of this paper is to illustrate for the first time gradient RP-HPLC chromatographic characterization of the peptide-rich fraction from tooth pulp tissue extract. This characterization component in our overall research program is consonant with our purposes of measurement of known opioid neuropeptides in biologic tissues and fluids in addition to structural elucidation of extracted peptides of unknown amino acid sequence. This study includes determination of metabolic profiles of peptides in tooth pulp tissue and presentation of data substantiating the hypothesis that specific RP-HPLC peaks are sensitive to proteolytic enzymolysis using immobilized alpha-chymotrypsin, carboxypeptidase A, and trypsin treatment in that tissue.

EXPERIMENTAL

Mongrel dogs (15-25 kg) are utilized for this study. After pentobarbital treatment, a femoral artery is catheterized for exsanguination. The four canine cuspid teeth are removed within minutes, tooth pulp obtained *in situ*, and tissue stored in liquid nitrogen to avoid chemical and/or enzymatic degradation of peptides and precursors. Typically, a total of 3-400 mg tooth pulp tissue is obtained from the four cuspid teeth of a 1-2 year old animal.

Extraction.

Combined tooth pulps are homogenized in cold 1.0 N HAc with a Polytron (17). Following centrifugation to remove protein precipitate, tissue extract dissolved in TFA (0.5%) is placed on a RP octadecylsilyl mini-column (Sep-Pak, Waters, Milford, MA) the peptide fraction is eluted with 80% acetonitrile and subjected to gradient RP-HPLC.

Enzymolysis.

Alpha-chymotrypsin on cellulose and carboxypeptidase A on agarose were purchased from Sigma Chemical Co. (St. Louis, MO). Trypsin (TPCK-treated) on 4% beaded agarose was purchased from Pierce Chemical Co. (Rockford, IL).

A 100 µl sample of lyophilized tooth pulp peptide extract (250 µg dry solid equivalent to 70 mg tissue) in TEAP

(0.06 M, pH 2.12) is adjusted to pH 8.5 with 1-2 µl concentrated ammonium hydroxide. Sample is stirred one hour with appropriate immobilized peptidase (1.5 units trypsin or 3 units of alpha-chymotrypsin or carboxypeptidase A). One-half of the sample is analyzed by RP-HPLC as described below.

Reverse-Phase High Performance Liquid Chromatography.

The HPLC chromatographic system is from Waters Associates (two Model 6000A pumps, a Model 660 solvent programmer, and a Model 450 variable wavelength detector). A µBondapak C-18 column, ten micron sphere size, follows a guard column packed with C-18 reverse phase packing (Corasil, 37-50 µ). Typical chromatography conditions are: UV detector - 200 nm and flow rate - 1.5 ml min^{-1} using either 0.25N, pH 2.12 triethylamine-phosphoric acid (TEAP) (20, 25) or 0.4M, pH 3.15 triethylamine-formic acid (TEAF) (26) buffer mixed with acetonitrile. The volatile TEAF buffer is used in isocratic RP-HPLC when sample collection is required for subsequent MS (15-20) or RIA analysis. Due to high absorbance of the TEAF buffer at 200 nm, its use in gradients is impractical due to large baseline shifts at detector sensitivities required. Thus TEAP, having much lower UV absorbance at 200 nm, is used in gradients. The gradient is

composed of sequential isocratic and gradient elution profiles (see figures): isocratic elution with 5% acetonitrile:TEAP (pH 2.12) for 15 minutes; gradient from 5-12% in seven minutes (1% min^{-1}); followed by isocratic elution at 12% to 50 minutes. The majority of peptides are eluted by a gradient from 12-50% in 76 minutes (0.5% min^{-1}). Hydrophobicity increases from 5% to 50% for the organic modifier acetonitrile over the entire elution profile.

RESULTS

Figure 1A contains the RP-HPLC chromatogram of a tooth pulp extract and demonstrates that most peptides elute at higher hydrophobicity (% organic modifier). This RP-HPLC chromatogram presents for the first time a metabolic profile of peptides found in tooth pulp extracts. Shaded areas in Figure 1A indicate peaks which disappear after treatment with trypsin. Figure 1B contains the RP-HPLC chromatogram of the same tooth pulp extract treated with trypsin. Shaded areas indicate peaks which appear after trypsin treatment.

Figure 2A contains the RP-HPLC chromatogram of another fraction of the same tooth pulp extract before treatment with alpha-chymotrypsin. (Figures 1A, 2A, and 3A are of one tooth pulp extract divided into three equal portions where

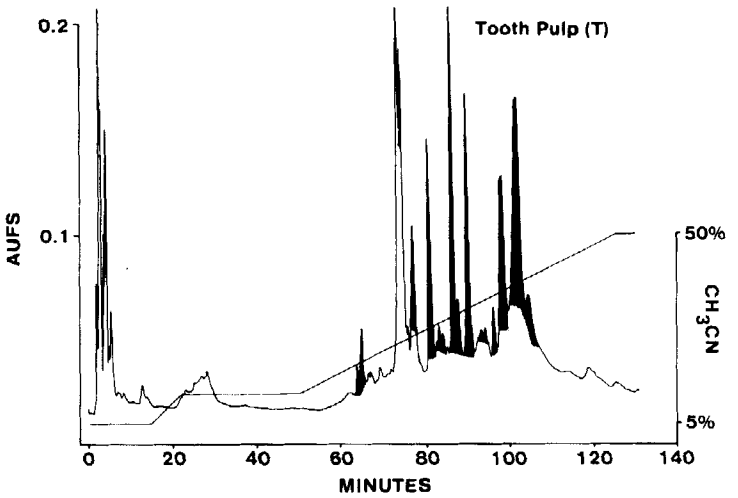

Figure 1A. RP-HPLC chromatogram of tooth pulp extract. Control sample which will be subjected to trypsin treatment. Experimental conditions: one μBondapak C_{18} column; 200 nm; 1.5 ml min^{-1}; 0.1 AUFS Gradient profile noted by line on chromatogram. Shaded peaks indicate peaks which disappear following trypsin treatment.

different shading illustrates specificity towards an individual proteolytic enzyme). An internal standard (YGGFM, for separate FDMS quantification study) is indicated at 54 minutes in Figure 2A. Shaded areas in Figure 2A indicate peaks which disappear after alpha-chymotrypsin treatment. Fewer peaks are hydrolyzed by alpha-chymotrypsin vis-a-vis trypsin. Figure 2B contains the HPLC chromatogram of α chymotrypsin-treated tissue extract. Shaded peaks indicate peaks appearing after chymotrypsin treatment.

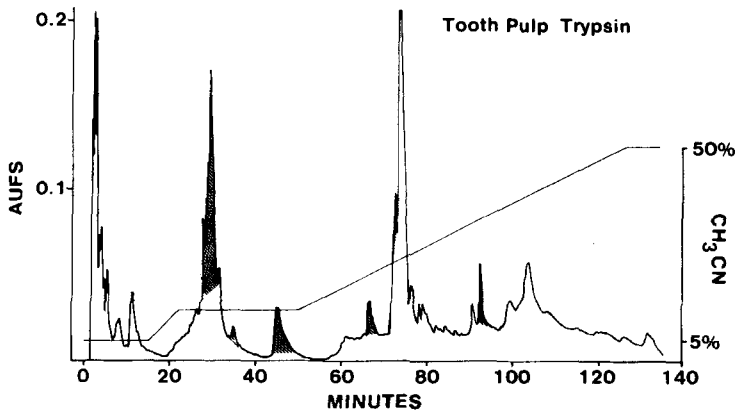

Figure 1B. RP-HPLC chromatogram of tooth pulp extract following trypsin treatment. Shaded areas indicate new peaks vis-a-vis Figure 1A. See Figure 1A legend for experimental details.

Figure 3A contains the HPLC chromatogram of tooth pulp tissue extract indicating peaks which disappear following carboxypeptidase A treatment. Figure 3B contains the HPLC chromatogram of carboxypeptidase A-treated tooth pulp tissue extract; hatched areas indicate new peaks.

DISCUSSION

For the first time, the definition of the previously employed "peptide-rich fraction" term (15-20) from biologic tissue is structurally substantiated and is based upon the fact that virtually every RP-HPLC peak in the untreated

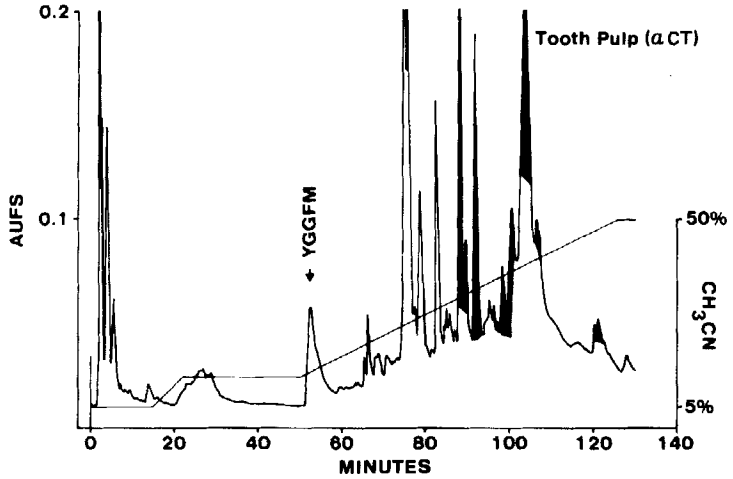

Figure 2A. RP-HPLC chromatogram of tooth pulp extract. Control sample which will be subjected to chymotrypsin (CT) treatment. Shaded areas indicate peaks which disappear following chymotrypsin treatment. See Figure 1A legend for experimental details.

peptide fraction of Figures 1A, 2A, and 3A shifts to a region of lower hydrophobicity on a RP-HPLC column following proteolytic enzyme alpha-chymotrypsin has less of an effect on this peptide fraction; fewer fragments result from treatment of this peptide extract which indicates fewer aromatic residues (Y, W, F) are present in that fraction compared to basic amino acids. Enzyme treatment with carboxypeptidase A produces fragmentation comparable in extent as with trypsin, which is not a surprising result because the C-terminus of these peptides will be continuously

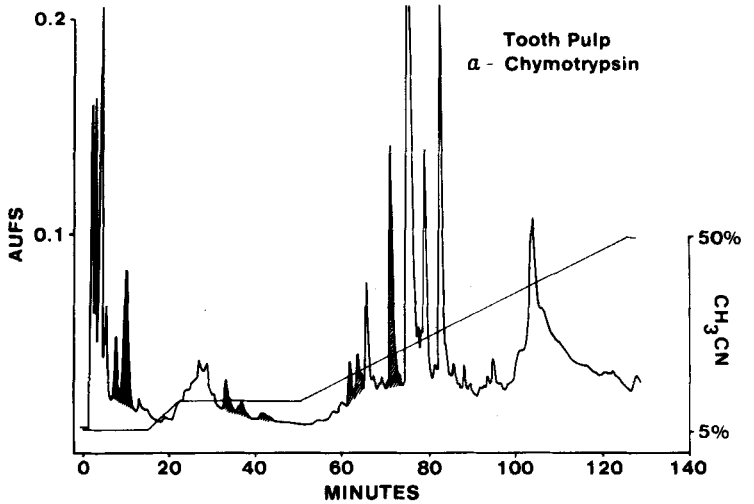

Figure 2B. RP-HPLC chromatogram of tooth pulp extract following chymotrypsin treatment. Shaded areas indicate new peaks following CT treatment. See Figure 1A legend for experimental details.

digested by an exopeptidase and therefore all peptides with an unblocked C-terminus will be affected.

Nothing further can be stated at this stage of our research concerning the genesis of the peptide peaks found in this study. These peptides may arise from neuropeptides, other peptides, or proteins (structural, enzymes, large fragments) not completely precipitated with acetic acid. Further studies underway are addressing this question. However, the endorphins LE and ME have been isolated and quantified in tooth pulp.

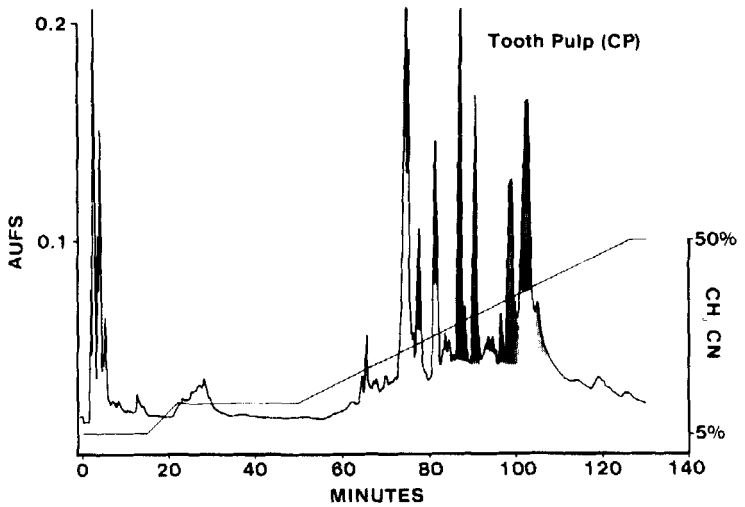

Figure 3A. RP-HPLC chromatogram of tooth pulp extract. Control to be subjected to carboxypeptidase A (CP) treatment. Shaded areas indicate peaks which disappear following CP treatment. See Figure 1A legend for experimental details.

It is also interesting to note the high resolution of the RP-HPLC buffer system utilized in this study. Good base-line resolution results for almost all peaks and sharp peaks occur throughout the chromatogram.

To assist in purification and separation of individual neuropeptides, RP-HPLC with an appropriate buffer plays a pivotal role due to speed, high resolution, and sensitivity. Femtomole amounts of somatostatin are determined by UV detection (210 nm) of the pentadecapeptide (26). However,

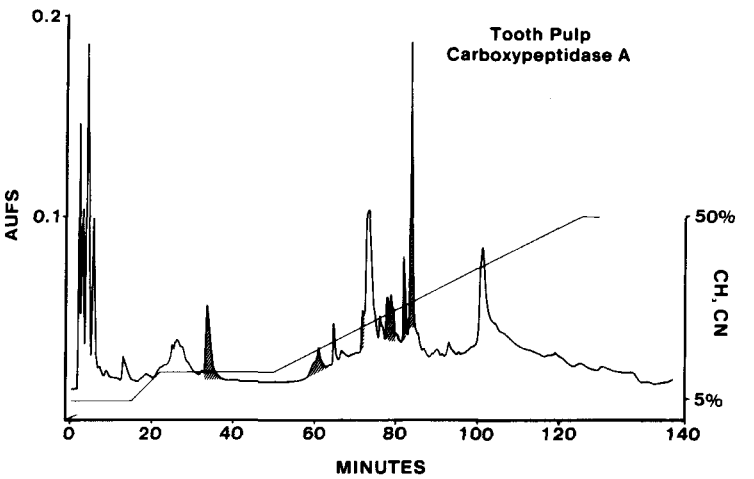

Figure 3B. RP-HPLC chromatogram of tooth pulp following CP treatment. Shaded areas indicate new peaks following CP treatment. See Figure 1A legend for experimentals details.

the philosophy of this laboratory is to attach a structurally unambiguous molecular parameter (molecular weight) to quantification of a peptide in a RP-HPLC peak (15-20). Towards this end, FD and/or FAB mass spectrometry is employed. With this combined RP-HPLC/MS methodology, pmol amounts of LE and ME g^{-1} of wet weight tissue have been quantified in several brain regions and tooth pulp tissue extracts, a measurement equivalent to part per billion sensitivity (15-20).

Even though molecular specificity of RIA, bioassay, and receptor analytical methods is presumed to be high, several

workers are discovering that this is not the case (13, 14, 28). For example, even though some bioassays exhibit high sensitivity towards a specific substrate, several disadvantages exist for this type of assay. Some bioassays are only semiquantitative at best: large variations occur between tissue preparations and within one tissue preparation. For example with leukotrienes, bioassay is not structurally specific; several agents evoke a bioassay response (28).

The advantage of MS methodology as a unique detector for HPLC versus other assay methods is structural certainty that the peptide we think we are measuring is the peptide actually being measured. This is a concept which is easily stated, but not readily employed. For those extracted peptides which are too large in molecular weight (for example, beta-endorphin, molecular weight 3624) for MS analysis of the intact peptide, immobilized enzymolysis treatment of the peptide, followed by gradient and isocratic RP-HPLC purification, will provide enzymic fractions amenable to MS quantification methods.

Another use for RP-HPLC/enzymic characterization of peptides extracted from tooth pulp tissue involves total molecular characterization of the peaks. Towards that end,

peaks to be characterized first are those displaying biologic activity or those HPLC peaks changing concentration following chosen physiologic alterations (croton oil, 27). A "screen" utilizing RIA antibodies raised against ME, LE, beta-endorphin, and other neuropeptides may be utilized (29).

The importance of this type of study can be realized by consideration of the following facts. Whenever separation is obtained from a peptide extract of a tissue by gradient elution, structural characterization and quantification will be performed on those biologically important HPLC peaks. Three methods exist for determining biologic activity of a HPLC peak: bioassay, cross-reactivity with antibodies raised to specific neuropeptides, and a combination of enzymic-chemical methodology. This paper illustrated the last methodology. For example, it is known that adrenal proenkephalins exist and have molecular weights between 5,000-30,000 daltons. Six ME to one LE sequence are found in adrenal proenkephalins (9). Brain proenkephalins have a molecular weight ranging from 5-90,000 in molecular weight and the ME to LE ratio is approximately one (30). It is also known that N-acetyl beta-endorphin$_{1-27}$ is the predominant molecular form in rat pituitary (31). This peptide is the highest immunoreactive species in that tissue. However, this

peptide is inactive as an opioid peptide. Tyrosine-sulphated leu-enkephalin may exist as 50% of the proenkephalin form in the brain proenkephalin (30). Furthermore, the primary structure of the bovine proenkephalin messenger RNA has been elucidated and the predicted amino acid sequence (Fig. 4) known (9). Six met-enkephalin residues are bracketed at both termini by either K-K, K-R, or R-R dipeptides indicating trypsin-like peptidase sensitivity would produce ME and LE opioid peptides. It is not known now whether tooth pulp tissue proenkephalins exist. We are now in a position to test the hypothesis that trypsin treatment of the tissue extract followed by cyanogen bromide treatment will produce the total available store of tooth pulp tissue of met-enkephalin. We propose to quantify the total tissue

MARFLGLCTWLLALGPGLLATVRAECSQDC-
ATCSYRLARPTDLNPLACTLESEGKLPSLK-
TWETCKELLQLTKLELPPDATSALSKQEES-
HLLAKK[YGGFM]KR[YGGFM]KKMDELYPLEVE-
EEANGGEVLGKR[YGGFM]KKDAEEDDGLGNS-
SNLLKELLGALDQREGSLHQEGSDAEDVSK-
R[YGGFMRGL]KRSPHLEDETKELQKR[YGGFM]-
RRVGRPEWWMDYQKR[YGGFL]KRFAEPLPSE-
EEGESYSKEVPEMEKR[YGGFMRF]

Bovine Adrenal preproenkephalin

Figure 4. Amino acid sequence data (single letter code) obtained for the gene product from DNA sequencing of bovine adrenal preproenkephalin.

store for bioavailability of met-enkephalin from brain proenkephalin. The ability to quantify available stores of met-enkephalin, which could be metabolized for bio-availability from brain proenkephalins, provides an indicator of that organism's tissue total capacity to deal with pain.

CONCLUSIONS

Mini-column (sep-pak) effluent treated with immobilized trypsin, chymotrypsin, or carboxypeptidase establishes for the first time the fact that these RP-HPLC peaks are peptides.

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