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THE COMBINED USE OF SIZE-EXCLUSION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR CHARACTERIZATION OF β-ENDORPHIN PROCESSING PATHWAYS

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SUMMARY

A method is described for the separation and analysis of multiple molecular forms of immunoreactive β-endorphin and its α-N-acetylated congeners by a combination of reversed-phase and size-exclusion high-performance liquid chromatography coupled with two specific radioimmunoassays. Both chromatographic procedures are fast (< 50 min per analysis) providing good resolution and high recovery (> 90%). The solvents used in both systems are ultraviolet transparent (< 214 nm), non-corrosive, low salt (< 0.05 *M*) and after evaporation fully compatible with subsequent radioimmunoassay. We have evaluated these techniques using both synthetic and purified peptide standards and have applied these procedures to characterize immunoreactive β-endorphin and α-N-acetylendorphin in rat and sheep pituitary extracts, and the low levels found in sheep hypothalamus and rat ovary. These chromatographic procedures are not only applicable to the study of pro-opiomelanocortin-derived peptides, but also could be employed to examine the processing pathways of other biologically active polypeptides, in both central and peripheral tissue extracts.

INTRODUCTION

The biogenesis of all biologically active peptides so far described, with the exception of the dipeptide β-carnosine, is via a high-molecular-mass polypeptide precursor. Typically, the active sequence within the precursor is flanked by a pair of basic amino acid residues and is liberated following a tryptic-like cleavage at these discrete sites. The maturation process may continue with a carboxypeptidase trimming of the COOH terminally extended basic residues and other minor or major structural modifications. For example, β-endorphin (βEP), the extreme COOH terminal 31 amino acid sequence of pro-opiomelanocortin (POMC), can undergo extensive post-translational processing which markedly affects the strong opiate activity of the peptide. These modifications occur as specific cleavages in

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the COOH terminal region of the peptide [1,2] and/or α-N-acetylation (Nac) of the NH_2 terminal tyrosyl residue [3]. Such processing pathways are usually tissue-specific [4–6] and often species-specific [7–9].

Studies to elucidate these complex POMC processing patterns in the pituitary gland, or in the central nervous system (CNS) and peripheral tissues where levels are much lower, are only possible by the use of specific radioimmunoassays (RIA). The successful identification and quantitation of these closely related species is invariably rendered difficult by the lack of uniquely specific antisera, and can therefore only be achieved after the chromatographic separation of cross-reacting components. Reversed-phase high-performance liquid chromatography (RP-HPLC) had been used extensively in both purifying multiple peptide forms [10–12] and in combination with RIA has proved an invaluable tool in the study of tissue- and species-specific peptide biosynthetic pathways [4,7,9,13–16]. The excellent resolving power and high recovery of bioactive peptides by RP-HPLC is well established [10–12,16–18], though the separation and recovery of larger precursor and intermediate forms by RP-HPLC can be difficult. These problems can be overcome to some extent by manipulating the ionic component in the mobile phase [10,12] and/or by using columns with increased pore size [19], altered alkyl chain length or type [20]. These separations are invariably achieved at the expense of resolution of smaller, subtly modified fragments; in addition the eluting buffers are frequently not totally compatible with the subsequent bioassay or RIA of collected fractions.

In this report we describe the combination of high-performance size-exclusion chromatography (HPSEC) and RP-HPLC for the rapid separation and high recovery of βEP, its molecular congeners and precursor polypeptides. We also describe the application of these procedures for the characterization of immunoreactive βEP (ir-βEP) and ir-NacEP in rat and sheep pituitary tissue, sheep hypothalamus, and also the low levels of ir-βEP in the immature rat ovary.

EXPERIMENTAL

Equipment

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system was used comprising two 510 pumps, a Model 680 gradient controller, U6K injector and a Model 441 fixed-wavelength detector (214 nm). The size exclusion column employed was a TSK S 2000 GW (60 cm$\times$7.5 mm I.D., Toyo Soda, Tokyo, Japan) and for reversed-phase, a Nova Pak C_{18} cartridge radially compressed in a Z-module compression system (Waters Assoc.). Fractions were collected using a LKB Helirac Model 2212 programmable fraction collector (LKB, Bromma, Sweden). Collected fractions were evaporated to dryness using a Speedivac centifugal vacuum evaporator (Savant Instruments, New York, NY, U.S.A.) and then reconstituted in RIA buffer.

Reagents

High-purity acetonitrile (UV cutoff 190 nm) was obtained from Waters Assoc. Disodium hydrogen phosphate and sodium dihydrogen phosphate (analytical grade) were obtained from Ajax Chemicals (Sydney, Australia). Trifluoroacetic

acid (TFA) and acetic acid (chromatography grade) were supplied by BDH (Poole, U.K.). Water was doubly glass-distilled and passed through a Milli-Q reagent water system (Millipore, Bedford, MA, U.S.A.). All the synthetic peptides were obtained from Peninsula Labs. (San Carlos, CA, U.S.A.). Ovine β-lipotropin (βLPH) (purified to homogeneity) was generously supplied by Dr. C.H. Li (San Francisco, CA, U.S.A.).

HPSEC

The mobile phase, 0.05 *M* sodium phosphate (Sørenson pH 5.8) containing 20% acetonitrile (to prevent both peptide aggregation and non-specific interactions with the column matrix) was degassed under vacuum, delivered at a flow-rate of 0.5 ml/min, and monitored at 214 nm (1.0 a.u.f.s.). Prior to injection both samples and standards were prepared in an aliquot of the mobile phase (500 μl) and applied to the system. The retention times of synthetic (βEP) and highly purified (βLPH) standards were calculated from the UV recording trace (0.5 cm/min). Fractions (0.5 min) were collected for either the radiolabelled standards ($\pm 10^5$ cpm ^{125}I) and counted, or for the tissue extracts which were dried and assayed for ir-βEP.

RP-HPLC

A 200-μl volume of the oxidized (50% acetic acid containing 10^{-3} *M* sodium periodate) standard βEP mixture containing αEP, Nacα EP, γEP, Nacγ EP, βEP_{1-31}, $NacEP_{1-31}$, βEP_{1-27} and $NacEP_{1-27}$ (each 5 μg) was applied to the column. The separations were achieved using a linear gradient of 30–80% of solvent B over 30 min at a flow-rate of 1 ml/min. Solvent A consisted of 11 m*M* TFA–2.6 m*M* acetic acid (added to balance the UV absorbance against that of the organic phase) and solvent B of 70% acetonitrile containing 11 m*M* TFA. Tissue extracts were reconstituted (500 μl) in oxidant (50% acetic acid containing 10^{-3} *M* sodium periodate) and injected (the anterior pituitary and neuro-intermediate lobe extracts from both rat and sheep were diluted prior to injection; details in figure legends); fractions (0.5 ml) were collected into a carrier solution (50 μl, 20% mannitol containing 100 μg polypep), dried and reconstituted in RIA buffer prior to assay. All solvents were filtered (0.45 μm) and degassed under vacuum immediately before use.

Tissue extraction

Tissues were rapidly removed and placed into liquid nitrogen prior to extraction in either ice-cold 2 *M* acetic acid [neuro-intermediate lobe (NIL), anterior pituitary (AP), hypothalamus (HT)] or 0.1 *M* hydrochloric acid (ovary). They were then homogenized (two 5-s bursts on a Polytron), centrifuged (20 000 *g*, 30 min) and the supernatants further purified by passage through C_{18} Sep-Pak cartridges (Waters Assoc.) as described previously [21]. Partial oxidation of the methionine residues in βEP often occurs during the extraction procedure; the differential elution of the reduced and oxidised forms of βEP complicates the chromatogram. Therefore, after drying, the Sep-Pak eluates were reconstituted in 500 μl 50% acetic acid containing 10^{-3} *M* sodium periodate and allowed to

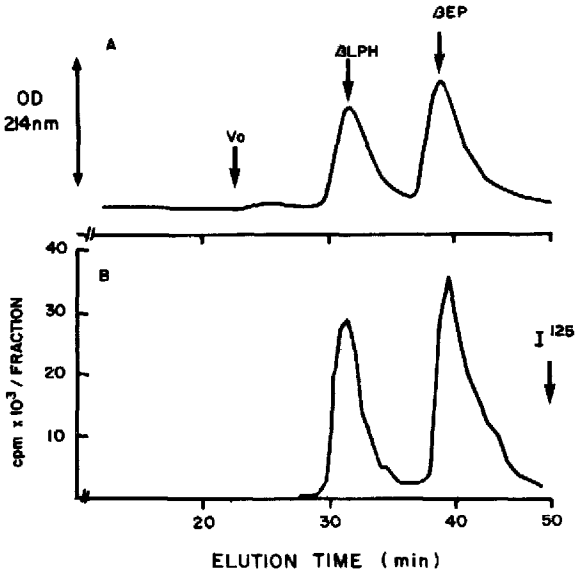

Fig. 1. Size-exclusion separation of synthetic ovine βEP (10 μg) and purified ovine βLPH (10 μg) (A) and ^{125}I-radiolabelled ovine βEP and ovine βLPH (B) using a TSK G 2000 SW column eluted with 0.05 *M* phosphate, pH 5.8, containing 20% acetonitrile (0.5 ml/min), monitored at 214 nm (0.5 a.u.f.s.). V_0 was calculated using Blue dextran.

stand at room temperature for 30 min, thus allowing the quantitative conversion of the methionine residues to their sulfoxide form prior to injection.

Radioimmunoassay

Two complementary antisera, R56 (βEP) and R92 (NacEP), raised against synthetic ovine βEP_{1-31} and ovine NacβEP_{1-27} respectively, were used for RIA as previously described [21]. R56 recognizes residues 18–26 of βEP and its α-N-acetylated derivatives, and thus βLPH equivalently on a molar basis, but fails to recognize αEP, γEP or the α-N-acetylated forms of these shorter peptides; sensitivity is routinely <4 pg per tube. The R92 antiserum does not recognize α-melanotropin (αMSH), any of the enkephalins or any non-acetylated POMC derived peptides, and specifically has a cross-reactivity of less than 0.15% for βEP_{1-31}, αEP or γEP. The antibody, however, cross-reacts with NacβEP and longer acetylated forms 100% on a molar basis; sensitivity is <2 pg per tube.

RESULTS

HPSEC

A low-salt (0.05 *M* phosphate buffer, pH 5.8) mobile phase containing 20% acetonitrile at a flow-rate of 0.5 ml/min on a TSK G 2000 SW (size-exclusion column) gave a reproducible, well resolved separation between synthetic ovine βEP and purified ovine βLPH (Fig. 1A) and also between low levels of radiolabelled ovine βEP and ovine βLPH applied similarly to the column (Fig. 1B). A calibration curve constructed with protein molecular weight standards within the

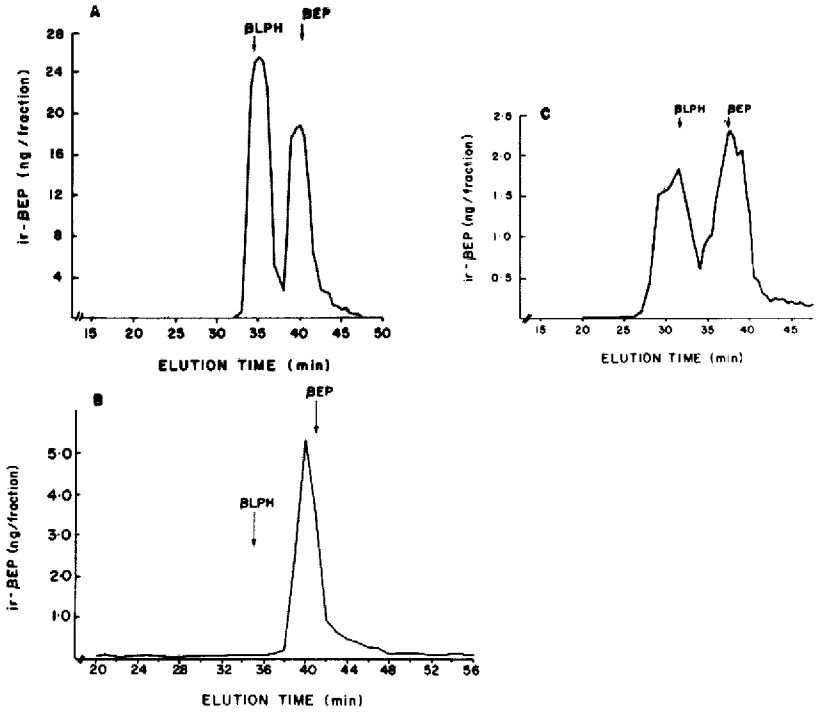

Fig. 2. Elution profiles of ir-βEP from extracts of rat AP (A), rat NIL (B) and rat ovary (C) chromatographed on a TSK G 2000 SW column (elution conditions as Fig. 1). The AP and NIL extracts were diluted 1:10 and 1:100, respectively ($n=3$), the ovarian extract was applied neat ($n=30$). The retention times of synthetic ovine βEP and purified ovine βLPH are arrowed.

recommended column fractionation range (500–60 000 daltons) was not completely linear, curving down in the lower molecular mass region (data not shown). This is possibly due to the non-denaturing nature of the mobile phase. However, our aim was the optimal separation of the two cross-reacting species βEP and βLPH (molecular mass 3500 and 11 500 daltons, respectively), which are poorly resolved by RP-HPLC [22]. The recovery of both non-radioactive and ^{125}I-radiolabelled βLPH and βEP was consistently greater than 90%.

Acid extracts of rat AP and NIL were applied to the column and fractions subsequently assayed for ir-βEP (Fig. 2A and B). Both tissues exhibited immunoreactive material corresponding in size to βEP, this being the only significant immunoreactive form in the rat NIL extract. The rat AP, however, contained two peaks of immunoreactivity, the major peak eluting in the position of βLPH with a slightly smaller peak migrating with βEP. To study POMC processing in a peripheral tissue we examined the immature (<30 days) rat ovary, which is also capable of POMC synthesis albeit at very low levels (ir-βEP <2 ng/g). The ovarian extract contained two quite similar sized peaks, one eluting as βEP and one as βLPH (Fig. 2C).

RP-HPLC

A linear gradient of 30–80% B over 30 min at 1 ml/min was sufficient to resolve all the synthetic ovine βEP standards (Fig. 3). In this system, however, βLPH

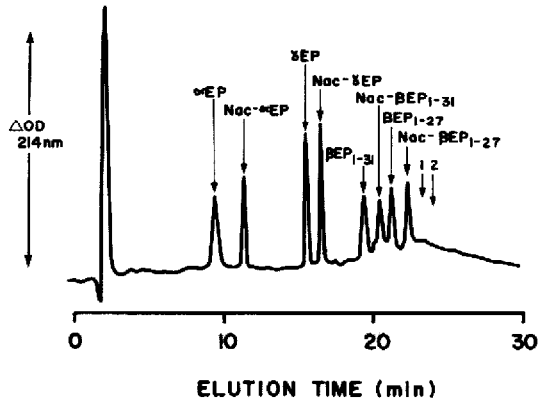

Fig. 3. Separation of synthetic ovine βEP-related peptide standards (all 5 μg) on a Nova Pak C_{18} radially compressed column, eluted with a linear gradient of 30–80% B (solvents A and B are described in Experimental) over 30 min at a flow-rate of 1 ml/min, monitored at 214 nm (0.5 a.u.f.s.). The predicted elution positions [23] of βEP_{1-23} and $NacEP_{1-26}$ are marked 1 and 2, respectively.

and βEP are not resolved from each other. Therefore, to further characterize the precise molecular nature of ir-βEP in the rat AP, NIL and ovary, either extracts (NIL), or a pool of the size exclusion column fractions corresponding to the area of eluting ir-βEP (AP and ovary) were subjected to RP-HPLC and the collected fractions assayed with the βEP antiserum (R56). The AP and NIL profiles were also assayed for NacEP (below detection limits in the ovarian extract) with the antiserum (R92), which specifically recognises the α-N-acetylated N-terminus. Extracts of sheep AP, NIL and HT were also applied directly to the RP-HPLC column and assayed for ir-NacEP (AP and NIL, non-detected in HT) and ir-βEP (HT).

The major immunoreactive βEP species in the rat AP extract (size exclusion fractions 38–45) were βEP_{1-31}, βEP_{1-27}, and βEP_{1-26} (Fig. 4A). In the rat NIL, however, using a similar size-exclusion cut, the profile showed the acetylated derivatives, $NacEP_{1-31}$, $NacEP_{1-27}$ and $NacEP_{1-26}$ as the dominant molecular forms (Fig. 4B); these data were later confirmed using the N-acetyl-specific RIA. Both the sheep hypothalamic extracts and the ovarian cut (fractions 38–45) had no detectable ir-NacEP, indicating perhaps that α-N-acetylation occurs principally in the pituitary. The ovarian profile (Fig. 4C) contained only one peak of immunoreactivity, eluting with βEP_{1-31}. Similarly the major immunoreactive species in the sheep HT extract was also βEP_{1-31} (Fig. 4D), though two minor peaks corresponding to βEP_{1-27} and βEP_{1-26} were also present in the chromatogram.

The ir-NacEP profile of both rat and sheep NIL (Fig. 5B and D) contained significant levels of the C terminally shortened forms, $NacEP_{1-17}$ (Nacγ EP) and $NacEP_{1-16}$ (Nacα EP), as well as the longer molecular forms $NacEP_{1-31}$, $NacEP_{1-27}$ and $NacEP_{1-26}$. The βEP in the rat anterior pituitary is only marginally acetylated (<5%), the only forms detected being $NacEP_{1-31}$, $NacEP_{1-27}$ and $NacEP_{1-26}$ (Fig. 5A). In the sheep, however, considerably more βEP-like mate-

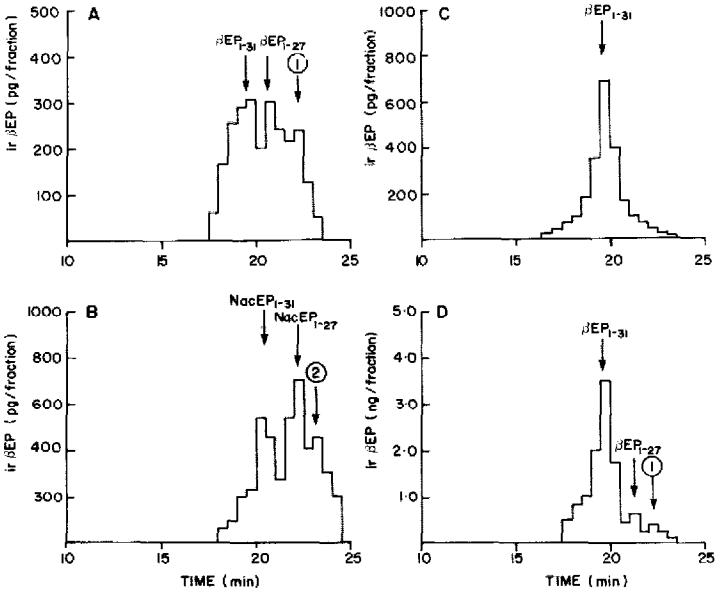

Fig. 4. RP-HPLC ir-βEP elution profiles of reconstituted HPSEC fractions 38-45 of rat AP (A), extract of rat NIL (diluted 1:100) (B), HPSEC fractions 38-45 of rat ovary (C) and sheep HT extract (D). The elution position of synthetic standards are arrowed 1 and 2 as in Fig. 3.

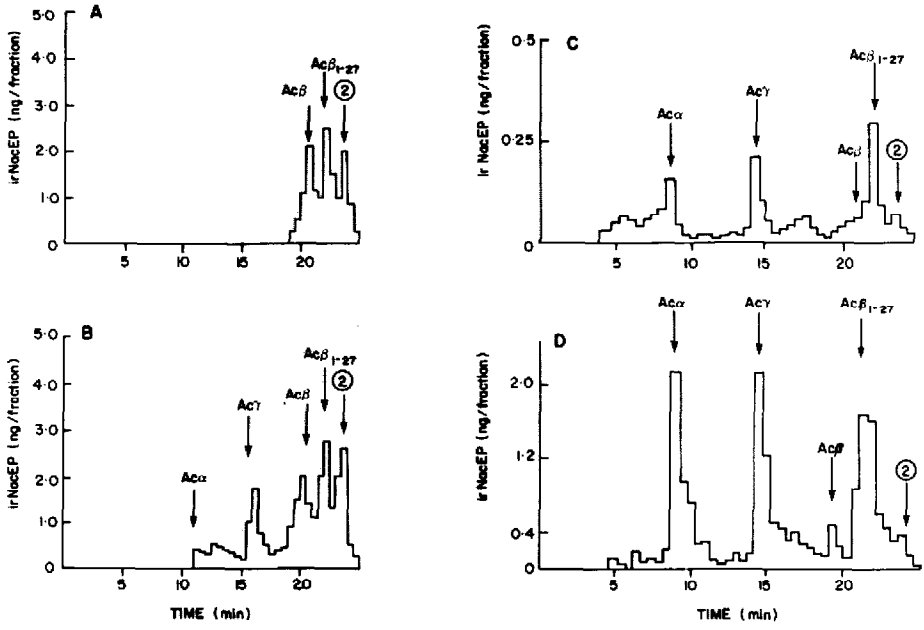

Fig. 5. RP-HPLC ir-NacEP elution profiles of extracts of rat AP (A), rat NIL (B), sheep AP (C) and sheep NIL (D); the rat NIL was diluted 1:100 and both sheep AP and NIL 1:1000 prior to injection. The elution positions of synthetic standards are arrowed (2 represents the predicted elution position of $NacEP_{1-26}$) [23].

rial is N-acetylated (> 25%) with NacαEP, NacγEP, NacEP$_{1-31}$, NacEP$_{1-27}$ and NacEP$_{1-26}$ all present in the chromatogram (Fig. 5C).

DISCUSSION

Previous reports describing post-translational processing of POMC in pituitary and extra-pituitary sites have utilized a variety of combinations, of conventional size-exclusion chromatography coupled with RP-HPLC [24–26] and/or ion-exchange chromatography [1,27], to separate the various molecular species derived from POMC. The rate-limiting step in such studies is invariably the classical open column size-exclusion chromatography stage, with runs typically taking up to 6 h to complete. Other groups have reported the use of HPSEC, reducing analysis times to less than 60 min [15,28]. These workers, however, have used strong protein denaturants in the mobile phase. Such denaturants may severely limit the sensitivity of such studies as collected fractions must be diluted or dialysed prior to RIA. We have described in this report a combined HPSEC–RP-HPLC scheme with analysis times less than 50 min, which allows the complete characterization of all βEP-like peptides, not only at the high tissue levels found in the pituitary but also at the low levels present in the CNS and periphery. The HPSEC mobile phase (0.05 *M* phosphate containing 20% acetonitrile) allows collected fractions to be dried, reconstituted in RIA buffer and assayed directly, there being no evidence for phosphate interference in any of the RIA used. Acetonitrile is a necessary mobile phase component, preventing both polypeptide aggregation and peak tailing due to non-specific interactions between the solute and the column matrix. Despite the non-linearity of peptide/protein molecular mass standards on the HPSEC column, we were able to achieve a good separation between βLPH and βEP. We were unable to detect any non-processed POMC in any of the tissue extracts, though the cross-reactivity of our antiserum (R56) with POMC has yet to be formally established. Protein standards of a similar size (31 000 daltons) are well resolved from βLPH and βEP in this system. The recovery of both non-radioactive and radiolabelled βLPH and βEP was consistently greater than 90%. The Nova Pak C_{18} column, eluted with a linear (30–80% B) gradient, was sufficient to reproducibly resolve all the synthetic acetylated and non-acetylated βEP standards in less than 30 min with recoveries greater than 95%.

The characterization of βEP-like material is complicated by the lack of a specific antiserum able to discriminate between βLPH and βEP and other possible intermediate-length polypeptides. However, the use of HPSEC coupled with RP-HPLC has enabled us to more fully characterize βEP processing pathways. We have assessed these procedures using rat pituitary tissue which has been already well described; our data are consistent with those already published [5]. POMC processing in both rat and sheep pituitary differs between the NIL and AP. In the NIL of both species the vast majority (> 95%) of the βEP-like material is α-N-acetylated [7]. In both rat and sheep NIL extracts we have demonstrated the presence of both the longer (NacEP$_{1-31}$, NacEP$_{1-27}$ and NacEP$_{1-26}$) and the shorter (NacαEP and NacγEP) N-acetylated forms of βEP. Unlike the NIL, in

rat only a very small portion of the βEP-like material is α-N-acetylated (<5%) [7], the principal molecular forms being βEP_{1-31}, βEP_{1-27} and βEP_{1-26}. βEP in the sheep AP is more extensively α-N-acetylated (±25%) [7] the molecular profile mirroring that observed the NIL, again the smaller NacαEP and Nac γEP representing more than half the total immunoreactivity.

The low salt concentration in the mobile phase for HPSEC allowed us to dry collected fractions, reconstitute directly in RIA buffer and therefore assay the total sample. This facilitates the study of tissue extracts with very low levels of POMC-derived peptides, e.g. the sheep HT and rat ovary. The HT extract did not contain any ir-NacEP, which is consistent with previous studies reporting low levels of α-N-acetyltransferase activity in the rat HT [29]. RP-HPLC analysis revealed a major peak of βEP_{1-31} with two much smaller peaks eluting in the position of βEP_{1-27} and βEP_{1-26}, again consistent with previous reports describing βEP in the rat HT [30].

Recent reports from this laboratory have described both the characterization and changes in levels of ovarian βEP during the estrous cycle [24] and pregnancy [31]. To further these studies we have applied these chromatographic procedures to characterize the low levels of ir-βEP in the immature rat ovary. Like the HT the ovary does not appear to α-N-acetylate βEP. The HPSEC profile showed two similarly sized ir peaks eluting in the position of synthetic βEP and βLPH. The RP-HPLC profile of the βEP cut revealed a single peak of immunoreactivity migrating with βEP_{1-31}.

In conclusion therefore, we have developed and validated a two-phase HPLC scheme which allows the rapid separation on the basis of both molecular size and hydrophobicity of βEP-like material at both high and low levels in tissue extracts. The application of these techniques in combination with specific RIA has enabled us to extend our studies on the post-translational processing of βEP. The elucidation of the biosynthetic pathways of POMC derivatives and other peptides will thus allow a clearer insight into processing regulation and the physiological roles of these peptides in the pituitary, CNS and peripheral tissues.

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