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ISOLATION OF $ACTH_{1-39}$, $ACTH_{1-38}$ AND CLIP FROM THE CALF ANTERIOR PITUITARY*

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SUMMARY

Calf anterior pituitaries were defatted and homogenized and peptides were adsorbed from the homogenate supernatant onto octadecylsilyl-silica. After elution, the resulting extract was subjected to gradient elution reversed-phase high pressure liquid chromatography (RP-HPLC) using aqueous acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA). Radioimmunoassay of column fractions for corticotropin (ACTH) revealed three major areas of immunoreactivity. Each was purified to homogeneity by gradient elution RP-HPLC employing aqueous acetonitrile containing either 0.13% heptafluorobutyric acid (v/v) or 0.1% TFA (v/v). Amino acid analysis and exopeptidase and trypsin digestions revealed the three forms of corticotropin to be $ACTH_{1-38}$, corticotropin-like intermediary lobe peptide, (CLIP, $ACTH_{18-39}$) and $ACTH_{1-39}$. ^{3}H-labeled $ACTH_{1-39}$ did not give rise to either ^{3}H-$ACTH_{1-38}$ or ^{3}H-CLIP during isolation.

INTRODUCTION

In order to gain some understanding of the pituitary-adrenal axis during pregnancy we have initiated a program of isolation of pituitary peptides with corticotropic activity. The amino acid sequence of the pituitary 31K corticotropin-β-lipotropin precursor derived from the sequence of cloned cDNA by Nakanishi et al (1) confirmed the structure of the precursor molecule proposed for the corticotropin (ACTH) and β-lipotropin family of peptides (2). Reversed-phase high pressure liquid chromatography (RP-HPLC) has been used recently in the isolation of some of these peptides from tissue extracts (3,4). We have developed RP-HPLC systems and applied them to the isolation of peptides from the calf anterior pituitary. We have isolated $ACTH_{1-38}$ and demonstrated that it is not artifactually formed from $ACTH_{1-39}$.

Abbreviations: RP-HPLC, reversed-phase high pressure liquid chromatography; ODS-silica, octadecylsilyl-silica; TFA, trifluoracetic acid; HFBA, heptafluorobutyric acid; $ACTH_{1-39}$, corticotropin; CLIP, corticotropin-like intermediary lobe peptide, $ACTH_{18-39}$. *Supported by MRC of Canada grant MT-1658, USPH grant HD04365 and National Foundation March of Dimes grant 1-694.
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MATERIALS AND METHODS

Trifluoroacetic acid (TFA) was purchased from BDH Chemicals and heptafluorobutyric acid (HFBA) from Pierce Chemical Co. Synthetic human corticotropin (ACTH) was kindly provided by Dr. W. Rittel (Ciba-Geigy Ltd., Basel, Switzerland) and [^{3}H-Tyr$_{23}$] hACTH$_{1-39}$ was supplied by Dr. R. Wade (Ciba-Geigy Ltd.). Carboxypeptidase-Y (bakers yeast), leucine-aminopeptidase (porcine kidney microsomes) and DPCC-treated trypsin were purchased from Sigma Chemical Co.; HPLC grade acetonitrile was obtained from Fisher Scientific and purified and filtered water was prepared as described previously (5). The HPLC apparatus (Waters Associates) consisted of two 6000A pumps and a 660 solvent programmer to generate gradients of aqueous acetonitrile containing either 0.1% TFA (v/v) or 0.1% HFBA (v/v). Column eluates were monitored for U.V. absorbance at 210 nm or 278 nm using an LC-75 or LC-55 flow-through spectrophotometer (Perkin-Elmer).

Calf pituitaries were collected at a local abbatoir within fifteen minutes of slaughter. The anterior lobes were dissected from the neurointermediary lobes and immediately frozen in liquid nitrogen. Tissue was stored at -40°C until processed. Forty anterior pituitary lobes (32 g wet weight) were ground to a powder with chips of dry ice using a pestle and mortar. This powder was defatted by addition to 600 ml of swirling acetone at -20°C. This was followed by similar treatment in 600 ml hexane and finally a second extraction in 600 ml acetone. The defatted tissue was extracted three times by homogenization in 50 ml 1N HCl containing 5% formic acid (v/v), 1% TFA (v/v) and 1% NaCl (w/v). This medium optimized extraction of corticotropin-like peptides (6). The homogenate supernatants were combined and passed through five octadecylsilyl-silica (ODS-silica) cartridges (C_{18} Sep-Pak, Waters Associates). Peptides, proteins and other components which had adsorbed to the ODS-silica surface were eluted with acetonitrile/water (80%, v/v) containing 0.1% TFA (v/v)(4 ml per cartridge). Aliquots were taken at each stage to estimate the efficiency of extraction and recovery. The ODS-silica eluates were combined, diluted one to five with aqueous TFA (0.1%, v/v) and pumped directly onto the RP-HPLC column (C_{18} μBondapak, Waters Associates) as described previously (5). The column was eluted using a linear gradient of 20 to 35.2% (v/v) aqueous acetonitrile containing 0.1% TFA (v/v) throughout over 1 hour at a flow rate of 1.5 ml/min. The solvent system used in this and subsequent steps was completely volatile. Aliquots from column fractions were dried in vacuo and ACTH immunoreactivity determined using an antiserum raised against porcine ACTH (Sigma Chemical Co.) which was specific for the 16-24 region of ACTH. Radioimmunoassays were performed using a modification of the method of Berson and Yalow (7). The various immunoreactive components were purified by diluting the appropriate fractions, pumping them back onto the same RP-HPLC column and eluting over 1 hr at 1.5 ml per minute with modified linear gradients and solvent systems. This was a three step procedure and involved (1) diluting one to two with 0.1% TFA (v/v), reloading and eluting with a 24 to 28.8% (v/v) acetonitrile gradient containing 0.1% TFA (v/v), (2) diluting one to two with 0.13% HFBA (v/v) reloading and eluting with a 20 to 45.6% (v/v) acetonitrile gradient containing 0.13% HFBA (v/v) and finally (3) diluting one to two with 0.1% TFA (v/v), reloading and eluting with a 20-35.2% (v/v) acetonitrile gradient containing 0.1% TFA (v/v).

Aliquots of column fractions were dried in vacuo for amino acid analysis (5). Peptide amino- and carboxyl-terminal amino acid residues were determined by leucine-aminopeptidase digestion (enzyme : peptide ratio of 1:100, w/w) in 50 ul of 0.01M sodium phosphate buffer, pH7 at 37°C for 4 hrs and carboxypeptidase-Y digestion (enzyme : peptide ratio of 1:100, w/w) in 50 ul of 0.05M sodium citrate pH7.8 at 20°C for 1 hr. Peptides were digested with trypsin (enzyme : peptide ratio of 1:50, w/w) in 100 ul of 0.05M ammonium bicarbonate, pH8.4 at 37°C for 12 hr. The fragments were separated

by HPLC using a concave gradient of 1.6 to 40% acetonitrile containing 0.1% TFA (v/v) throughout. Amino acid analyses of the peptides and enzyme digests were performed on a Durrum Series D500 amino acid analyzer.

RESULTS

The loss of ACTH immunoreactivity during acetone and hexane extraction was estimated to be less than 5%. Approximately 20% of the extractable ACTH would not bind to the ODS-silica cartridges despite repeated batch extraction. The ODS-silica has a limited capacity for proteins (8) and it was assumed that ACTH immunoreactivity lost at this stage represented high molecular weight forms. Of the remaining immunoreactive material, greater than 75% was recovered at the end of the purification procedure.

Fig. 1a shows the RP-HPLC elution profile of the ODS-silica eluate. Three major peaks of ACTH immunoreactivity were detected (A,B,C). These peptides eluted considerably earlier than the elution position of synthetic human ACTH (Fraction No. 37) and this was assumed to be due to column overloading. The peptides contained in peaks A (fractions 17-19), B (fractions 20-22) and C (fractions 23-30) were purified on the same RP-HPLC column. The purification process is illustrated in Fig. 1b, c and d for the purification of material in peak A. In the final chromatographic step for each peptide, the isolated material emerged as a single peak. Fractions 26-28 from 1d were combined and submitted for amino acid analysis. Amino acid analyses of the peptide A and those from B and C which were obtained after submitting these materials to the same purification procedure are shown in Table 1.

Digestion of peptides A and C with Leu-aminopeptidase released equimolar amounts of serine and tyrosine, the first two amino acid residues of $ACTH_{1-39}$. No free amino acids were detected after enzyme digestion of peptide B. Carboxypeptidase-Y digestion of all three proceeded to at least amino acid residue 34. Amino acid analysis of the carboxypeptidase-Y digest of peptide C, believed to be $ACTH_{1-39}$ based upon amino acid composition, indicated the release of 1.7 residues of phenylalanine. This indicated

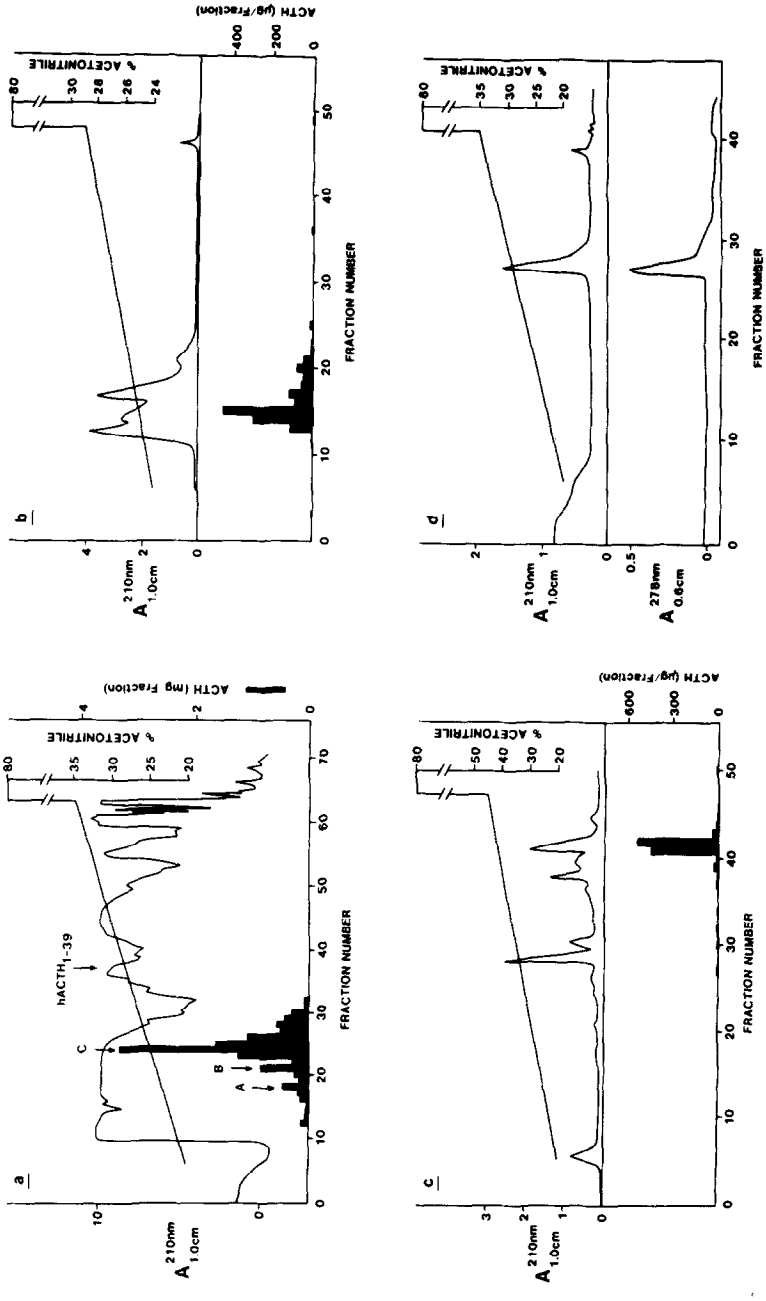

Figure 1: a) HPLC of an extract of 40 calf anterior pituitaries using a linear gradient of 20-35.2% (v/v) acetonitrile containing 0.1% TFA (v/v) throughout. Peaks A, B and C were detected by radioimmunoassay of ACTH. b) HPLC of peak A (fractions 17-19) from 1a using a linear gradient of 24-28.8% (v/v) acetonitrile containing 0.1% TFA (v/v) throughout. Fractions 13-16 were re-chromatographed as shown in c) using a linear gradient 20-45.6% (v/v) acetonitrile containing 0.13% HFBA (v/v) throughout. d) HPLC of fractions 41-43 from Fig. 1c using a gradient of 20-35.2% (v/v) acetonitrile containing 0.1% TFA (v/v) throughout. Amino acid analysis was performed on aliquots from fractions 26-28 which contained the pure peptide A (Table 1).

Table 1 Amino acid composition of peptides A,B and C after purification by HPLC

	Peptide A	Peptide B	Expected Values For CLIP	Peptide C	Expected Values For $ACTH_{1-39}$
ASX	1.7	1.9	2	2.0	2
THR	0	0	0	0	0
SER	2.8	0.8	1	2.7	3
GLX	4.9	4.1	4	5.4	5
PRO	4.0	3.1	3	4.1	4
CYS	0	0	0	0	0
GLY	2.8	0.8	1	2.9	3
ALA	3.2	2.6	3	2.9	3
VAL	3.0*	1.9	2	3.0*	3
MET	0.9	0	0	0.8	1
ILE	0.1	0	0	0	0
LEU	0.8	0.9	1	1.0	1
TYR	2.3	0.9	1	1.9	2
PHE	1.8	1.6	2	2.8	3
HIS	1.1	0	0	1.0	1
LYS	3.9	1.0*	1	3.8	4
ARG	3.0	0.9	1	2.9	3
TRP**	1	0	0	1	1

* Used to calculate amino acid ratios.
** Presence indicated by U.V. absorbance at 278nm.

the total cleavage of the C-terminal amino acid and partial release of phenylalanine residue 35. Similar results were obtained from the digest of peptide B. Only 0.7 residues of phenylalanine were released by carboxypeptidase-Y digestion of peptide A indicating the absence of residue 39. Peptides A, B and C were digested with trypsin, the resulting peptides were separated by HPLC and amino acid analysis carried out on each fragment. Peptide A gave fragments corresponding to amino acids 1-8, 9-15, 17-21, 18-21 and 22-38 of $ACTH_{1-39}$, peptide B to 18-21 and 22-39 and peptide C to 1-8, 9-15, 17-21, 18-21 and 22-39.

From these results it was concluded that the identity of peptide A is $ACTH_{1-38}$, B is bovine corticotropin-like intermediary lobe peptide (CLIP, $ACTH_{18-39}$) and C is bovine $ACTH_{1-39}$. Starting with 32 g of pituitary tissue the yield of each peptide estimated from amino acid analyses was 0.5 mg of $ACTH_{1-38}$, 1.0 mg of CLIP and 6.0 mg of $ACTH_{1-39}$.

DISCUSSION

In recent years evidence has been accumulating to indicate that the adult and fetal pituitaries contain corticotropic factors other than $ACTH_{1-39}$

(9,10), but to date none of these have been identified. We have isolated a novel corticotropin from the calf anterior pituitary and have identified it as $ACTH_{1-38}$.

RP-HPLC was used to isolate $ACTH_{1-38}$, CLIP and $ACTH_{1-39}$ from an extract of 40 calf anterior pituitaries as shown in Fig. 1 for $ACTH_{1-38}$ (peptide A). The initial HPLC of the ODS-silica extract (Fig 1a) used an aqueous acetonitrile solvent system containing 0.1% TFA (v/v). It can be seen from the absorbance profile that the column had been overloaded. The peptides contained in peaks A, B and C were re-chromatographed separately using a shallow acetonitrile gradient with 0.1% TFA (v/v) to achieve partial purification (Fig. 1b). Each peptide was then submitted to gradient elution with aqueous acetonitrile containing 0.13% HFBA (v/v). HFBA radically changes the retention times of most peptides relative to the TFA system (11). This is illustrated in Fig. 1c which shows the ACTH-immunoreactive material in peak A being eluted at the end of the gradient. The peptides contained in peaks B and C exhibited similar behaviour at this purification step (data not shown). Each peptide was re-chromatographed in the original TFA solvent system before amino acid analysis since this acid yields a cleaner final extract. The similarity of the amino acid composition (Table 1) of peptides B and C with those expected for CLIP and $ACTH_{1-38}$ respectively, indicated that the peptides had been purified to homogeneity by HPLC. Peptides A, B and C were identified as $ACTH_{1-38}$, CLIP and $ACTH_{1-39}$ based on the data obtained from amino acid analysis, exopeptidase and trypsin digestion.

^{3}H-$ACTH_{1-39}$ (12) was added to calf anterior pituitary tissue and the radioactivity analysed after HPLC in control experiments. We have been unable to detect any conversion of the labeled material to $ACTH_{1-38}$ or CLIP during extraction and chromatography. Immunoreactive-ACTH was found in calf plasma and in media taken from fetal calf anterior pituitary cells in monolayer culture. This immunoreactive-ACTH was demonstrated to elute with

the extraction and HPLC procedures. It therefore seems that $ACTH_{1-38}$ is a normal constituent of the calf anterior pituitary.

The abilities of $ACTH_{1-38}$, CLIP and $ACTH_{1-39}$ to stimulate adrenal steroidogenesis have been tested using fetal calf cells in monolayer culture. Preliminary experiments indicated that $ACTH_{1-38}$ is equipotent to $ACTH_{1-39}$ in stimulating the production of cortisol, 11-deoxycortisol and Δ^4 - androstenedione by the adrenal cells, but CLIP had no effect at any of the doses tested (0.1 - 100 ng/150,000 cells). The presence of substantial amounts of CLIP in the anterior pituitary is therefore of interest in view of its apparent lack of steroidogenic activity.

The origin of $ACTH_{1-38}$ in the calf pituitary is unknown. $ACTH_{1-38}$ may arise from the same precursor molecule as $ACTH_{1-39}$(2) but the possible existence of a distinct precursor for this peptide cannot be ruled out. The presence of two different insulins which are products of nonallelic genes has been shown for the rat, mouse and several fish species (14,15). The possibility of a similar gene duplication event for the ACTH 31K precursor molecule in the bovine pituitary cannot be ruled out. The identification of $ACTH_{1-38}$ demonstrates that the bovine pituitary contains corticotropic peptides other than $ACTH_{1-39}$.

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