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USE OF REVERSED-PHASE AND ION-EXCHANGE BATCH EXTRACTION IN THE PURIFICATION OF BOVINE PITUITARY PEPTIDES

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SUMMARY

Bovine posterior pituitaries were extracted with an acidic medium designed to maximize solubilization of peptides while precipitating high-molecular-weight protein. The supernatant was then extracted with C_{18} reversed-phase cartridges to generate a peptide-enriched fraction. Cartridge eluates were subjected to ion-exchange extraction, using a batch procedure which fractionated the peptides into basic, acidic, and neutral pools. Amino-terminal fragments of bovine pro-opiomelanocortin were found to be resolved into separate pools by this method. The 1 to 49 fragment was eluted in the acidic pool while the 1 to 77 fragment was eluted in the basic pool. The 1 to 77 fragment was purified by reversed-phase high-performance liquid chromatography. Amino acid analysis of the fragments, generated from trypsin and V_8 protease digestion of the 1 to 77 fragment, permitted assignment of cystine bridges between residues 2 and 24 and between residues 8 and 20. Results from amino sugar analysis were consistent with the presence of an O-linked oligosaccharide at threonine$_{45}$ and an N-linked oligosaccharide at asparagine$_{65}$.

INTRODUCTION

Previous studies from this laboratory have been concerned with maximizing the resolving power of reversed-phase high-performance liquid chromatography (RP-HPLC) columns in the purification of pituitary peptides. This can be achieved by manipulating pH and the nature of the ion-pairing reagent[1–3]. Such alterations in solvent properties can introduce elements of cation- and anion-exchange chromatography into a purification scheme[3]. When the task is the purification of a major tissue component (*e.g.*, pituitary corticotropin), the use of such methods makes the exercise very simple, especially when they are combined with a reversed-phase tissue extraction procedure[4]. Excellent recoveries can be maintained during the purification scheme, if fractions are loaded and reloaded directly onto the reversed-phase column by using the HPLC pump[2]. In this manner wasteful procedures, such as lyophilization and de-salting, can be avoided.

When the task is the purification of a minor component, the problem of column overloading arises. If the proportion of unwanted peptide and protein becomes very

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large, the chromatographic behaviour of minor components is distorted. They are no longer eluted as discrete peaks, but tend to streak and are eluted from the column in large volumes. These kinds of problems are not confined to RP-HPLC. They are encountered in other forms of chromatography and have made some peptide isolation, such as the purification of the hypothalamic releasing factors, extremely difficult. Reversed-phase cartridges and HPLC columns do not discriminate between peptides of basic, acidic, or neutral charge other than in terms of how the polarity affects the overall hydrophobicity. This paper describes how high-performance ion-exchangers can be used, in a batch procedure, to fractionate peptides extracted from bovine pituitaries into basic, acidic, and neutral pools. The method greatly reduces the amount of peptide loaded, since a large proportion of unwanted material is removed prior to RP-HPLC. The method has been applied to the purification of the 1 to 77 fragment of pro-opiomelanocortin (POMC)[5]. This pituitary peptide shares a common biosynthetic origin with corticotropin and β-lipotropin and is located at the amino-terminal portion of the precursor. It was originally identified as an approximately 16000-dalton (16 kilodalton) glycopeptide in the course of biosynthetic studies[6,7]. For this reason, it is frequently referred to as the 16K fragment. Two variants, $16K_{1-77}$ and $16K_{1-49}$, have been identified in posterior pituitary extracts.

MATERIALS AND METHODS

High-performance liquid chromatography

Chromatography was performed with a Waters Assoc. (Milford, MA, U.S.A.) HPLC system. Column eluates were monitored for UV absorbance at 210 and 280 nm by a Waters Model 450 variable-wavelength detector, and an LDC (Riviera Beach, FL, U.S.A.) fixed-wavelength detector, connected in series. HPLC solvents and reagents were prepared as described previously[2,3].

Tissue extraction

Twenty lyophilyzed bovine posterior pituitaries (approximately 1.5 g dry weight, Pel-Freez Biologicals, Rogers, AR, U.S.A.) were defatted as described previously[8]. The resulting pituitary powder was extracted twice with 25 ml of an acidic medium, consisting of 1 *M* hydrochloric acid, containing 5% (v/v) formic acid, 1% (w/v) sodium chloride and 1% (v/v) trifluoroacetic acid (TFA)[1,2]. The combined supernatants (*i.e.*, 50 ml) were subjected to repeated batchwise reversed-phase extraction with a total of ten octadecylsilyl-silica (ODS-silica) cartridges (C_{18} Sep-Pak cartridges, Waters), as described previously[2,8].

Ion-exchange fractionation

The eluates from the ten ODS-silica cartridges were combined in a 100-ml conical flask (total volume of 45 ml of 80% aqueous acetonitrile, containing 0.1% TFA) and the volume was reduced to approximately 10 ml by briskly bubbling a stream of nitrogen through it. One-twentieth of this aqueous residue was saved in order to provide an RP-HPLC analysis of the total peptide content (Fig. 1). The pH of the rest of the aqueous residue was raised to approximately pH 5 by the drop-wise addition of 1 *M* sodium hydroxide solution. This initial peptide fraction (approximately 50 m*M* in terms of sodium ions) was further fractionated by batchwise ion-

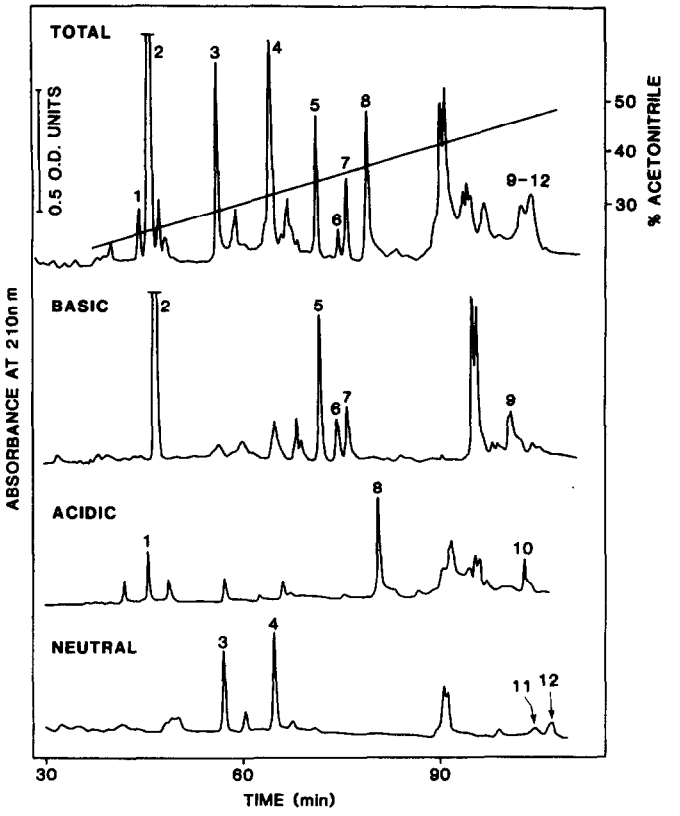

Fig. 1. Analytical RP-HPLC of peptides extracted from bovine posterior pituitaries. Twenty posterior pituitaries were subjected to reversed-phase extraction with ODS-silica cartridges. The top panel (total) shows the result obtained from the RP-HPLC of the equivalent of one posterior pituitary. The ODS-silica extract was fractionated into basic, acidic, and neutral pools on CM and DEAE ion-exchange columns. The lower three panels (basic, acidic, and neutral) show the results obtained from the RP-HPLC of the equivalent of one posterior pituitary for each of the three ion-exchange pools. For experimental details see the Materials and methods section. Peptides numbered 1 to 12 were identified by elution position and amino acid analysis. Peaks: 1 = $POMC_{80-103}$, the acidic joining peptide of bovine pro-opiomelanocortin; 2 = arginine-vasopressin; 3 = oxytocin; 4 = β-melanotropin; 5 = monoacetyl α-melanotropin; 6 = $Lys^1\gamma_3$-melanotropin; 8 = corticotropin-like intermediate lobe peptide; 9 = $16K_{1-77}$ fragment of bovine POMC; 10 = $16K_{1-49}$ fragment of bovine POMC; 11, 12 = bovine posterior pituitary glycopeptides.

exchange extraction on Bio-Sil TSK CM-3SW and DEAE-3SW HPLC columns, as follows. The ion-exchange columns were connected in series (with the CM column first) and equilibrated with 50 m*M* ammonium acetate (pH 5.5). The peptide fraction was pumped through both columns, which were then washed with 20 ml of 50 m*M* ammonium acetate (pH 5.5). Material which passed through both columns was combined with the washings and designated as the neutral pool. The two ion exchangers were then detached from each other and each one was eluted with 20 ml of 1 *M* ammonium acetate (pH 5.5). The above operations were carried out at a flow-rate of 2 ml/min. The eluate from the CM ion exchanger was designated as the basic pool while the eluate from the DEAE ion exchanger was designated as the acidic pool.

The pH of the basic, acidic, and neutral pools was reduced to approximately 2 by drop-wise addition of TFA.

Analytical RP-HPLC

Analytical RP-HPLC was performed on the sample of the total peptide extract and on one-nineteenth of the basic, acidic, and neutral peptide pools (Fig. 1). In each instance, the column (μBondapak C_{18}, Waters) was eluted with a linear gradient of from 1.6% to 61.6% aqueous acetonitrile, containing 0.1% TFA, over a 3-h period at a flow-rate of 1.5 ml per min. The methods of column loading and elution have been described in detail elsewhere[2,3].

Preparative RP-HPLC

Preparative RP-HPLC was performed on the rest of the basic peptide pool by using two μBondapak C_{18} columns, connected in series. The columns were eluted with a gradient of from 1.6% to 61.6% aqueous acetonitrile, containing 0.1% TFA over a 6-h period at a flow-rate of 1 ml/min; 2-min fractions were collected. The $16K_{1-77}$ fragment of bovine POMC (*i.e.* peak 9 in Fig. 1) was eluted in fractions 65 to 67. This material was repurified by using the same two μBondapak C_{18} columns, essentially as described previously[2]. Purification was achieved with the use of solvents consisting of acetonitrile first containing 0.13% heptafluorobutyric acid and then 0.1% TFA.

Characterization of bovine $16K_{1-77}$

The purified bovine $16K_{1-77}$ was subjected to trypsin and V_8 protease digestion under conditions designed to minimize cleavage and oxidation of cystine residues[9]. Peptide (56.4 nmol) was incubated with 60 μg trypsin (Type X1, diphenylcarbomoyl chloride treated, Sigma) at 37°C for 2 h in 200 μl of 50 m*M* ammonium bicarbonate buffer (pH 8.0) which had previously been saturated with argon. The tryptic fragment corresponding to the 1 to 49 portion of the $16K_{1-77}$ sequence (*i.e.* TP_7 in Fig. 2) was then digested with V_8 protease (*Staphylococcus aureus,* Miles Labs., Rexdale, Canada). Peptide (36.0 nmol) was incubated with 28 μg V_8 protease exactly as described for the trypsin digestion.

Tryptic and V_8 protease digests were subjected to RP-HPLC on a μBondapak C_{18} column, eluted with linear gradients of from 1.6% to 48% aqueous acetonitrile, containing 0.1% TFA over a 1-h period at a flow-rate of 1.5 ml/min. Peptide fragments were collected by hand as they emerged from the column.

Amino acid and amino sugar analysis of the purified 16K fragment and its tryptic and v_8 protease fragments was performed with a System 6300 high-performance analyzer (Beckman Instruments, Palo, Alto, CA, U.S.A.).

RESULTS AND DISCUSSION

Analytical RP-HPLC of extracts of bovine posterior pituitaries

To determine the effectiveness of the ion-exchange fractionation procedure, portions from each pool were subjected to RP-HPLC. Fig. 1 shows the results of the RP-HPLC of the equivalent of one pituitary from the starting material (total) and from the basic, acidic, and neutral pools. Peaks 1 to 12 were identified by amino acid

compositions (results not shown). They all represent fragments of pro-opiomelanocortin[10], pro-oxyphysin, or pro-pressophysin[11]. The polarity of each peptide was determined by examining their amino acid sequences (Table I). The distribution of most of the peptides within the three ion-exchange pools is fairly predictable. For instance, joining peptide, CLIP and $16K_{1-49}$ have overall negative charges of 7, 2, and 5 respectively, and are found in the acidic pool. While the theoretical consideration of overall charge is accurate for some peptides, it does not explain the behaviour of others. For instance, the bovine posterior pituitary glycopeptides, with a theoretical negative charge of 2 are found in the neutral pool and $16K_{1-77}$ with an overall neutral charge collects cleanly in the basic pool. This apparently anomalous behaviour is extremely useful in the preparative isolation of bovine 16K fragments. $16K_{1-77}$ is found in the basic pool, $16K_{1-49}$ is found in the acidic pool; and a major contaminant, the posterior pituitary glycopeptide, is found in the neutral pool. The isolation of $16K_{1-49}$ has been described previously[9]. This paper is concerned with the isolation and characterization of bovine $16K_{1-77}$.

Isolation and characterization of the amino-terminal 1–77 sequence of bovine POMC (i.e. $16K_{1-77}$)

Preparative RP-HPLC was performed on the remainder of the basic ion-exchange pool. Using methods described previously[2,3], $16K_{1-77}$ was purified to apparent homogeneity (results not shown). The yield from eighteen posterior pituitaries was approximately 60 nmoles (*i.e.*, 3.3 nmoles per pituitary). Amino acid analysis revealed a composition close to that expected for the 1–77 sequence of bovine POMC (Table II).

To characterize the 16K fragment further, the purified peptide was digested with trypsin. RP-HPLC analysis of the digestion products is shown in Fig. 2. Each of the tryptic peptides (TP_1 to TP_7) was subjected to amino acid analysis (Table II). TP_2 and TP_6 were shown to be glycopeptides and corresponded in amino acid composition to the 65 to 77 and 1 to 49 sequences of $16K_{1-77}$, respectively. These findings suggest that $16K_{1-77}$ has two glycosylation sites (see Fig. 3). Furthermore, glucosamine is the predominant sugar in TP_2, and galactosamine is the predominant sugar in TP_6 (Table II). This would suggest that TP_2 is N-glycosylated, and TP_6 is O-glycosylated[12]. Similar observations have been made for the amino-terminal fragments of human[13] and porcine[14] POMC. From these studies it was concluded that the site of N-glycosylation was asparagine residue 65 and the site of O-glycosylation was threonine residue 45. $Asparagine_{65}$ is part of the tripeptide sequence –Asn–Gly–Ser– which is a classical recognition site for the enzyme that brings about N-glycosylation[12]. No such simple sequence requirements apply to O-glycosylation. However, they have been observed in proline-rich sequences[12] and the $threonine_{45}$ residue does fulfill this criterion. We have recently confirmed the site of O-glycosylation is $threonine_{45}$ by careful structural analysis[15]. Tryptic fragment TP_7 also corresponded in amino acid composition to the 1 to 49 sequence of $16K_{1-77}$. However, this fragment contained no amino sugars. This suggests that the original $16K_{1-77}$ preparation consisted of two components (*i.e.*, with or without the O-linked oligosaccharide at $threonine_{45}$).

Seidah *et al.*[13], in their study of the structure of the amino-terminal fragment of human POMC (*i.e.*, $16K_{1-77}$), assigned cystine bridges between residues 2 and 8

TABLE I

SUMMARY OF THE THEORETICAL CHARGES AND THE ACTUAL ION-EXCHANGE PROPERTIES OF PEPTIDES EXTRACTED FROM BOVINE POSTERIOR PITUITARIES

Abbreviations: $POMC_{80-103}$ = the acidic joining peptide of pro-opiomelanocortin; AVP = arginine-vasopressin; α-, β- and γ-MSH = α-, β- and γ-melanotropin; CLIP = corticotropin-like intermediate lobe peptide (*i.e.*, $ACTH_{18-39}$); $16K_{1-77}$ and $16K_{1-49}$ = amino-terminal fragments of pro-opiomelanocortin. Posterior pituitary glycopeptides = the 109 to 147 sequence of propressophysin (peptides 11 and 12 differ only in terms of their apparent extent of glycosylation). Using the sequence information from references 10 and 11, the overall charge for each peptide was determined. The overall charge represents the difference between the number of acidic residues (*i.e.*, Asp, Glu and free carboxyl-termini) and the number of basic residues (*i.e.*, Lys, Arg, His and free amino-termini). The polarity depends on which group is in excess.

Peptide No. (see Fig. 1)	1	2	3	4	5	6	7	8	9	10	11 and 12
Peptide identity	$POMC_{80-103}$	AVP	Oxytocin	β-MSH	Monoacetyl α-MSH	Lys^1 γ_3-MSH	Di-acetyl α-MSH	CLIP	$16K_{1-77}$	$16K_{1-49}$	Posterior pituitary glycopeptides
Overall charge	7−	2+	1+	0	2+	4+	2+	2−	0	5−	2−
Fractionation pool*	Acidic	Basic	Neutral	Neutral	Basic	Basic	Basic	Acidic	Basic	Acidic	Neutral

* See Fig. 1.

TABLE II

AMINO ACID AND AMINO SUGAR COMPOSITION OF PURIFIED BOVINE $16K_{1-77}$, ITS TRYPTIC FRAGMENTS (TP) AND THE V_8 PROTEASE FRAGMENTS (VP) OF TRYPTIC PEPTIDE TP_6

The expected values for each fragment are shown in parenthesis assuming the identity shown in each of the headings. CysO = Cystine sulphoxide; GalN = galactosamine; GlcN = glucosamine; ND = not determined because of breakdown during hydrolysis.

Amino acid/amino sugar	*Fragment*													
	16K, 1–77	*TP_1*, 56–57*	*TP_2, 65–77*	*TP_3, 61–63*	*TP_4, 58–60*	*TP_5*, 50–55*	*TP_6, 1–49*	*TP_7, 1–49*	*VP_1, 1–4*	*VP_2, 23–49*	*VP_3, 5–14, 15–22*	*VP_4, 23–49*	*VP_5, 1–4, 23–49*	*VP_6, 1–4*
CysO**	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.5(1)	0.4(1)	0 (0)	0 (0)	0 (0)	0 (0)
Asx	8.8(8)	0 (0)	1.1(1)	0 (0)	1.0(1)	0 (0)	5.4(6)	5.1(6)	0 (0)	4.2(4)	1.9(2)	3.8(4)	4.1(4)	0 (0)
Thr***	3.7(4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3.4(4)	2.6(4)	0 (0)	1.8(2)	1.7(2)	1.9(2)	2.1(2)	0 (0)
Ser***	6.3(8)	0 (0)	2.6(4)	0 (0)	0 (0)	0 (0)	2.8(4)	1.9(4)	0 (0)	1.0(1)	1.4(3)	0.7(1)	0.7(1)	0 (0)
Glx	10.7(10)	0 (0)	1.2(1)	0 (0)	0 (0)	0 (0)	8.3(8)	7.5(8)	1.0(0)	4.3(4)	2.8(3)	3.8(4)	5.1(5)	1.0(1)
Pro	5.7(5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	5.4(5)	5.3(5)	0 (0)	4.8(5)	0 (0)	5.0(5)	5.1(5)	0 (0)
Gly	7.1(7)	0 (0)	3.5(4)	1.0(1)	0 (0)	1.2(1)	2.5(2)	2.2(2)	0 (0)	1.6(2)	0 (0)	1.7(2)	1.7(2)	0 (0)
Ala	5.6(5)	0 (0)	1.9(2)	0 (0)	0 (0)	0 (0)	3.5(3)	2.6(3)	0 (0)	1.9(2)	1.0(1)	1.8(2)	1.9(2)	0 (0)
Cys	2.5(4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1.5(4)	1.9(4)	0 (0)	0 (0)	1.3(2)	0.8(1)	1.5(2)	0.8(1)
Val	2.3(3)	0 (0)	0.9(1)	0 (0)	0 (0)	1.1(1)	0.6(1)	1.0(1)	0 (0)	0.6(1)	0 (0)	0.8(1)	0.9(1)	0 (0)
Met	0.8(1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.6(1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ile	0.3(1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.5(1)	0.8(1)	0 (0)	0 (0)	0.4(1)	0 (0)	0 (0)
Leu	6.3(6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	6.1(6)	6.1(6)	1.0(1)	2.3(2)	3.0(3)	2.3(2)	2.9(3)	1.0(1)
Tyr	0.8(1)	0 (0)	0 (0)	0 (0)	0 (0)	1.0(1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Phe	2.9(3)	1.0(1)	0 (0)	1.0(1)	0 (0)	0.8(1)	1.0(1)	1.1(1)	0 (0)	0.8(1)	0 (0)	0.9(1)	0.9(1)	0 (0)
His	0.9(1)	0 (0)	0 (0)	0 (0)	0 (0)	0.7(1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Lys	2.1(2)	0 (0)	0 (0)	0 (0)	0 (0)	1.0(1)	1.1(1)	1.2(1)	0 (0)	1.0(1)	0 (0)	0.8(1)	1.0(1)	0 (0)
Arg	5.6(6)	1.0(1)	0 (0)	1.0(1)	1.0(1)	0 (0)	1.5(2)	1.7(2)	0 (0)	1.0(1)	0.6(1)	1.0(1)	1.1(1)	0 (0)
Trp	ND(2)	ND(0)	ND(0)	ND(0)	ND(1)	ND(0)	ND(1)	ND(1)	ND(1)	ND(0)	ND(0)	ND(0)	ND(0)	ND(1)
GlcN	3.1	0	1.9	0	0	0	0.7	0	0	0.9	0	0.9	0.5	0
GalN	1.2	0	0.4	0	0	0	1.3	0	0	1.4	0	1.3	0.9	0

* TP_1 and TP_5 have been generated by cleavage at His_{55}–Phe_{56}. This is not a tryptic cleavage site and must be a result of endopeptidase contamination of the trypsin preparation.

** The oxidized form of cystine which is generated during peptidase digestion elutes from the analyzer in the same position as cysteic acid (*i.e.*, unretained). However, it is more likely that it is in a lower oxidation state such as cystine sulphoxide.

*** Serine and threonine values have not been adjusted for breakdown during hydrolysis.

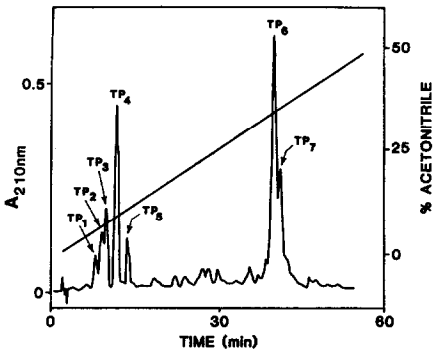

Fig. 2. RP-HPLC analysis of the trypsin digest of $16K_{1-77}$ purified from an extract of 18 bovine posterior pituitaries (see Materials and methods section for details). Tryptic peptides TP_1 to TP_7 were subjected to amino acid and amino sugar analysis (Table II). All other peaks were also present in an enzyme blank and represent contaminants in the trypsin preparation.

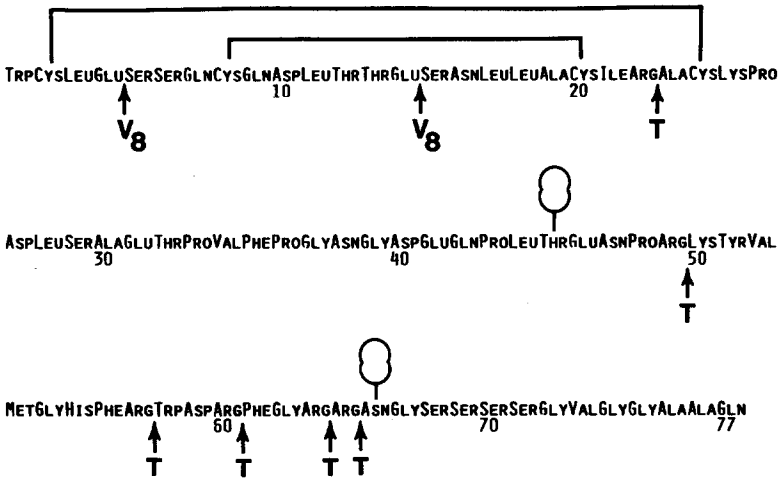

Fig. 3. The primary structure of 1–77 amino-terminal sequence of bovine POMC, as predicted by recombinant DNA methods[10]. The present study has established the presence of cystine bridges between residues 2 and 24 and between residues 8 and 20. The results are compatible with the presence of oligosaccarides, linked to threonine residue 45 and asparagine residue 65. The arrows indicate trypsin (T) and V_8 protease (V_8) cleavage sites.

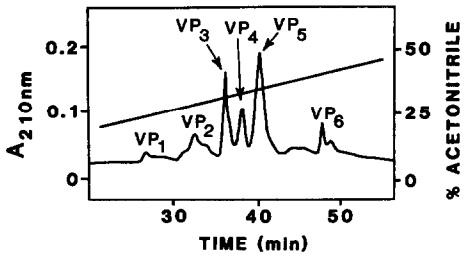

Fig. 4. RP-HPLC analysis of the V_8 protease digest of TP_6 derived from a trypsin digest of bovine $16K_{1-77}$ (Fig. 1, see Materials and methods section for details). V_8 protease digestion products VP_1 to VP_6 were subjected to amino acid and amino sugar analysis (Table II).

and between residues 20 and 24. Recently we have shown[9] that the amino-terminal 1 to 49 sequence of POMC, purified from bovine posterior pituitaries had cystine bridges, linking residues 2 and 24 and linking residues 8 and 20. The human $16K_{1-77}$ originated from anterior lobe tissue, while the bovine $16K_{1-77}$ was from intermediate lobe tissue. It is necessary to re-investigate the sulphur bridging in every molecular form of the amino-terminal fragment of POMC in order to determine whether alternatively bridged forms actually exist. The $16K_{1-77}$, purified in this study was also of intermediate lobe origin. To investigate the nature of the cystine bridging, the tryptic fragment TP_6 was digested with V_8 protease. Our previous study[9] showed that this peptidase cleaved only after glutamate residues 4 and 14 within bovine $16K_{1-49}$ (Fig. 3). If the previous trypsinization has cleaved the $-Arg_{22}-Ala_{23}-$ bond, then the V_8 protease digestion should generate two fragments, irrespective of the orientation of the cystine bridging. Fig. 4 shows the RP-HPLC of the fragments resulting from the V_8 protease digest of TP_6. The amino acid analyses of VP_1 to VP_6 are shown in Table II. The amino acid composition of VP_3 indicates that the 5 to 14 sequence is linked to the 15 to 22 sequence. Similarly, the amino acid composition of VP_5 indicates that the 1 to 4 sequence is linked to the 23 to 49 sequence. Fragments VP_1, VP_2, VP_4, and VP_6 appear to be the reduced and oxidized forms of the 1 to 49 and 23 to 49 sequences.

We have frequently encountered cleavage and oxidation of cystine bridges during peptidase digestions of fragments of the amino-terminal sequence of POMC. This can result in ambiguous results with respect to assigning cystine bridging. In an attempt to minimize this problem during endopeptidase digestions, we have increased the enzyme concentration, shortened the incubation times, and used buffer saturated with argon. Despite these precautions, it is clear from Fig. 4 that considerable cleavage of the cystine bridges linking the 1–4 and 23–49 sequences has occurred. Our findings suggest that the problem of determining the sulphur bridging in novel peptides and proteins must be approached with caution.

CONCLUSIONS

This study has demonstrated the usefulness of ion-exchange fractionation in combination with reversed-phase tissue extraction in the RP-HPLC purification of peptides from bovine pituitary tissue. The reduction in the column loading that the ion-exchange fractionation provides is of considerable assistance in the purification of bovine $16K_{1-77}$. Peptidase digestion of this material provided evidence for O-linked glycosylation at $threonine_{45}$, N-linked glycosylation at $asparagine_{65}$ and cystine bridging between residues 2 and 24 and between residues 8 and 20.

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