C-Terminal sequencing of peptide hormones using carboxypeptidase Y and SELDI-TOF mass spectrometry

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The identification and precise analysis of peptides expressed and processed under different conditions is an integral part of the new and growing field of proteomics (1–5). Of particular interest is the proper identification of the C termini of peptide hormones. Prohormones are cleaved at the C termini of paired-basic residues. Thus, the N-terminal amino acids may be the same, whereas the C-terminal amino acids may be modified or extended and cause altered bioactivity or even inhibit the cleavage of the prohormone by endoproteases (6–11). Recently, a method was described in which peptides were digested with the enzyme carboxypeptidase Y (CPY) on the sample plate and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to determine the amino acid sequence of the peptides (12). Here, we describe further development of this method for sequencing peptides in both simple and complex mixtures using the unique properties of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) (1,4).

For this analysis, we first sequenced the peptide hormone, adrenocorticotrophic hormone (ACTH₁₋₃₉) (Phoenix Pharmaceuticals, Belmont, CA, USA). ACTH₁₋₃₉ is endogenously expressed in the anterior pituitary and is the precursor to other peptide hormones [e.g., α -melanocyte stimulating-hormone (α -MSH)]. ACTH₁₋₃₉ (1 μ g/ μ L) was diluted in deionized water to 100 ng/ μ L. From this, 1 μ L was applied to a spot on an H4 (hydrophobic) ProteinChip® array (Ciphergen Biosystems, Fremont, CA, USA) and air-dried. The array was placed in a “moist chamber” (i.e., pipet tip box containing a wet paper towel).

For the sequencing reaction, CPY (30 U/ μ L; Pierce, Rockford, IL USA) was diluted to 2.4×10^{-3} , 1.2×10^{-3} , and 8×10^{-4} U/ μ L in 50 mM MES, pH 6.5. One microliter of each dilution was added to the peptide on the array, and the moist chamber was placed in a 37°C incubator for 5 min. After incubation, 1 μ L of matrix, α -cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile containing 0.1% trifluoroacetic acid, was added to each spot to stop the reaction. The array was analyzed using a PBSII ProteinChip reader (Ciphergen Biosystems).

The C-terminal amino acid sequence of ACTH₁₋₃₉ was derived using this protocol (Figure 1A). For the 2.4×10^{-3} dilution of CPY, the spectra revealed a “ladder” of 11 ion peaks, beginning with the original ion peak for ACTH₁₋₃₉ [mass/charge (M/Z) of 4578.7]. Each successively smaller ion peak represents the removal of an amino acid from the C terminus of the peptide. The difference in mass for each peak represents the mass of an amino acid. The single nomenclature amino acid sequence identified by the Ciphergen Biosystems 3.1 software was ESAEAFPi/IEF (Figure 1A). Since isoleucine and leucine have the same mass (approximately 113.1 Da), the software identified the third amino acid from the C terminus as either isoleucine or leucine. The known sequence for ACTH₁₋₃₉ is: ESAEAFPLEF, which represents a 100% homology with the sequence generated on the ProteinChip. Further dilution of CPY yielded similar results, the only exception being that only 9 and 5 amino acids could be easily identified at

Figure 1. On-chip C-terminal sequencing of simple and complex mixtures of peptides by enzyme digestion. M/Z indicates mass/charge and is represented as daltons in the spectra. Representative spectra generated by digestion of synthetic adrenocorticotrophic hormone (ACTH₁₋₃₉) with carboxypeptidase Y (CPY). (A) ACTH₁₋₃₉ (4578.7 Da) is labeled by an arrow. Each ion peak is labeled with the mass for the peptide and on the line between ion peaks with the single amino acid code for the amino acid derived from subtracting the smaller peak from the larger peak and comparing with a table of the mass for each amino acid. The number beside each amino acid indicates the difference in daltons between the derived amino acid mass and the mass of the true amino acid. (B) The spectrum represents the region around 4578 Da (ACTH₁₋₃₉) from the anterior pituitary prior to treatment. (C) After treating the sample with CPY, the same area was scanned, and new ion peaks were identified. Note that after the incubation, ACTH₁₋₃₉ was completely absent, and ACTH₁₋₃₈ was diminished in intensity.

Table 1. Expected Versus Observed Ion Peaks for α -MSH

Sequence	des-acetyl- α -MSH		α -MSH		di-acetyl- α -MSH		Amino Acid
	Exp	Obs	Exp	Obs	Exp	Obs	
SYSMEHFRWGKPVG	1679	1679.72	1721	1721.93	1763	1766.66	—
SYSMEHFRWGKPV	1622	1621.81	1664	1664.1	1706	1706.05	G
SYSMEHFRWGKVP	1523	1523.13	1565	1565.77	1607	1607.57	V
SYSMEHFRWGK	1426	—	1468	1470.98	1510	1511.43	P
SYSMEHFRWG	1298	—	1340	—	1382	1379.08	K
SYSMEHFRW	1241	1243.57	1283	1282.66	1325	1323.47	G

All ion peaks are in daltons. The single amino acid code is used for the amino acid sequence. Exp indicates expected ion peak and Obs indicates observed ion peak. Not all ions were observed (i.e., 1426 and 1298 Da). The amino acid column indicates the amino acid removed from the C terminus. α -MSH, α -melanocyte stimulating-hormone; G, glycine; V, valine; P, proline; K, lysine.

1.2×10^{-3} and 8×10^{-4} U/ μ L dilutions, respectively. Longer incubation times or a higher concentration of CPY resulted in the generation of smaller peptide fragments (i.e., 20–26 amino acids long) (data not shown).

The analysis of complex samples (e.g., pituitary homogenates) is made more difficult due to the presence of other peptides and proteins. However, in areas without adjacent ion peaks, important sequence data can be obtained. Mouse anterior pituitary tissue was suspended in 30 μ L 0.1 M

HCl and homogenized with a plastic 0.5-mL microcentrifuge homogenizer, centrifuged at $15,000\times g$ for 5 min, and the soluble protein was adjusted to 1 μ g/ μ L. One microgram of protein was applied to an H4 ProteinChip, dried, washed with 5 μ L distilled water, dried again, and then 1 μ L of CPY (0.0024 U/ μ L) was added in the moist chamber. After 15 min, CHCA was added to stop the reaction, the chip was air-dried and scanned in the ProteinChip reader. A separate spot was analyzed without CPY digestion to determine the origi-

nal ion peaks for comparison (Figure 1B). As an internal calibration, after the entire ProteinChip had been analyzed, ACTH₁₋₃₉ (10 ng) was added to each spot, the ProteinChip was analyzed again, and the spectra was calibrated to the new ACTH₁₋₃₉ ion peak.

Based on the known mass for ACTH₁₋₃₉ (4578 Da), initial examination of this region of the mass spectra revealed several ion peaks. In contrast to the synthetic ACTH₁₋₃₉ used in Figure 1A, the intensity of the ion peak representing endogenously expressed ACTH₁₋₃₉ in the anterior pituitary was very small, while the ion peak representative of ACTH₁₋₃₈ was larger, indicating that it was the major form of ACTH in this sample. After digestion with CPY, eight ion peaks were formed that were identified as ACTH₃₀₋₃₈, respectively (Figure 1C), as compared with the ion peaks generated from CPY treatment of synthetic ACTH₁₋₃₉. The identity of the amino acids was determined manually, rather than relying entirely on the software package. The C-terminal amino acid sequence was similar to the sequence derived with the standard in

Figure 2. On-chip C-terminal analysis of peptides in the region representative of α -MSH. (A) The spectrum indicates three peptide peaks tentatively identified based on mass as des-acetyl- α -MSH, α -MSH, and di-acetyl- α -MSH. (B) Following treatment with carboxypeptidase Y (CPY), a complex ladder of ion peaks was generated. From the right, wide dashed lines indicate a glycine (G) residue (from ACTH₁₋₁₄) was removed, and solid lines connecting peaks indicate a valine (V) residue was removed. Other dashed lines indicate the removal of proline (P) and lysine (K) residues. ACTH, adrenocorticotrophic hormone; α -MSH, α -melanocyte stimulating-hormone. M/Z, mass/charge.

Figure 1A (i.e., SAEAFPi/LEF).

To show that this technique can be used to sequence other anterior pituitary peptides on the same chip simultaneously, we examined the anterior pituitary peptide hormone, α -MSH, which represents the first 13 amino acids of ACTH. α -MSH is interesting because it is acetylated but can exist in the nonacetylated form (i.e., des-acetyl- α -MSH). Acetylation adds 42 Da to the peptide mass. α -MSH is predicted to be 1664 Da, and when the spectra were reviewed, a major peak at M/Z of 1664.17 was identified (Figure 2A). A minor ion peak representative of des-acetyl- α -MSH, at M/Z of 1621.91, was also identified. Another major ion peak was observed at M/Z of 1706.31, which potentially represented a di-acetylated form of α -MSH. When the ProteinChip spot containing anterior pituitary extracts was incubated with CPY, the three original ion peaks were diminished, and a new series of peaks was observed (Figure

2B and Table 1). The difference in mass for each of the largest of the new ion peaks was nearly the same (i.e., 99.1 Da less than the original, representing a valine residue). The last amino acid of α -MSH, des-acetyl- α -MSH, and di-acetyl- α -MSH is a valine. Analysis of the CPY digests revealed smaller peaks, representing further amino acid removal from the des, mono-, or di-acetylated forms of α -MSH (Figure 2B and Table 1). The entire sequence generated by CPY digestion is: GKPVG. This is 100% homologous with the corresponding sequence for α -MSH and clearly suggests that these ion peaks represent α -MSH in its acetylated, di-acetylated, or des-acetylated form.

Advanced qualitative and quantitative proteomic analysis of simple and complex mixtures of peptide hormones is critical to understanding the physiological role that prohormone processing enzymes and peptide hormones play in regulating the endocrine system in the body. By using "on-chip" C-terminal amino acid sequencing with the CPY enzyme followed by SELDI-TOF MS analysis, we provide evidence that important peptide amino acid sequence data can be derived from complex mixtures of peptides and proteins.

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