DIONEX

Application Note 129

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Separation of Tryptophan and Methionine Oxidized Peptides from Their Unoxidized Forms

INTRODUCTION

Protein and peptide microheterogeneity can sometimes be attributed to oxidation of tryptophan (Trp) or methionine (Met) residues. Amino acid oxidation in proteins and peptides is a common post-translational modification. *In vivo* oxidation is caused by oxygen radicals and other biological factors (e.g., exposure to certain oxidizing drugs or other compounds). *In vitro* oxidation can be due to conditions encountered during purification or formulation. Oxidation can also occur during storage and from frequent freeze-thawing cycles. Protein chemists in process development and quality control are concerned with oxidation because it can adversely impact the activity and stability of biotherapeutics.[1]

In this Application Note, luteinizing hormone-releasing hormone (LH-RH), a peptide containing Trp, was forcibly oxidized with dimethyl sulfoxide/HCl.[2,3] α-Melanocyte stimulating hormone (α-MSH), a peptide containing Met was forcibly oxidized with hydrogen peroxide/ammonium bicarbonate.[3,4] The resulting peptides were separated using the ProPac™ WCX-10 weak cation exchange column.

EQUIPMENT

DX-500 BioLC® liquid chromatography system consisting of:

- GP50 gradient pump (PEEK)
- AD20 variable wavelength absorbance detector
- AS50 autosampler
- LC30 or LC25 chromatography oven or AS50 thermal compartment

PeakNet Chromatography Workstation

Dionex ProPac WCX-10 cation-exchange column, 250 x 4 mm i.d. (P/N 54993)

REAGENTS AND SAMPLES

Deionized water

Monobasic sodium phosphate, monohydrate, crystal (J.T. Baker, Inc.)

Dibasic sodium phosphate, anhydrous, powder (J.T. Baker, Inc.)

Sodium chloride, ACS grade (VWR Scientific)

Ammonium bicarbonate, HPLC grade (J.T. Baker, Inc.)

Ethylenediaminetetraacetic acid (EDTA), disodium, dihydrate (Sigma Chemical Co.)

Hydrogen peroxide, 30% (Sigma Chemical Co.)

Dimethyl sulfoxide (DMSO), ACS reagent (Sigma Chemical Co.)

Hydrochloric acid (HCl), 11–12 N, Ultrex II Ultrapure Reagent (J.T. Baker, Inc.)

Acetic acid, glacial, HPLC grade (J.T. Baker, Inc.)

Luteinizing hormone-releasing hormone (LH-RH), Human; 84% of dry weight is peptide, 97% of peptide contains the following sequence: *p*-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Sigma Chemical Co.)

α-Melanocyte Stimulating Hormone (α-MSH), 78% of dry weight is peptide, 98% of peptide contains the following sequence: *N*-Acetyl-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 (Sigma Chemical Co.)

CONDITIONS

Column:	Dionex ProPac WCX-10 cation-exchange column, 250 x 4 mm (P/N 54993)
Temperature:	30 °C
Flow Rate:	1 mL/min
Inj. Volume:	10 µL
Detection:	UV, 254 nm
Eluents:	A: 10 mM Sodium phosphate (pH 6.0) B: 10 mM Sodium phosphate with 500 mM Sodium chloride (pH 6.0)

Gradient Program:

Time (min)	A (%)	B (%)
0.0	98	2
30.0	84	16
30.1	98	2
45.0	98	2

Autosampler Cycle Time: 45 min

PREPARATION OF SOLUTIONS AND REAGENTS

Two eluents are used for the chromatography: 10 mM sodium phosphate (pH 6.0) and 10 mM sodium phosphate (pH 6.0) with 500 mM sodium chloride (NaCl). The sodium phosphate buffer system was prepared by diluting appropriate quantities of monobasic and dibasic sodium phosphate concentrate solutions with water to attain the desired pH 6.0. The relative amount of monobasic and dibasic sodium phosphate solution added differed between the eluents with and without sodium chloride because NaCl is not normally pH neutral. The following procedure is a recommended starting point for obtaining the desired eluents, but some deviation from this formula may be necessary to achieve the desired pH. If the pH is not 6.0, then adjust the proportions of monobasic and dibasic solutions added. The combined total volume should remain 100 mL to produce 10 mM sodium phosphate for 2 L of eluent.

1 M Sodium Chloride

Dissolve 58.45 g of sodium chloride in water and fill to a final volume of 1.0 L. Filter through a 0.22-µm filter.

200 mM Sodium Phosphate, Dibasic

Dissolve 28.38 g anhydrous dibasic sodium phosphate in 1.0 L of water. Filter through a 0.22-µm filter.

200 mM Sodium Phosphate, Monobasic

Dissolve 27.60 g monohydrate monobasic sodium phosphate in 1.0 L of water. Filter through a 0.22-µm filter.

10 mM Sodium Phosphate, pH 6.0

Combine 14 mL of 200 mM dibasic sodium phosphate, 86 mL of 200 mM monobasic sodium phosphate, and 1900 mL of water. If a 6.0 pH is not attained, adjust relative proportions of 200 mM dibasic sodium phosphate used, maintaining a total volume of 100 mL.

10 mM Sodium Phosphate with 500 mM Sodium Chloride, pH 6.0

Combine 35 mL of 200 mM dibasic sodium phosphate, 65 mL of 200 mM monobasic sodium phosphate, 1000 mL of 1 M sodium chloride, 900 mL of water. If pH 6.0 is not attained, adjust relative proportions of 200 mM mono- and dibasic sodium phosphate used, maintaining a total volume of 100 mL.

1 M Ammonium Bicarbonate, pH 8.8

Combine 7.91 g ammonium bicarbonate with 90 mL water. Adjust the pH to 8.8 with an 11–12 N HCl solution. Adjust to 100 mL with water, and filter through a 0.22-µm filter.

75 mM EDTA, pH 8.0

Combine 78.8 mg ethylenediaminetetraacetic acid (EDTA), disodium, dihydrate with 900 mL water. Adjust the pH to 8.0 with 0.05% (w/w NaOH). Adjust to a final volume of 1 L with water, and filter through a 0.22-µm filter.

400 mM Hydrogen Peroxide

Combine 0.455 mL hydrogen peroxide (30%, 8.79 M) with 9.55 mL water. Make a fresh solution the day of use.

SAMPLE PREPARATION

Oxidation of Tryptophan in LH-RH

Reconstitute a vial containing 5 mg of the LH-RH preparation (4.2 mg peptide content) with 243 µL glacial acetic acid, 32.7 µL of 11–12 N HCl, and 6.4 µL water. Non-oxidized buffer control is prepared by combining 41.7 µL glacial acetic acid, 5.6 µL 11–12 N HCl, and 1.1 µL water. Oxidized buffer control is prepared by combining 41.7 µL glacial acetic acid, 5.6 µL 11–12 N HCl, and 1.1 µL DMSO. Transfer two 48.4 µL aliquots of the LH-RH into two microinjection vials, add 1.1 µL of

DMSO to one of the vials for oxidation (oxy-LH-RH), and 1.1 µL of water to the other vial. Incubate all vials at room temperature for 15 min. No action is taken to stop oxidation. Add 94.7 µL of water to all vials. Dilute 100-fold by transferring 10 µL of each vial into 990 µL of water. Mix and inject 10 µL for analysis of the 0.05 mg/mL LH-RH and oxy-LH-RH, and the two buffer controls.

The peptides are detected at 254 nm. These peptides can also be detected at 210 nm and 280 nm. After Trp oxidation, LH-RH's response increases at 254 nm, and decreases at 280 nm. For this reason, it is not possible to obtain accurate mass balance of peptides based on peak area or height measurements without characterizing the response of the products.

Oxidation of Methionine in Melanocyte Stimulating Hormone (MSH)

Reconstitute 1 mg of the MSH preparation (~0.78 mg peptide) with 533 µL water to make 1.5 mg peptide/mL. Prepare 1 mg/mL untreated peptide control by combining 105 µL of the 1.5 mg peptide/mL with 15 µL of 1 M ammonium bicarbonate, 10 µL 75 mM EDTA, and 20 µL water. Prepare 1 mg/mL Met-oxidized peptide sample by combining 105 µL of 1.5 mg peptide/mL with 15 µL of 1 M ammonium bicarbonate, 10 µL of 75 mM EDTA, and 20 µL 400 mM hydrogen peroxide. Prepare a buffer control by combining 105 µL of water with 15 µL of 1 M ammonium bicarbonate, 10 µL of 75 mM EDTA, and 20 µL of 400 mM hydrogen peroxide. This control and all oxidized samples (oxy-MSH) had final concentrations of 0.1 M ammonium bicarbonate, 5 mM EDTA, and 53 mM hydrogen peroxide. Incubate all samples for 30 min in an icewater bath. No steps are taken to stop oxidation. After incubation, analyze 10 µL of the 1 mg/mL MSH, oxy-MSH, and the buffer control.

RESULTS AND DISCUSSION

Figure 1A shows the elution of the non-oxidized LH-RH at 16 min on the ProPac WCX-10 column. The two peaks eluting within the first 1.5 min are also seen in the buffer control (data not shown). After Trp oxidation, the peptide elutes earlier at 11–12 min (Figure 1B), completely resolved from the native peptide. A small peak at 16 min (peak 5) suggests that LH-RH is not completely oxidized. Two unidentified peaks are observed at 2 and 19 min (peaks 3 and 6).

Figure 2 shows MSH before (Panel A) and after oxidation (Panel B) with hydrogen peroxide. These are conditions that have been reported to oxidize methionine in Substance P.[3] MSH elutes at 23–24 min (peak 7) before oxidation, and at 16–17 min (peak 6) after oxidation. MSH contains a single Trp residue. Minor peaks are also observed at 11, 12, and 13 min (peaks 3, 4, and 5, respectively)

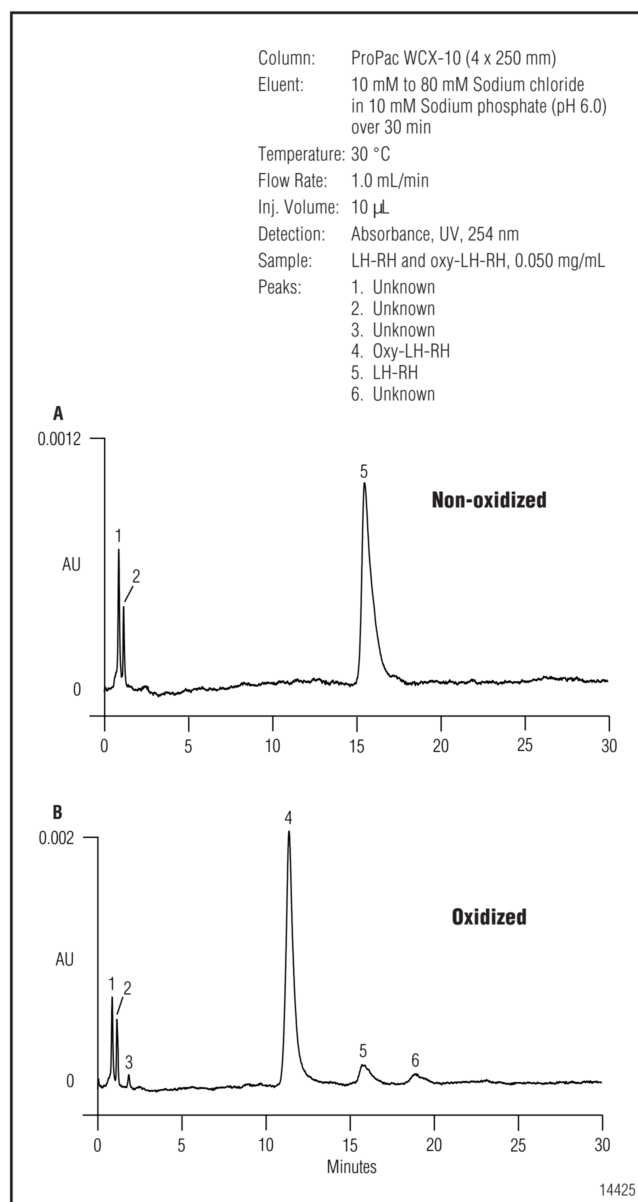

Figure 1. *Separation of (A) LH-RH and (B) Oxy-LH-RH using the ProPac WXC-10 column with detection at 254 nm.*

that could be Trp oxidation products. Experiments using the same Trp oxidation conditions described for LH-RH showed the evolution of peaks at these retention times (data not presented), but the interpretation of this experiment was complicated by an additional peak between 10–20 min in the peptide control (results not presented). Figure 2 shows that MSH and oxy-MSH are easily separated on the ProPac WCX-10 column.

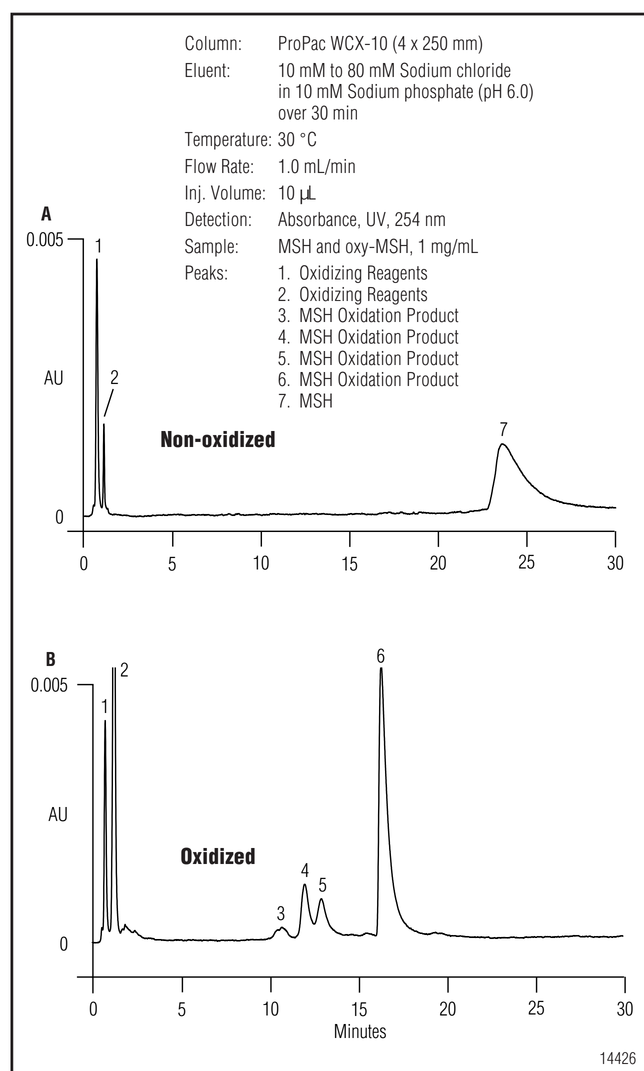

Figure 2. Separation of (A) MSH and (B) Oxy-MSH using the ProPac WCX-10 column with detection at 254 nm.

CONCLUSION

These separations demonstrate the resolving ability of the ProPac WCX-10 column to separate peptides with differences as small as the oxidation of a single amino acid. These separations use easily disposable aqueous eluents and are complimentary or an alternative to reversed-phase chromatography for characterizing peptides.

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* Designed, developed, and manufactured under an NSAI registered ISO 9001 Quality System.

LPN 1053-01 3.5M 6/02

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