

Hydrophobic-Interaction Chromatography for the Separation of Tryptophan and Methionine Oxidized Peptides from Their Unoxidized Forms

Now sold under the
Thermo Scientific brand

Thermo
SCIENTIFIC

Pranathi R. Perati, Jinyuan Wang, Valoran P. Hanko, and Jeffrey S. Rohrer, Dionex Corporation, Sunnyvale, CA, USA

ABSTRACT

Oxidation of proteins is a natural posttranslational event that is a serious concern in the biopharmaceutical industry. Amino acids are susceptible to oxidation during processing and storage of proteins in pharmaceuticals, these oxidations can alter the protein's biological activity, half-life, and immunogenicity.¹ Traditionally reversed-phase HPLC has been used for preparative and analytical separation of peptides and low molecular weight proteins that have a stable primary structure in aqueous-organic mobile phases. Hydrophobic-interaction chromatography (HIC) is an alternative method that exploits the hydrophobic properties of these molecules in aqueous mobile phases.

Here we discuss the use of HIC to separate non-oxidized Luteinizing Hormone-Releasing Hormone (LH-RH), a tryptophan-containing peptide, and α -Melanocyte Stimulating Hormone (α -MSH), a methionine-containing peptide, from their forcibly oxidized variants. HIC can separate peptides with differences as small as the oxidation of a single amino acid residue. The HIC column separation of methionine oxidized α -MSH showed a new peak for oxidized α -MSH and the original non-oxidized peak. The separation of oxidized LH-RH revealed four peaks in addition to the original non-oxidized peaks. In order to identify these peaks, a mass spectrometric analysis of the oxidized LH-RH peaks was performed along with the native peptide. The analysis of non-oxidized LH-RH revealed only a single major component with a mass-to-charge ratio equal to that expected for LH-RH. MS analysis of the forcibly oxidized LH-RH peaks identified two tryptophan oxidation products, hydroxytryptophan and *N*-formylkynurenine.

INTRODUCTION

Hydrophobic interaction chromatography (HIC) is a technique used to separate peptides, proteins, and other biological molecules based on their degree of hydrophobicity. The ProPac[®] HIC-10 stationary phase is based on ultrahigh-purity spherical silica gel particles with 300 Å pores. Multiple points of attachment between the functional layer and the silica substrate are used to ensure enhanced hydrolytic stability in highly aqueous media. In addition, a balanced ratio between the hydrophilic (amide) and hydrophobic (alkyl) groups for protein retention results in a high-efficiency, high-capacity, multipurpose chromatographic HIC media

for bioseparations. The unique chemistry of the ProPac HIC-10 column offers exceptional selectivity and stability over the range of pH 2.5 to 7.5. The mobile phase consists of a high concentration of a chaotrope, usually ammonium sulfate, that increases the hydrophobic interaction between the solute and the stationary phase.²

HIC and reversed-phase chromatography are closely related techniques. Both are based upon interactions between solvent-accessible non-polar groups (hydrophobic patches) on the surface of the solute and the hydrophobic ligands of the stationary phase. In practice, however, HIC and reversed-phase are different, as reversed-phase stationary phases are more highly substituted with hydrophobic ligands than HIC stationary phases and the techniques use very different mobile phases. Protein binding to reversed-phase stationary-phases is usually very strong, requiring strong polar solvents for elution, often denaturing the proteins during the separation. Reversed-phase HPLC has found extensive applications in analytical and preparative separations of mainly peptides and low molecular weight proteins that have stable primary structure in aqueous-organic mobile phases. HIC is an alternative way of exploiting the hydrophobic properties of proteins, working in a more polar, non-denaturing environment.³

Proteins and peptides are sensitive to oxidative damage. Natural biological oxidants and environmental oxidants have been suggested as causative or contributory factors in many diseases. Oxidation of proteins has also been reported as natural posttranslational events mediated enzymatically by amines and oxidases.³ According to the literature, methionine, cysteine, histidine, tryptophan, and tyrosine residues are most susceptible to oxidation. Methionine (Met) is easily oxidized by atmospheric oxygen to form methionine sulfoxide.⁴ Tryptophan (Trp) can be oxidized by peroxide. Peroxide is an impurity generated during protein storage, or from polysorbates that are commonly used for protein purification and solubilization. Other peroxide-contaminated materials include polyethylene glycol or silicon rubber from vial stoppers. When proteins are used as pharmaceuticals, Met and Trp oxidations during processing or storage can be of serious concern due to the possible effect on protein activity.⁵

Here, we separate oxidized and non-oxidized variants of a methionine-containing peptide and a tryptophan-containing peptide using a ProPac HIC-10 hydrophobic interaction column.

EXPERIMENTAL

Equipment

ICS-3000 liquid chromatography system consisting of:

- ICS-3000 SP single pump
- ICS-3000 VWD variable wavelength detector
- ICS-3000 DC Detector/Chromatography compartment
- AS autosampler
- Dionex ProPac HIC-10 column (4.6 × 100 mm)

This application can also be performed using the Dionex UltiMate® 3000 Titanium liquid chromatography system consisting of:

- SRD-3600 Solvent Rack with six degasser channels
- LPG-3400AB Quaternary Analytical Pump, or a DPG-3600AB Dual Ternary Analytical Pump
- WPS-3000TBPL Biocompatible Analytical Autosampler
- TCC-3200B Column Compartment with two PEEK™ 10-port, 2-position valves, or a TCC-3000 Column Compartment without switching valve
- VWD-3400 Variable Wavelength Detector, or PDA-3000 Photodiode Array Detector
- Biocompatible Analytical Flow Cell for PDA
- Chromeleon® Chromatography Data Management System software
- MSQ Plus® Mass Spectrometer with Data System
- AXP-MS Auxiliary Pump Kit
- Chromeleon MS Support (MSQ MS control)

Sample Preparation

Oxidation of Tryptophan in LH-RH

A vial containing 1 mg of LH-RH was reconstituted in a mix of glacial acetic acid, 11–12 N HCl, and water. DMSO was added to the mix in order to force oxidation. All vials were incubated at room temperature for 15 min, after which water was added to each vial to stop the reaction. The samples were diluted by 100 with 50% eluent A and 50% eluent B. The samples were maintained at room temperature and used within 48 h. For more extensive details of the method, refer to the application in Reference 7.

Oxidation of Methionine in α -MSH

1 M Ammonium bicarbonate, 75 mM EDTA, and 400 mM hydrogen peroxide were added to a 1.5 mg/mL MSH solution in water. All vials were incubated for 30 min in an ice water bath. The samples were diluted by tenfold with 50% Eluent A and 50% Eluent B. The samples were maintained at room temperature and used within 48 h. For more extensive details of the method, refer to the application in Reference 7.

Method Conditions

- Column: ProPac HIC-10 column, 4.6 × 100 mm (P/N 063655)
- Flow Rate: 1.0 mL/min
- Temperature: 30 °C
- Injection Volume: 10 μ L
- Detection: UV at 214, 254, and 280 nm
- Eluents: (A) 2 M ammonium sulfate in 0.1 M sodium phosphate (pH 7.0)
(B) 0.1 M sodium phosphate, monobasic (pH 7.0)

Table 1. Gradient Program

Time (min)	A%	B%	Comments
-10.0	100.0	0.0	Preconditioning of the column before sample injection
0.0	100.0	0.0	Sample injection
2.0	100.0	0.0	Avoid gradients during sample injection
17.0	0.0	100.0	Gradient
20.0/26.0	0.0	100.0	Wash
22.0/28.0	100.0	0.0	Re-equilibration to prepare for the next sample injection

Prewash with 100% B for 15 min prior to the first injection

RESULTS AND DISCUSSION

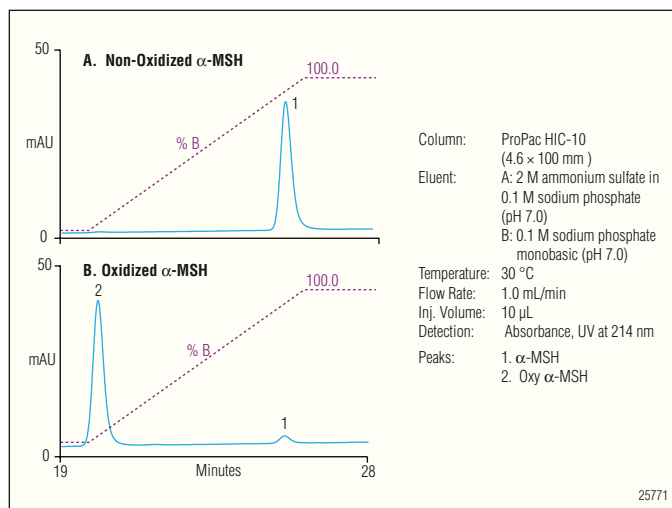


Figure 1. Separation of (A) non-oxidized α -MSH and (B) oxidized α -MSH using the ProPac HIC-10 column.

2 Hydrophobic-Interaction Chromatography for the Separation of Tryptophan and Methionine Oxidized Peptides from Their Unoxidized Forms

Figure 1A shows non-oxidized α -MSH (peak 1) at 26 min on the HIC-10 column. After forced oxidation of the Met in α -MSH, a new peak for the oxidized α -MSH (peak 2) was observed at 20 min (Figure 1B). The presence of a remaining non-oxidized MSH peak at 26 min indicates that α -MSH was not completely oxidized.

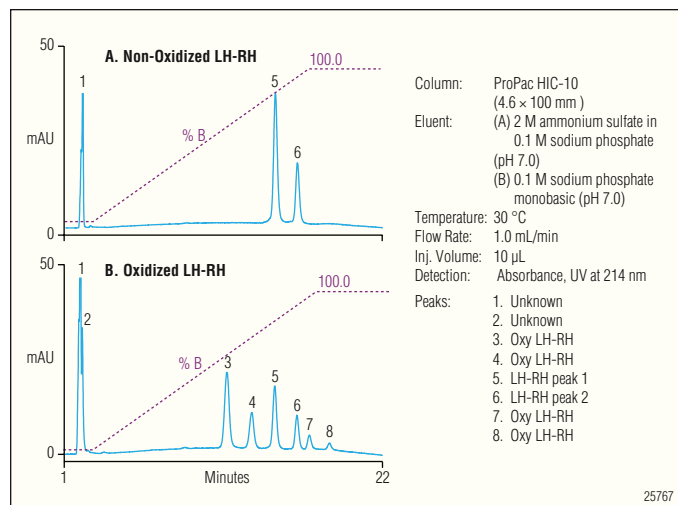


Figure 2. The separation of (A) non-oxidized LH-RH and (B) oxidized LH-RH using the ProPac HIC-10 column.

Figure 2A shows the elution of two non-oxidized LH-RH peaks on the HIC-10 column at 11 and 18 min (peaks 5 and 6, respectively). Figure 2B shows peak 5 and 6 as seen in the unoxidized material and four additional peaks when the Trp in LH-RH is forcibly oxidized with DMSO. The presence of peaks 5 and 6 with reduced peak areas in the oxidized LH-RH indicated incomplete oxidation. We hypothesized that peak 3 and 4 are oxidized variants of peak 5, and peak 7 and 8 are oxidized variants of peak 6.

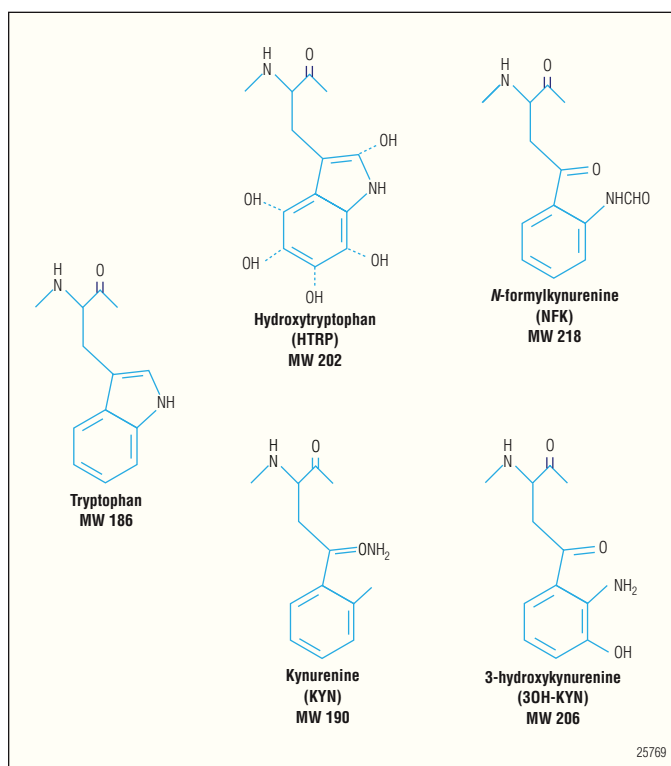


Figure 3. The chemical structure of tryptophan and its major oxidation products.⁶

Figure 3 shows a variety of Trp oxidation products described by E.L. Finley et al.⁶ The expected products of Trp oxidation are hydroxytryptophan (HTRP), *N*-formylkynurenine (NFK), Kynurenine (KYN), and 3-hydroxykynurenine (3OH-KYN).

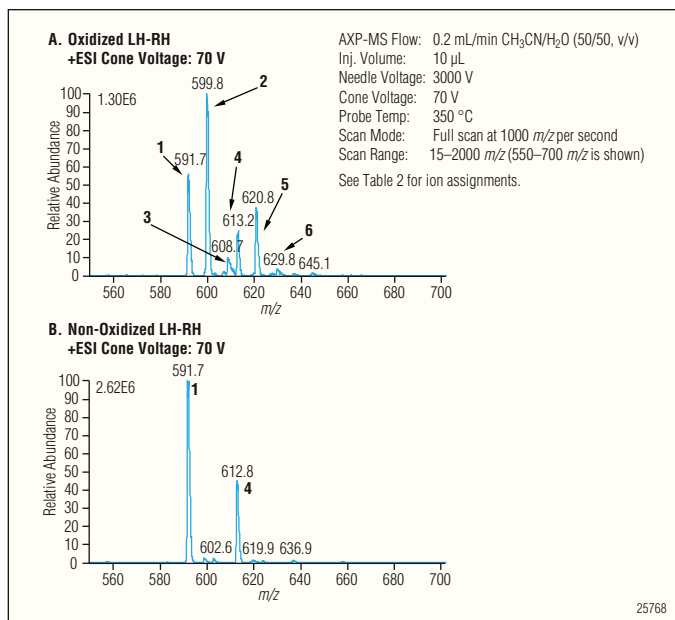


Figure 4. Mass spectra of (A) oxidized LH-RH and (B) non-oxidized LH-RH. See Table 2 for ion assignments.

MS analysis of the oxidized LH-RH (Figure 4A) shows the presence of four additional major ions. Mass spectrometric (MS) analysis of the non-oxidized LH-RH (Figure 4B) revealed a single major component with a mass-to-charge (m/z) ratio equal to that expected for LH-RH. We were able to putatively identify two of the oxidation products and the assignments in Table 2 were made based on mass/charge.

Table 2. Ion Assignments from Figure 4

Ion #	Mass/charge (m/z)	Adduct	Assignment
1	591.1	[M+2H] ²⁺	LH-RH
2	599.8	[(M+16)+2H] ²⁺	Hydroxytryptophan (HTRP)
3	608.7	[(M+32)+2H] ²⁺	<i>N</i> -formylkynurenine (NFK)
4	613.2	[LHRH+CH ₃ CN+2H] ²⁺	Acetonitrile adduct of LH-RH
5	620.8	[HTRP+CH ₃ CN+2H] ²⁺	Acetonitrile adduct of Hydroxytryptophan (HTRP)
6	629.8	[NFK+CH ₃ CN+2H] ²⁺	Acetonitrile adduct of <i>N</i> -formylkynurenine

CONCLUSION

- This study shows that the ProPac HIC-10 column can separate methionine- and tryptophan-oxidized peptides from their native forms.
- The ProPac HIC-10 column is a high-capacity column with enhanced hydrolytic stability that can separate peptides with a single amino acid residue difference.

The separations presented here occur in a non-denaturing environment, without the use of organic solvents, and show that HIC is a good alternative to RP-HPLC for the separation of peptide variants.

REFERENCES

- Walsh, C.T. *Posttranslational Modification of Proteins*. Roberts & Co., Englewood, CO, USA 2006, pps 358–365.
- Rao, S.; Bordunov, A.; Pohl, C. High-Resolution Silica Hydrophobic Interaction Chromatography (HIC) Column for Protein/Peptide Separations with Improved Hydrolytic Stability, LPN 1864-01, Dionex Corporation, Sunnyvale, CA.
- Jennissen, H. P. Hydrophobic Interaction Chromatography, *Nature Encyclopedia of Life Sciences*. 2002, Vol.9, 353–361.
- Kim, Y.H.; Berry, A.H.; Spencer, D.S.; Sites, W. E. Comparing the Effect of Protein Stability of Methionine Oxidation versus Mutagenesis: Steps Toward Engineering Oxidative Resistance Proteins, *Protein Engineering* **2001**, 14 (5), 343–347.
- Zang, J.; Kalonia, D.S. The Effect of Neighboring Amino Acid Residues And Solution Environment On The Oxidative Stability Of Tyrosine In Small Peptides, *AAPS PharmSci. Tech.* **2007**, 8 (4), Article 102
- Finley, E.L.; Dillon, J.; Crouch, R.K.; Schey, K.L. Identification of Tryptophan Oxidation Products in Bovine α -Crystalline, *Protein Science* **1998**, 7, 2391–2397
- Dionex Corporation. Use of Hydrophobic Interaction Chromatography for the Separation of Tryptophan and Methionine Oxidized Peptides from their Unoxidized Forms, Application Note 211, LPN 2110, Sunnyvale, CA.

PEEK is a trademark of Victrex PLC.

MSQ Plus is a registered trademark of Thermo Fisher Scientific.

Chromeleon, ProPac, and UltiMate are registered trademarks of Dionex Corporation.

Passion. Power. Productivity.



Dionex Corporation

1228 Titan Way
 P.O. Box 3603
 Sunnyvale, CA
 94088-3603
 (408) 737-0700

North America

U.S./Canada (847) 295-7500

South America

Brazil (55) 11 3731 5140

Europe

Austria (43) 1 616 51 25 Benelux (31) 20 683 9768 (32) 3 353 4294
 Denmark (45) 36 36 90 90 France (33) 1 39 30 01 10 Germany (49) 6126 991 0
 Ireland (353) 1 644 0064 Italy (39) 02 51 62 1267 Sweden (46) 8 473 3380
 Switzerland (41) 62 205 9966 United Kingdom (44) 1276 691722

Asia Pacific

Australia (61) 2 9420 5233 China (852) 2428 3282 India (91) 22 2764 2735
 Japan (81) 6 6885 1213 Korea (82) 2 2653 2580 Singapore (65) 6289 1190
 Taiwan (886) 2 8751 6655

www.dionex.com



LPN 2246-01 3/09
 ©2009 Dionex Corporation