Development of a Convenient Peptide-Based Assay for Lysyl Hydroxylase

Mare Cudic,[1] Deepak A. Patel,[1] Janelle L. Lauer-Fields,[1,2] Keith Brew,[2] Gregg B. Fields[1]
[1]*Department of Chemistry and Biochemistry, Florida Atlantic University, 777 Glades Road, Boca Raton, FL 33431-0991*
[2]*College of Biomedical Science, Florida Atlantic University, 777 Glades Road, Boca Raton, FL 33431-0991*

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ABSTRACT:

Hydroxylysine (Hyl) is a posttranslationally modified amino acid found mainly in collagens, the most abundant protein in mammals. Lysyl hydroxylase (LH) catalyzes the hydroxylation of the C-5 position of a Lys residue, resulting in the production of Hyl. Mechanistically, LH incorporates one oxygen atom into both Lys and 2-oxoglutarate; the latter is decarboxylated to form succinate and CO_2. To develop a convenient, RP-HPLC based LH assay, we used Fmoc solid-phase methodology to synthesize three different peptides designed as LH substrates and one peptide corresponding to an LH product. Peptides were characterized by RP-HPLC, MALDI-TOF mass spectrometry and CD spectroscopy. Separation of peptides was examined under a variety of RP-HPLC conditions. The best results were achieved using peptide derivatization (1-anthroylnitrile for organic phase and dansyl chloride for aqueous phase) prior to RP-HPLC analysis. The products (di- and tetra-substituted Lys- and Hyl-containing peptides) were well resolved by RP-HPLC. The resolution of each peak allows for quantification of peak areas, which in turn, when examined as a function of time, can be utilized for studying the kinetics of LH catalyzed reactions. Most significantly, the RP-HPLC assay directly monitors the Hyl containing product. Prior LH assay methods are multi-step, require radio-labeled substrates, and/or measure depletion of 2-oxoglutarate or formation of CO_2. Since the LH reaction with 2-oxoglutarate is uncoupled from Lys hydroxylation, the most accurate assay of LH activity should monitor the formation of Hyl. © *2007 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 90: 330–338, 2008.*

Keywords: *collagen; hydroxylation; enzyme assay; lysyl hydroxylase*

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INTRODUCTION

Collagens are a superfamily of glycoproteins, found in the extracellular matrix throughout the body. Collagens play an important role in maintaining the integrity of various tissues and organs, and in regulating cellular behavior.[1–3] The characteristic structural feature of collagen is a triple helix of high mechanical strength.

The collagen triple-helix has an unusual amino acid composition and sequence. An Xxx-Yyy-Gly repeating motif is charac-

Correspondence to: Gregg B. Fields; e-mail: fieldsg@fau.edu
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teristic of triple-helical structures. Two post-translationally modified amino acids are commonly found: 4-hydroxyproline (Hyp) and 5-hydroxylysine (Hyl). The hydroxyl group of Hyl has two important functions. First, the Hyl hydroxyl group serves as an attachment site for galactose or glucosylgalactose.[4] The extent of Hyl glycosylation has a direct effect on collagen fibril assembly, in that increased carbohydrate content decreases fibril diameter.[5] Hyl glycoslyation levels may also modulate mineralization of collagen triple-helices[6] and cellular interactions with collagen.[7] Second, the Hyl hydroxyl group participates in collagen cross-link formation, which occurs between an aldehyde derived from a Lys or Hyl residue and the ε-amino group of a second Lys or Hyl.[4] Cross-links formed from a Hyl-derived aldehyde are more stable than those formed from a Lys-derived aldehyde.[4] Hyl hydroxy groups can also be phosphorylated,[8] but the significance of this modification has not been described.

Lysyl hydroxylase (LH; EC 1.14.11.4) catalyzes the modification of Lys residues in collagens and other proteins with collagen-like sequences.[4,9] The reaction occurs as a post-translational event in the endoplasmic reticulum prior to triple-helix formation. LH activity in the human, mouse and rat tissues is present in four isoenzymes, LH1, LH2a, LH2b, and LH3.[10,11] LH1 and LH3 hydroxylate Lys residues within the triple-helical region of collagen, while LH2 functions in the telopeptide region.[9] LH3 differs from LH1 and LH2 in that it is multifunctional and able to catalyze, in addition to Lys hydroxylation, sugar transfer reactions, the subsequent steps in the formation of glucosylgalactosyl-Hyl residues.[12–14] Only one isoform for LH is present in lower species such as *Caenorhabditis elegans*.[9,15] This ancestral *C. elegans* LH is also able to glycosylate Hyl residues and, thus, is functionally similar to LH3.[9,13] Recently, Salo et al.[16] found that LH3 is located in two compartments in tissues, in the endoplasmic reticulum and the extracellular space, and the partitioning varies with tissue type. Thus, LH3 may be functional both inside and outside of the cell.

The LH isoforms belong to the family of 2-oxoglutarate dioxygenases. They require ferrous ion, 2-oxoglutarate, molecular oxygen and ascorbate for activity. One atom of oxygen is incorporated at the C-5 position of a Lys residue and another one into 2-oxoglutarate, which is decarboxylated to form succinate and CO_2 (Figure 1).[4,5,9] Ascorbate is needed to regenerate the enzyme after several reaction cycles.[9]

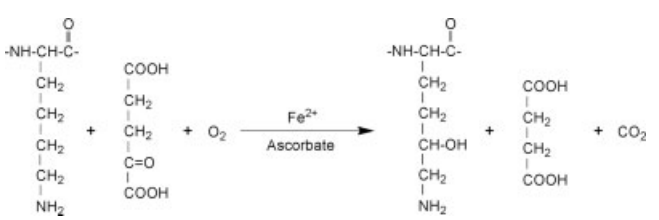

FIGURE 1 Hydroxylation reaction catalyzed by lysyl hydroxylase (LH).

LH1 and LH2 gene mutations have been linked to Ehlers-Danlos syndrome type VI and Bruck syndrome, respectively.[17,18] LH3 null mice have defective type IV collagen biosynthesis, leading to embryonic lethality.[19] Thus, studies of LH isoforms are of interest not only for their physiological functions but also for their roles in inheritable human diseases.

The present study focused on the development of a new assay for LH activity that is convenient and directly monitors Hyl formation. Prior LH assay methods are multi-step, require radio-labeled substrates, and/or measure depletion of 2-oxoglutarate or formation of CO_2.[5,20] Since the LH reaction with 2-oxoglutarate is uncoupled from Lys hydroxylation,[9,21,22] the most accurate assay of LH activity should monitor the formation of Hyl. Conceptually, we sought to efficiently separate Lys-containing peptide substrates from Hyl-containing peptide products using RP-HPLC. Conditions have been examined for resolving non-derivatized and derivatized peptides. Baseline separation has been achieved in both organic and aqueous phases using a derivatizing reagent prior to RP-HPLC analysis. The products (di- or tetra-substituted Lys- and Hyl-containing peptides) have been isolated by RP-HPLC and analyzed by MALDI-TOF MS.

MATERIALS AND METHODS

Synthesis of Peptides

All standard peptide synthesis chemicals and solvents were analytical reagent grade or better and purchased from VWR (Atlanta, GA). 9-Fluorenylmethoxycarbonyl (Fmoc)-4-((2′,4′-dimethoxyphenyl)aminomethyl)phenoxy resin (substitution level = 0.72 mmol/g) and *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU) were purchased from EMD Biosciences (La Jolla, CA). All Fmoc-amino acid derivatives [except Fmoc-Hyl(*O*-TBDMS)] were from EMD Biosciences and are of L-configuration. Fmoc-Hyl(*O*-TBDMS) was synthesized as described.[23]

Peptide synthesis was performed by Fmoc solid-phase chemistry on an Applied Biosystems 433A peptide synthesizer as described.[23,24] Peptides were cleaved from the resin with thioanisole-H_2O-TFA (1:1:18) for 2 h. Cleavage solutions were extracted with methyl *tert*-butyl ether and centrifuged. The pellet was washed with methyl *tert*-butyl ether and air-dried. Four different peptides were synthesized:

a. Gly-Phe-Hyp-Gly-Leu-Hyp-Gly-Ala-Lys-Gly-Glu-NH_2 (peptide **1**);
b. Gly-Phe-Hyp-Gly-Leu-Hyp-Gly-Ala-Hyl-Gly-Glu-NH_2 (peptide **2**);
c. Pro-Hyp-Gly-Arg-Lys-Gly-Ala-Lys-Gly-Lys-Arg-Gly-Pro-Hyp-Gly-NH_2 (peptide **3**)
d. Ile-Lys-Gly-Ile-Lys-Gly-Ile-Lys-Gly-NH_2 (peptide **4**).

Peptide Purification and Analysis

RP-HPLC purification was performed on a Ranin Autoprep system with a Vydac 218TP152022 protein and peptide C_{18} column (15–20 μm particle size, 300 Å pore size, 250 × 22 mm) at a flow rate of 10 ml/min and with detection at $\lambda = 220$ nm. A gradient of 0–100% B in 75 min was found to be effective for peptides **1**, **2**, and **4**. For peptide **3**, the elution gradient was 0% B for the first 5 min followed by 0–75% B in 75 min. The mobile phases were 0.1% TFA in H_2O (A) and 0.1% TFA in acetonitrile (B). Fractions were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Those with the correct mass were pooled together and lyophilized. The purity of peptides was confirmed by RP-HPLC and MALDI-TOF MS (see following).

Analytical RP-HPLC was performed on a Hewlett-Packard 1100 Liquid Chromatograph equipped with a Vydac 218TP5415 protein and peptide C_{18} column (15–10 μm particle size, 300 Å pore size, 150 × 4.1 mm) at a flow rate of 1 ml/min. The other columns used were a (a) Vydac 214TP5415 protein and peptide C_4 column (15–10 μm particle size, 300 Å pore size, 150 × 4.1 mm), (b) Vydac 219TP5415 protein and peptide diphenyl column (15–10 μm particle size, 300 Å pore size, 150 × 4.1 mm), and (c) Vydac 208TP5415 protein and peptide C_8 column (15–10 μm particle size, 300 Å pore size, 150 × 4.1 mm). The mobile phases were 0.1% TFA in H_2O (A) and 0.1% TFA in acetonitrile (B). Detection was at $\lambda = 220$, 254, and 280 nm.

MALDI-TOF MS was performed on an Applied Biosystems Voyager-DE STR using α-cyano-4-hydroxycinnamic acid matrix. Mass values for the peptides were as follows: peptide **1**, $[M+H]^+ = 1060.6$ Da (calculated 1060); peptide **2**, $[M+H]^+ = 1076.5$ Da (calculated 1076); peptide **3**, $[M+H]^+ = 1491.5$ Da (calculated 1491); and peptide **4**, $[M+H]^+ = 913.3$ Da (calculated 913).

Circular Dichroism Spectroscopy

Circular dichroism (CD) spectra were obtained using a JASCO J-810 CD spectropolarimeter at room temperature with 0.1 mg/ml peptide in a 0.2 cm path-length quartz cuvette. The spectra were recorded over a range of $\lambda = 190$–250 nm.

Peptide Derivatization and Separation

Prior to derivatization, several RP-HPLC methods for separation of Lys- and Hyl-containing peptides were examined using different stationary phases, temperatures, flow rates, gradients, and mobile phases, including varying mobile phase A pH. The stationary phases used were C_{18}, C_8, C_4, and diphenyl. The temperatures used were 25, 40, and 50°C. Mobile phase A was 0.1% TFA in H_2O, 0.1% acetic acid (AcOH) in H_2O, 0.1% H_3PO_4 in H_2O, 10 m*M* hexanesulfonic acid (HSA) in H_2O, 25 m*M* KH_2PO_4 in H_2O, or 25 m*M* NaH_2PO_4 in H_2O. Mobile phase B was 0.1% TFA in acetonitrile, 0.1% TFA in acetonitrile/isopropanol, methanol/H_2O, or methanol. The pH values for mobile phase A were 2, 4, and 6. The flow rates were 0.5, 0.7, and 1 ml/min.

Derivatization reactions were performed in both organic and aqueous phases. In organic phase (*N*,*N*-dimethylformamide; DMF), 1-anthroyl nitrile (1-AN) in the presence of base catalyst 4-dimethylaminopyridine (DMAP) was used to derivatize peptides prior to RP-HPLC analysis. In aqueous phase, dansyl chloride (Dns-Cl) in combination with one of two buffers (tricine or sodium carbonate) was used to derivatize peptides prior to RP-HPLC analysis. These buffers were chosen to replace Tris•HCl, which is commonly used in the study of LH activities but is not compatible with Dns-Cl due to the presence of primary amino groups. Both derivatization reactions are described in detail in the following.

Organic Phase. 1-AN reactions were performed with a molar ratio of 1:1:4 (peptide:1-AN:DMAP). Stock solutions were prepared as follows: 0.88 m*M* peptide **1** or **2** in DMF; 17.3 m*M* of 1-AN in DMF; and 164 m*M* of DMAP in DMF. Two hundred microliters of peptide solution was added to a 5 ml amber colored glass vial followed by 10 μl of 1-AN solution and 4 μl of DMAP solution. The reaction vessel was mixed and an aliquot was withdrawn immediately for analytical RP-HPLC and MALDI-TOF MS. The remaining solution was incubated at 37°C. Aliquots were withdrawn at pre-determined times and analyzed by RP-HPLC and MALDI-TOF MS. For RP-HPLC, C_{18} was the stationary phase, the flow rate = 1 ml/min, and two gradients were utilized: the standard gradient consisted of 5% B for the first 5 min and 5–95% B in 20 min, while the extended gradient consisted of 5% B for the first 5 min, 5–23% B in 4 min, 23–50% B in 27 min, and 50–95% B in 10 min. The mobile phases were 0.1% TFA in H_2O (A) and 0.1% TFA in acetonitrile (B).

Aqueous Phase. Dns-Cl reactions were performed in 0.1*M* sodium carbonate or 0.1*M* tricine with a molar ratio of 1:5 (peptide:Dns-Cl). Stock solutions were prepared as follows: 1.16 m*M* of peptide **1** or **2** in H_2O; 0.671 m*M* of peptide **3** in H_2O; 1.1 m*M* of peptide **4** in H_2O; and 9.07 m*M* of Dns-Cl in acetonitrile. One hundred microliters of peptide solution was added to a 5 ml amber colored glass vial followed by 400 μl of tricine or sodium carbonate buffer and 500 μl of Dns-Cl. The reaction vessel was mixed and an aliquot was withdrawn immediately for analytical RP-HPLC and MALDI-TOF MS. The remaining solution was incubated at 37°C. Aliquots were withdrawn at pre-determined times and analyzed by RP-HPLC (using gradients above) and MALDI-TOF MS.

RESULTS

Design, Synthesis, and Characterization of Peptide Substrates

Three potential LH substrates were utilized in the present work. Substrate design was based on the location of Hyl residues in only the Yyy positions of repeating Xxx-Yyy-Gly sequences of collagenous polypeptide chains. In the early 1990s, Ananthanarayan and coworkers examined the substrate specificity of LH1.[25,26] LH1 activity was correlated to substrate sequences and secondary structure. The LH1 kinetic data indicated improved hydroxylation rates in peptides having relatively higher β-turn content and improved binding in peptides with higher contents of polyPro II helices.[25] It was proposed that the conformational criteria for Lys hydroxylation in collagen-related peptides was the presence of a γ- or β-turn in the substrate which interacted with the catalytic site of LH1 and an extended polyPro II type structure in the substrate which interacted with the binding site(s) of the enzyme's active site.[25] Based on CD and NMR spectro-

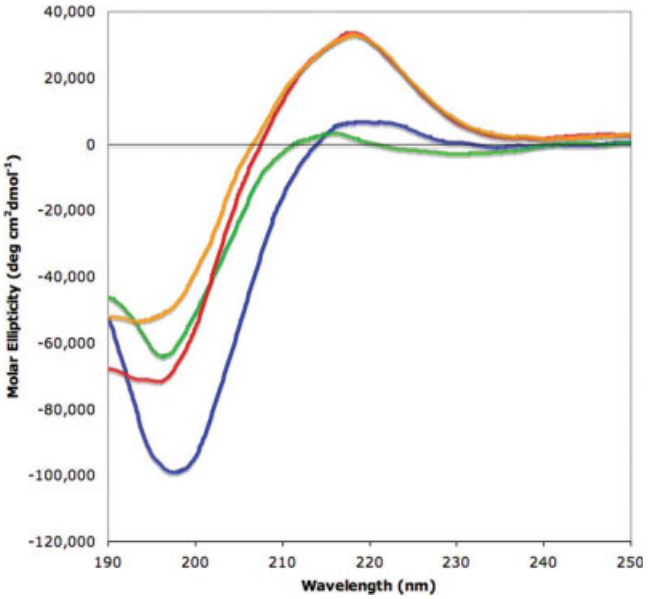

FIGURE 2 CD spectra of peptides **1** (red), **2** (orange), **3** (blue), and **4** (green). Conditions are given in Materials and Methods. The spectra for peptides **1** and **2** show a mixture of β-turn and polyPro II structures, as previously described.[25] The spectra of peptides **3** and **4** are indicative of lower contents of polyPro II structure compared with peptides **1** and **2**, based on the decease in ellipticity at $\lambda = 218$ nm.[25]

scopic studies, peptide **1** (Gly-Phe-Hyp-Gly-Leu-Hyp-Gly-Ala-Lys-Gly-Glu-NH_2) was found to satisfy these conformational criteria, and to be a good LH1 substrate.[25,26] Peptide **2** (Gly-Phe-Hyp-Gly-Leu-Hyp-Gly-Ala-Hyl-Gly-Glu-NH_2) was synthesized to create a model for the peptide product resulting from LH catalyzed hydroxylation of peptide **1**.

Peptide **3** (Pro-Hyp-Gly-Arg-Lys-Gly-Ala-Lys-Gly-Lys-Arg-Gly-Pro-Hyp-Gly-NH_2) was constructed based on the work of Risteli et al.[27] Their study utilized the template Xxx^1-Yyy^2-Gly-Xxx^4-Lys-Gly-Xxx^7-Yyy^8-Gly to screen a collagen-based peptide library and correlate different sequences to LH catalyzed activity. The results suggested that, in general, the LH binding site is not a deep hydrophobic pocket, but rather open and hydrophilic, and where acidic residues can play an important role in substrate binding. Certain LH isoforms exhibited clear preferences for specific peptide sequences. For example, Arg, Lys, Ala, Lys, and Arg at positions 1, 2, 4, 7, and 8, respectively, were favored for LH3 activity. In light of these results, peptide **3** was designed as a potential LH3 substrate.

Peptide **4** (Ile-Lys-Gly-Ile-Lys-Gly-Ile-Lys-Gly-NH_2) was synthesized based on the initial studies of Kivirikko et al.[28] Hydroxylation of Ile-Lys-Gly-Ile-Lys-Gly catalyzed by LH1 was found to proceed 15 times faster than for Ile-Lys-Gly. Subsequent work expanded on these findings, utilizing three repeats of Ile-Lys-Gly as a substrate for all LH isoenzymes.[10,11,14]

Peptides **1**–**4** were obtained in high purity, as indicated by homogenous RP-HPLC elution profiles (data not shown) and MALDI-TOF MS values (see Materials and Methods). The CD spectrum for peptide **1** (Figure 2) was virtually identical to that previously reported.[25] Peptide **1** possesses a mixture of both β-turn and polyPro II structures, based on CD and NMR spectroscopic studies.[25] Replacement of Lys^9 by Hyl (peptide **2**) had virtually no affect on the peptide secondary structure (Figure 2). CD spectra of peptides **3** and **4** (Figure 2) suggested lower contents of polyPro II structure compared with peptides **1** and **2**, based on the decease in molar ellipticity at $\lambda = 218$ nm.[25]

Peptide Separation Without Derivatization

Separation of peptides **1** and **2** was initially attempted using C_{18} RP-HPLC, 0.1 m*M* peptide in H_2O, with a flow rate = 1 ml/min and mobile phases of 0.1% TFA in H_2O (A) and 0.1% TFA in acetonitrile (B). The elution gradient was 0% B for first 2 min then 0–100% B in 20 min. Peptides were

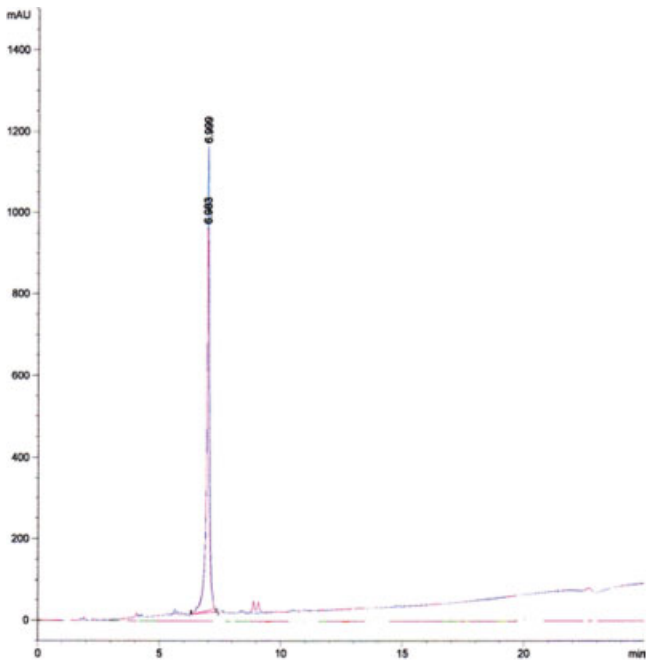

FIGURE 3 Analytical RP-HPLC spectrum of a mixture of peptides **1** and **2** using a C_{18} stationary phase, flow rate = 1 ml/min, and elution gradient of 0% B for the first 2 min and 0–100% B in 20 min. The mobile phases were 0.1% TFA in H_2O (A) and 0.1% TFA in acetonitrile (B). The peptides co-eluted, with retention times of 6.983 min for peptide **2** and 6.999 min for peptide **1**.

Table I Conditions Utilized for Separation of Peptides 1 and 2 by RP-HPLC[a]

Mobile Phase A	Mobile Phase B	Columns	pH	Difference in Elution Times of Two Peptides (min)
0.1% TFA in H_2O	0.1% TFA in ACN	C_{18}, C_8, C_4, diphenyl	2,4,6	0.0
0.1% TFA in H_2O	0.1% TFA in ACN/IPA	C_{18}, C_8, C_4, diphenyl	2,4,6	0.0
0.1% AcOH in H_2O	ACN/MeOH	C_{18}, C_8, C_4, **diphenyl**	2,**4**,6	0.9
0.1% AcOH in H_2O	MeOH	C_{18}, C_8, C_4, **diphenyl**	2,**4**,6	1.9
0.1% H_3PO_4 in H_2O	MeOH	C_{18}, diphenyl	2,4,6	0.0
50 *mM* HSA in H_2O	MeOH	C_{18}, C_4, diphenyl	2,4,6	0.0
25–50 *mM* KH_2PO_4 in H_2O	MeOH	C_{18}, C_4, diphenyl	2,4,6	0.0
25–50 *mM* NaH_2PO_4 in H_2O	MeOH	C_{18}, C_4, diphenyl	2,4,6	0.0

[a] Entries in boldface indicate conditions under which peptides **1** and **2** were separated.
ACN, acetonitrile; IPA, isopropanol; MeOH, methanol.

injected individually and also mixed together. Both peptides eluted at ~7.0 min (Figure 3).

Following initial RP-HPLC analysis, a variety of different conditions were examined to improve separation. The best separation between peptides **1** and **2** was observed using diphenyl RP-HPLC with mobile phases of (A) 0.1% AcOH in H_2O (pH = 4, adjusted with triethylamine) and (B) methanol (Table I). The elution gradient was 20% B for first 2 min, 20–30% B in 60 min, and 30–80% B in 5 min. The retention time for peptide **2** was 30.5 min and peptide **1** was 32.4 min, resulting in a difference of 1.9 min (Figure 4). Unfortunately, peptides **1** and **2** were not baseline resolved, even under the best separation conditions. The lack of baseline separation suggested that peptide derivatization was needed prior to RP-HPLC analysis.

Peptide Separation With Derivatization

1-AN was chosen as the initial peptide derivatization agent, as the resulting products could be monitored by relatively sensitive fluorescence detection. 1-AN is capable of reacting efficiently with amino and primary hydroxyl groups in organic solvents, but more slowly with secondary and tertiary hydroxyl groups due to the steric hindrance of the anthracene ring.[29–32] Under mild conditions, 1-AN reacts with amino and hydroxyl groups to form stable amide and ester bonds, respectively.

The 1-AN derivatization reaction was carried out for peptides **1** and **2** in DMF in the presence of DMAP at 37°C for 1 h. The products were separated under standard gradient RP-HPLC conditions (Figure 5) and evaluated by MALDI-TOF MS analysis (data not shown). 1-AN reacted with peptides **1** and **2** in two positions, presumably the Lys (in peptide **1**) or Hyl (in peptide **2**) ε-amino group and the peptide α-amino group. As peptide **1** contains 2 secondary hydroxyl groups and peptide **2** contains 3 secondary hydroxyl groups, if 1-AN reacted with secondary hydroxyls there would have been different levels of 1-AN modification for the peptides. Since both peptides were modified in two positions by 1-AN, the reaction likely occurred at amino groups, as both peptides have two free amino groups. No additional derivatization (with the secondary hydroxyl groups) was observed after

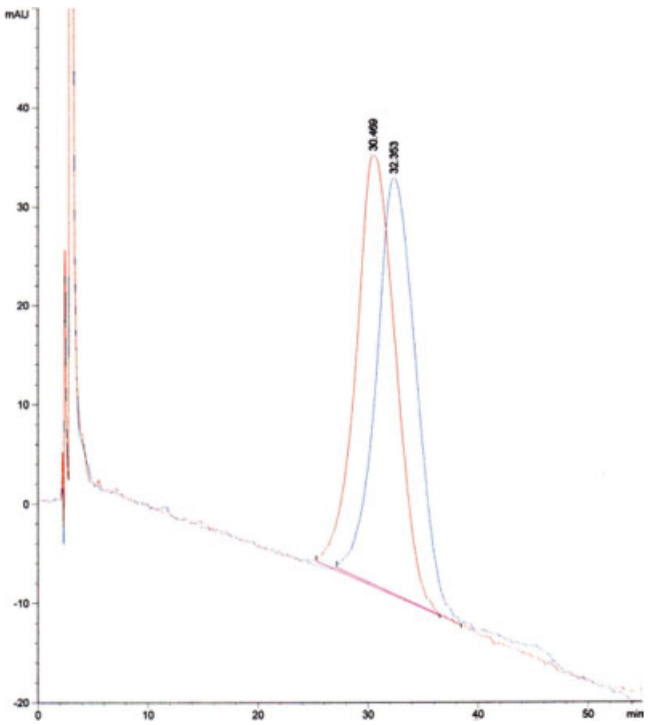

FIGURE 4 Analytical RP-HPLC spectrum of a mixture of peptides **1** and **2** using a diphenyl stationary phase, flow rate of 1 ml/min, and elution gradient of 20% B for the first 2 min, 20–30% B in 60 min, and 30–80% B in 5 min. The mobile phases were 0.1% AcOH in H_2O (A) and methanol (B). Peptide **1** eluted at 32.4 min, and peptide **2** eluted at 30.5 min.

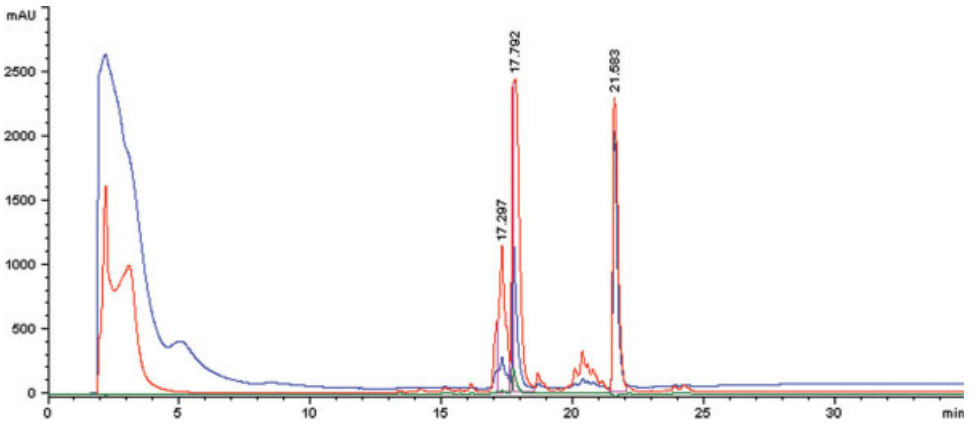

FIGURE 5 Analytical RP-HPLC spectra of mixtures of peptides **1** and **2** after derivatization with 1-AN for 40 min. RP-HPLC conditions were: C_{18} stationary phase, flow rate = 1 ml/min, and elution gradient of 5% B for the first 5 min and 5–95% B in 20 min. The mobile phases were 0.1% TFA in H_2O (A) and 0.1% TFA in acetonitrile (B). Peptide **1**, modified by two anthroyl groups, eluted at 17.8 min, while peptide **2**, modified by two anthroyl groups, eluted at 17.3 min. The peak at 21.6 min is a non-peptide product of 1-AN.

40 min. Derivatized peptide **1** eluted at 17.8 min and peptide **2** at 17.3 min (Figure 5).

The results from the 1-AN derivatization study in organic phase led us to consider a potentially more convenient aqueous phase reaction in which peptide amino groups were modified. Dns-Cl was examined for this purpose as, under mild conditions in aqueous phases, amino groups react with Dns-Cl to form stable conjugates.[33,34] Also, like 1-AN, the products of Dns-Cl reactions can be monitored by fluorescence.

The Dns-Cl derivatization reaction was carried out for peptides **1** and **2** in tricine buffer at 37°C for 1 h. The products were separated under extended gradient RP-HPLC conditions (Figure 6), and structures evaluated by MALDI-TOF MS analysis (data not shown). Dns-Cl reacted with peptides **1** and **2** in two positions, presumably the ε-amino and α-amino groups. Derivatized peptide **1** eluted at 18.0 min and peptide **2** at 17.1 min, with baseline separation between the peaks (Figure 6).

The Dns-Cl reaction in aqueous phase was repeated by using sodium carbonate buffer. The result was identical to that observed with tricine buffer, in that Dns-Cl reacted with peptides **1** and **2** presumably at the ε-amino and α-amino

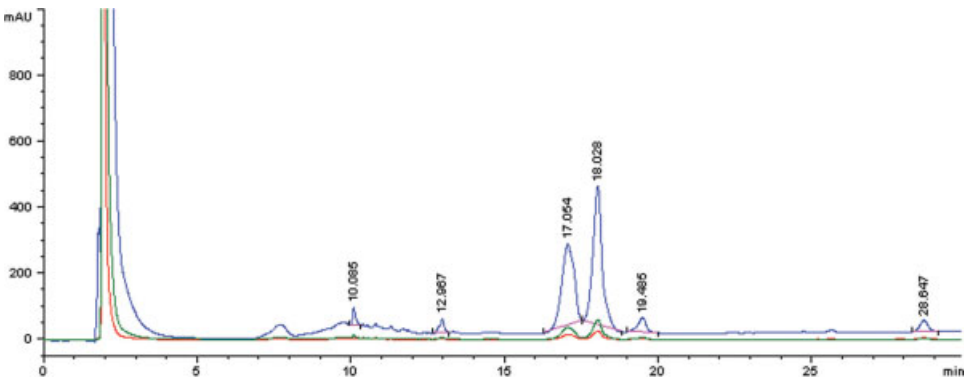

FIGURE 6 Analytical RP-HPLC spectra of mixtures of peptides **1** and **2** after derivatization with Dns-Cl using tricine buffer for 1 h. RP-HPLC conditions were: C_{18} stationary phase, flow rate = 1 ml/min, and elution gradient of 5% B for the first 5 min, 5–23% B in 4 min, 23–50% B in 27 min, and 50–95% B in 10 min. The mobile phases were 0.1% TFA in H_2O (A) and 0.1% TFA in acetonitrile (B). Peptide **1**, modified by two dansyl groups, eluted at 18.0 min, while peptide **2**, modified by two dansyl groups, eluted at 17.1 min. The peak at 28.6 min is Dns-OH.

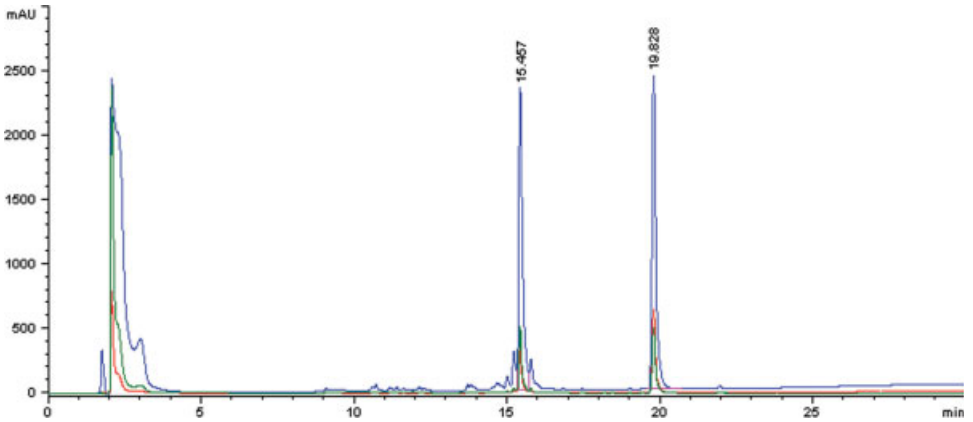

FIGURE 7 Analytical RP-HPLC spectra of peptide **3** after derivatization with Dns-Cl using tricine buffer for 1 h. RP-HPLC conditions were: C_{18} stationary phase, flow rate = 1 ml/min, and elution gradient of 5% B for the first 5 min and 5–95% B in 20 min. The mobile phases were 0.1% TFA in H_2O (A) and 0.1% TFA in acetonitrile (B). Peptide **3**, modified by four dansyl groups, eluted at 15.5 min. The peak at 19.8 min is Dns-OH.

groups and the derivatized peptides were well resolved (data not shown).

To determine whether the Dns-Cl reaction is generally applicable, peptides **3** and **4** were treated with Dns-Cl in tricine buffer. Based on MALDI-TOF MS analysis (data not shown), Dns-Cl reacted with peptides **3** and **4** in four positions each, the three Lys ε-amino groups and the peptide α-amino group. Even though the peptides each possessed four Dns groups, the products were well retained under standard gradient RP-HPLC conditions (Figures 7 and 8). The reaction was efficient, as no intermediate species (one, two, or three amino groups modified) were detected after 1 h.

DISCUSSION

There are two well-documented methods for monitoring LH activity: (a) determination of hydroxy[^{14}C]lysine; and (b) determination of 2-oxo-[1-^{14}C]glutarate.[5] The first method requires preparation of a radio-labeled substrate ([^{14}C]lysine) and radiochemical determination of the hydroxy[^{14}C]lysine product in a three step process. The second method requires preparation of a radio-labeled substrate (2-oxo-[1-^{14}C]glutarate) and radiochemical determination of the decarboxylation reaction. Filter paper saturated with the nitro catechol sulfate solution is used for trapping the $^{14}CO_2$.

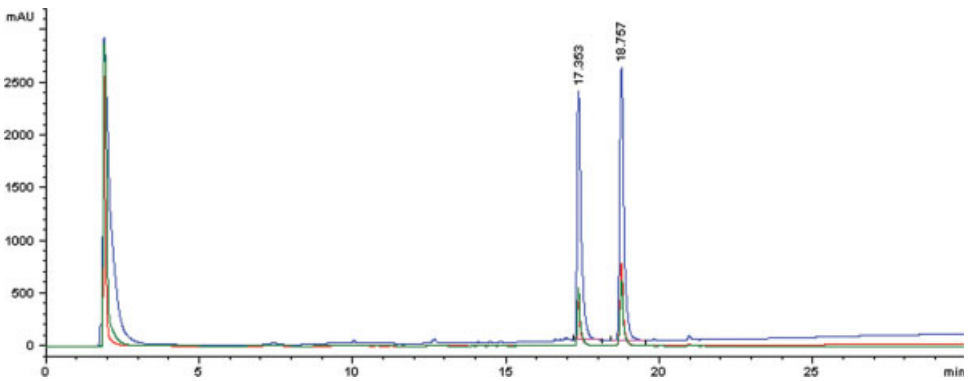

FIGURE 8 Analytical RP-HPLC spectra of peptide **4** after derivatization with Dns-Cl using tricine buffer for 1 h. RP-HPLC conditions were: C_{18} stationary phase, flow rate = 1 ml/min, and elution gradient of 5% B for the first 5 min and 5–95% B in 20 min. The mobile phases were 0.1% TFA in H_2O (A) and 0.1% TFA in acetonitrile (B). Peptide **4**, modified by four dansyl groups, eluted at 17.4 min. The peak at 18.8 min is Dns-OH.

Recently, McNeill et al.[20] reported an assay in which depletion of 2-oxoglutarate is monitored by its derivatization with *o*-phenylenediamine to form 3-(2-carboxyethyl)-2(1*H*)-quinoxalinone, which is amenable to fluorescence analysis. The assay can be utilized for a variety of 2-oxoglutarate-dependent oxygenases, as exemplified by application with FIH (factor-inhibiting hypoxia-inducible factor) and PHD2 (prolyl hydroxylase domain).

The assay based on analysis of hydroxy[^{14}C]lysine is specific for LH activity but is multi-step and requires radioactivity. The assay based on measurement of $^{14}CO_2$ requires fewer steps, but is non-specific, does not directly measure the Hyl content in a substrate, and also utilizes radioactivity. The *o*-phenylenediamine fluorescence assay is the most convenient, but is non-specific and does not directly measure the Hyl content in a substrate. Direct measurement of Hyl content may be critical for accurate evaluation of LH activity, as for many 2-oxoglutarate dioxygenases (including LH), decarboxylation of 2-oxoglutarate is uncoupled from substrate hydroxylation.[9,21,22] Following the 2-oxoglutarate decarboxylation step, the resulting enzyme-oxygen species (a) can hydroxylate the substrate, (b) be quenched by H_2O, generating hydrogen peroxide and inactive enzyme [35], or (c) be regenerated by ascorbate.[9,21,22] Thus, monitoring the depletion of 2-oxoglutarate or formation of CO_2 may provide an inaccurate assessment of LH activity towards peptide and protein substrates.

The present study has focused on RP-HPLC analysis of an LH substrate (peptide **1**) and the resulting product (peptide **2**). Following initial RP-HPLC analysis under standard peptide RP-HPLC conditions, which resulted in co-elution of peptides **1** and **2**, a variety of different conditions were examined to improve separation. These involved changing stationary phases, temperatures, flow rates, and mobile phases, including mobile phase pH. The rationales were as follows. Anionic counter-ions such as trifluoroacetate (TFA^-) or phosphate ($H_2PO_4^-$) will interact with the protonated basic residues of a peptide (Lys, Arg, His, or a free α-amino group). A hydrophobic counter-ion such as TFA^-, through interaction with the positively charged groups in the peptide, will increase the affinity of the peptides for the hydrophobic stationary phase. In contrast, a polar hydrophilic counter-ion such as $H_2PO_4^-$, following ion-pair formation, will neutralize the positive charge on the peptides (thereby decreasing the peptide hydrophilicity) but would be unlikely to interact with the non-polar stationary phase.[36,37] Thus, we compared TFA, acetic acid (AcOH), H_3PO_4, hexanesulfonic acid (HAS), KH_2PO_4, and NaH_2PO_4 as counter-ions (Table I).

Manipulation of mobile phase pH can be utilized to optimize peptide separations.[37] For example, the acidic side chains of Glu and Asp go from fully protonated at pH 2 to fully ionized at pH 6.5. This, in turn, can alter the secondary structure of peptides, modulating interaction of the peptides with the stationary phase. By varying pH values from 2 to 6, better resolution of peptides with acidic side-chains (such as peptides **1** and **2**) may be achieved.

Heat may denature or otherwise alter the secondary structure of peptides. As a consequence, interaction with the stationary phase will change and resolution may be improved or degraded. At various temperatures (5–80°C), regions within the peptide can unfold to different extents and, hence, interact with the stationary phase to differing extents, thus effecting retention time. Although in most cases the effect of temperature on a peptide separation may be small, critical separations may be affected by small changes in temperature.[37,38]

Although optimization of peptide separations during RP-HPLC has generally been achieved via manipulation of the mobile phase on a given RP column, the employment of different stationary phases can also improve separation. Generally, for silica-based stationary phases, useful selectivity differences for peptides have been noted for long chain *n*-alkyl bonded-phases (C_8, C_{18}) versus shorter *n*-alkyl functionalities (C_3, C_4).[38]

Ultimately, separation of peptides **1** and **2** was achieved using a diphenyl stationary phase, AcOH as the counter-ion, and methanol in mobile phase B. There are several possible reasons why this method separated the two peptides (Figure 4), compared with our standard approach (Figure 3): (a) AcO^- served as the counter-ion, creating a slightly less hydrophobic peptide than TFA^-; (b) the mobile phase pH of 4 altered peptide ionization compared with TFA; (c) the diphenyl stationary phase was not as non-polar as C_{18}; and/or (d) a very slow gradient for the elution of both peptides was utilized. The difference between peptides **1** and **2** is only a single hydroxyl group, and thus it appeared that separation required intermediate hydrophobic interactions between the peptides and stationary phases.

The lack of baseline separation between peptides **1** and **2** prevents accurate integration of each peak area, and thus could not be utilized for a quantitative LH assay. A second method was thus developed for efficient RP-HPLC separation of Lys- from Hyl-containing peptides using a derivatizing reagent to modify amino groups, in either organic or aqueous phase, prior to RP-HPLC analysis. The products (di-substituted Lys- and Hyl-containing peptides) were well resolved by RP-HPLC (Figures 5 and 6). The modification of free amino groups by 1-AN or Dns resulted in peptides with greater hydrophobic character, which in turn allowed for enhanced interaction with the C_{18} stationary phase (compare the retention times in Figures 3 and 5). Ultimately, the stronger interaction with the stationary phase resulted in baseline

separation of peptides differing by only one hydroxyl group (Figures 5 and 6), whereas the weaker interaction with the C_{18} stationary phase observed for the unmodified peptide resulted in no separation of peptides differing by a single hydroxyl (Figure 3). The 1-AN and Dns modified peptides had slightly different hydrophobicities, and thus the gradients required to achieve optimal separation differed slightly (Figures 5 and 6).

The resolution of Lys- and Hyl-containing peptide peaks allows for quantification of peak areas, which in turn, when examined as a function of time, can be utilized for studying the kinetics of LH catalyzed reactions. The automation of RP-HPLC should allow for convenient and fast measurement of LH activity. Compared with other LH assays, the present RP-HPLC assay has several distinct advantages. The derivatization reagents are commercially available, and yield products that may be monitored by fluorescence spectroscopy ($\lambda_{excitation}$ = 361 nm and $\lambda_{emission}$ = 470 nm for 1-AN modified peptides, $\lambda_{excitation}$ = 335 nm and $\lambda_{emission}$ = 557 nm for Dns-peptides in H_2O). The RP-HPLC method is sensitive, reproducible, and utilizes simple derivatization conditions, yielding a complete reaction within 1 h at 37°C. Most significantly, the assay monitors the Hyl content in the product. Depending upon solubility of the substrate to be tested, organic or aqueous conditions can be utilized. Finally, different buffer systems can be applied depending upon the LH isoform preference.

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