Consideration of Conformational Transitions and Racemization during Process Development of Recombinant Glucagon-like Peptide-1

RICHARD I. SENDEROFF*, KATHLEEN M. KONTOR, LOTTE KREILGAARD, JIN JYI CHANG, SUNIL PATEL, JONATHON KRAKOVER, JANE K. HEFFERNAN, LEO B. SNELL†, AND GARY B. ROSENBERG

Contribution from *ZymoGenetics Corporation, 1201 Eastlake Ave. East, Seattle, Washington 98102, and Novo Nordisk A/S, Novo Alle, DK-2880, Bagsvaerd, DK.*

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Abstract □ Physicochemical characterization of dry, excipient-free recombinant glucagon-like peptide-1 (rGLP-1) indicates the conformation and purity of the bulk peptide is dependent on the purification scheme and the in-process storage and handling. The recombinant peptide preparations were highly pure and consistent with the expected primary structure and bioactivity. However, variations in solubility were observed for preparations processed by different methods. The differences in solubility were shown to be due to conformational differences induced during purification. A processing scheme was identified to produce rGLP-1 in its native, soluble form, which exhibits FT-IR spectra, consistent with glucagon-like peptide-1 synthesized by solid-state peptide synthesis. rGLP-1 was also found to undergo base-catalyzed amino acid racemization. Racemization can impact the yield and impurity profile of bulk rGLP-1, since the peptide is exposed to alkali during its purification. A combination of enzymatic digestion using leucine aminopeptidase (which cleaves N-terminal L-amino acids ≫ D-amino acids) and matrix-assisted laser desorption ionization mass spectrometry was used to identify racemization as a degradation pathway. The racemization rate increased with increasing temperature and base concentration, but decreased with increasing peptide concentration. The racemized peptides were shown to be less bioactive than rGLP-1.

Advances in biotechnology are leading to the discovery of an ever increasing number of proteins and peptides with pharmaceutical applications. Biopharmaceutical development and realization of the therapeutic potential of these proteins and peptides is dependent on their production and purification in commercially viable quantities. Unfortunately, proteins possess multiple functional groups in addition to three-dimensional structure, making them inherently unstable molecules. This complicates the recombinant production and purification of homogeneous protein preparations having the desired biological and physicochemical characteristics.[1,2] Our studies involving glucagon-like peptide-1 (GLP-1) address these issues and illustrate the importance of physicochemical characterization in support of process development (in addition to formulation and analytical development) of recombinant proteins and peptides.

Glucagon-like peptide-1 (Figure 1) is a 31 amino acid peptide (3356 Da, p*I* 5.4) which promotes the secretion of insulin and has been identified as a mediator of satiety.[3–5] Much of its biological evaluation has been conducted with peptide synthesized by solid-state peptide synthesis (sGLP-1). Clinical evaluation and commercialization of GLP-1 requires a recombinant production process, making a pharmaceutical product economically feasible. Both the physicochemical properties and the biological properties of the recombinant peptide must be equivalent to the synthetic peptide, so that the formulation, administration protocols, and clinical data established for sGLP-1 can be applied toward the development of a recombinant GLP-1 (rGLP-1) product. A production process which yields a dry, excipient-free bulk peptide intermediate simplifies characterization studies (i.e. stability, conformation), dosage form development (e.g. immediate release vs sustained release formulations), in vivo considerations (e.g. dosing protocols), and storage issues (e.g. temperature, capacity).[6] We conducted studies to characterize the physicochemical behavior of rGLP-1 in support of process development of dry, excipient-free bulk peptide. Our results indicate that the conformation and purity of the bulk peptide is dependent on the purification scheme and the in-process storage and handling.

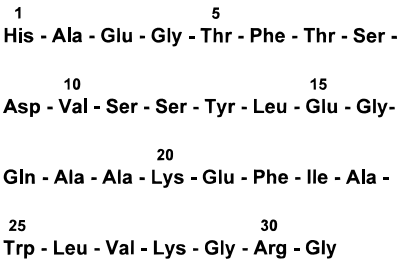

Figure 1—Amino acid sequence of GLP-1.

Experimental Section

GLP-1 Preparations—sGLP-1 was synthesized by solid-state peptide synthesis and was obtained from Bachem California (lot #FGLP9201BR). s(D-Ser[8])GLP-1 was synthesized by solid-state peptide synthesis at ZymoGenetics. rGLP-1 was overexpressed in yeast (Yarrowia lipolytica), purified from culture media, and subsequently lyophilized from either an ammonium hydroxide solution or a suspension in water. rGLP-1(A) was the original batch and was purified by a combination of hydroxylapatite chromatography, reverse phase chromatography, and precipitation (Figure 2A). Prior to lyophilization, rGLP-1(A) was precipitated (by pH adjustment) to remove organic solvent from the reverse phase eluate, resolubilized (≈10 mg/mL) in 0.05 M ammonium hydroxide, and subsequently lyophilized. Portions of rGLP-1(A) were reprocessed by three different schemes (Figure 2B), yielding rGLP-1(B), rGLP-1(C), and rGLP-1(D), to determine the effects of processing on the resultant peptide structure and solubility behavior. Bulk lyophilization was performed using Lyoguard freeze-drying bags (W. L. Gore & Associates) in a Dura-Stop MP Tray Dryer/20 Liter Dura-Dry MP (−85 °C) freeze-drying system (FTS Systems, Stone Ridge, NY) equipped with a liquid nitrogen trap.

* To whom correspondence should be addressed (tel, 206-442-6766; fax, 206-442-6608; e-mail, senderor@zgi.com).
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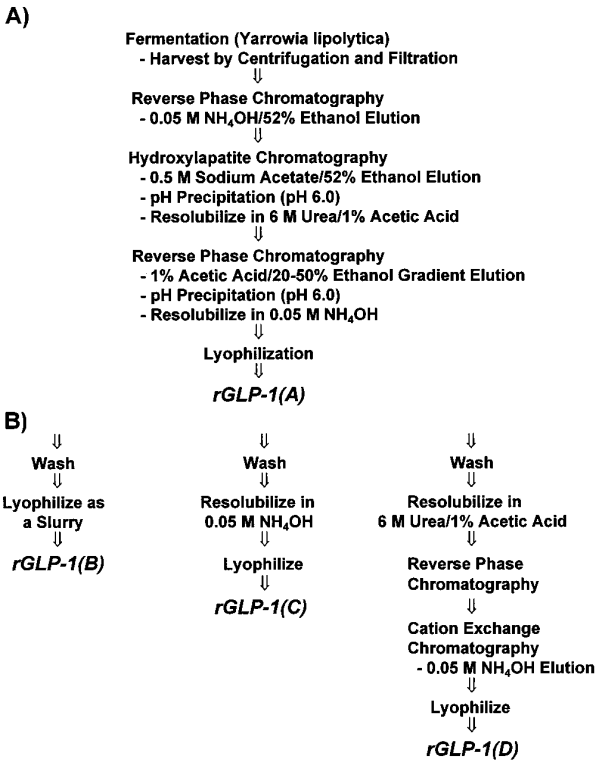

Figure 2—Summary of processing schemes for rGLP-1 variants. Key: (A) purification of rGLP-1(A) and (B) reprocessing of rGLP-1(A) yielding rGLP-1(B), rGLP-1(C), and rGLP-1(D).

Fourier Transform Infrared Spectroscopy (FTIR)—FTIR analysis was used to characterize conformation differences between GLP-1 variants. FTIR spectra were collected at 25 °C using a Magna 550 IR spectrometer (Nicolet Instrument Corp.) equipped with a KBr beam splitter and a DTGS detector. Samples were prepared by mixing 0.5−1 mg of GLP-1 with 300 mg of KBr, which was then compressed into a pellet. Each spectra was obtained by collecting a 256-scan interferogram using a single-beam mode with a 4-cm^{-1} resolution. Background and water vapor spectra were subtracted from each sample spectra. The resultant difference spectra were smoothed using a seven-point Savitsky−Golay function to remove the possible white noise. The inverted second-derivative GLP-1 spectra (OMNIC software; Nicolet Instrument Corp.) were curve-fit using Guassian band profiles (GRAMS software; Galactic Industries). The individual secondary structural elements (amide I band region) were assigned to wavenumbers on the basis of carbonyl stretching in the protein backbone structure according to the method of Dong and Caughey.[7] Semi-quantitative comparison of spectra from rGLP-1 preparations to sGLP-1 (reference) spectra were made be estimating the area of overlap.[8]

HPLC Analysis—The analytical HPLC method was used to estimate the purity of bulk peptide, assay samples from kinetic studies, isolate degradation products, and estimate the concentration and purity of isolated peak fractions. The HPLC system was composed of a Hewlett-Packard HP1090 with diode array detection. Samples in the autosampler were maintained at ≈5 °C during analyses. Hewlett-Packard Chemstation software was used for data aquisition and integration. [Column: PLRP−S (Polymer Labs), 250 × 4.6 mm i.d., 8-μm particle size, 300 Å pore size. Conditions: load at 40% B (1 min) and then linear AB ramp to 65% B (25% B/min) and elution using a linear AB gradient from 65% to 80% (0.5% B/min), where eluent A was 0.1% TFA in water and eluent B was 0.1% TFA in acetonitrile/water (50:50, v/v); flow rate, 1 mL/min; detection wavelength, 215 nm.]

Luciferase Bioassay—The luciferase bioassay was used to evaluate in vitro biologic activity of GLP-1 and its aqueous degradation products. The assay uses baby hamster kidney (BHK) cells that express the human GLP-1 receptor and contain a luciferase gene controlled by a cyclic AMP response element. Exogenous GLP-1 binds to the receptor and activates adenylate cyclase resulting in an increase in intracellular cyclic AMP. Cyclic AMP then activates transcription of the luciferase gene. The expressed luciferase is capable of catalyzing oxidation of luciferin, generating photons which are quantitated in a scintillation counter (LucLite Luciferase Reporter Gene Assay Kit, TopCount Microplate Scintillation Counter; Packard). Luciferase expression is directly proportional to the concentration of GLP-1 binding to the receptor and a standard four-parameter curve can be constructed from known concentrations of sGLP-1 for quantitation of unknown samples. The assay is carried out in a deep-well titer plate (Beckman #267007) format.

Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS)—A combination of N-terminal enzymatic digestion using leucine aminopeptidase (LAP) and MALDI-MS of GLP-1 variants and isolated aqueous degradation products was used to identify the base-catalyzed aqueous degradation pathway(s). Samples were prepared at concentrations of ≈0.1 mg/mL. The N-terminal enzymatic digestion was initiated by adding 20 μL of sample solution to 80 μL of Tris (50 mM)/$MgCl_2$ (50 mM) buffer (pH 8.5), 4 μL of *n*-octyl glucopyranoside (5 mg/mL), and 10 μL of leucine aminopeptidase suspension (Sigma, L-5658, 3 mg/mL; 750 units/mg; 1 unit will hydrolyze 1 μmol of substrate per minute at pH 8.5 at 25 °C). The reaction mixture was incubated for 48 h at 40 °C, and was then stopped by adding 10 μL of acetonitrile/water/trifluoroacetic acid (50/50/0.1). MALDI-MS was performed using a Micromass TofSpec SE in linear mode. A 1.5-μL portion of the digested sample was mixed with 1.5 μL of α-cyano-4-hydroxycinnamic acid solution (Hewlett-Packard) containing 2% trifluoroacetic acid, and the resulting mixture was spotted onto the target and allowed to air-dry.[9,10] Spectra were accumulated several times from random places on the spot to verify consistent relative peak intensity thereby ensuring a homogeneous sample spot.

Results and Discussion

The process by which rGLP-1(A) was produced resulted in a highly pure (1.5% peptide contaminants by RP-HPLC, 3.5% residual acetate, 5.9% residual moisture) white powder which was consistent with sGLP-1 in terms of amino acid sequence, molecular weight, and in vitro bioactivity. However, rGLP-1(A) did not exibit the expected solubility in neutral phosphate buffer based on solubility studies conducted with sGLP-1. Equilibrium solubility determinations by pH titration of a 1.7 mg/mL sGLP-1 solution (23 °C) resulted in a pH−solubility profile consistent with expectations for a peptide having a p*I* 5.4. A minimum solubility of 0.2 mg/mL at approximately pH 6.0 and a precipitation zone (solubility < 1.7 mg/mL) between approximately pH 4.5 and 6.5 was observed. The solubility of sGLP-1 allowed for preparation of solutions at concentrations >10 mg/mL in neutral phosphate buffers, which was required for in vivo biological evaluations. However, equivalent preparations could not be prepared with rGLP-1(A) due to its limited solubility in neutral phosphate buffers (<1 mg/mL). Both sGLP-1 and rGLP-1(A) are readily soluble (>10 mg/mL) in acidic (50 mM phosphoric acid) and basic (50 mM ammonium hydroxide) solvents, which allowed for in vitro bioactivity comparisons. Even though the in vitro bioactivities of sGLP-1 and acid-solubilized rGLP-1(A) were equivalent, the differences in solubility behavior in neutral phosphate buffers revealed inconsistencies between the sGLP-1 and rGLP-1(A) bulk peptides which impacted formulation and subsequent in vivo biological comparisons.

It has been previously shown that chemical and shear pertubations can induce precipitation of sGLP-1 which is related to conformational transitions.[11] Secondary structure transitions in proteins have also been associated with salt-induced precipitation and dehydration.[12,13] FTIR spectra of rGLP-1(A) revealed significant differences (area of overlap = 73%) in the conformationally sensitive amide I

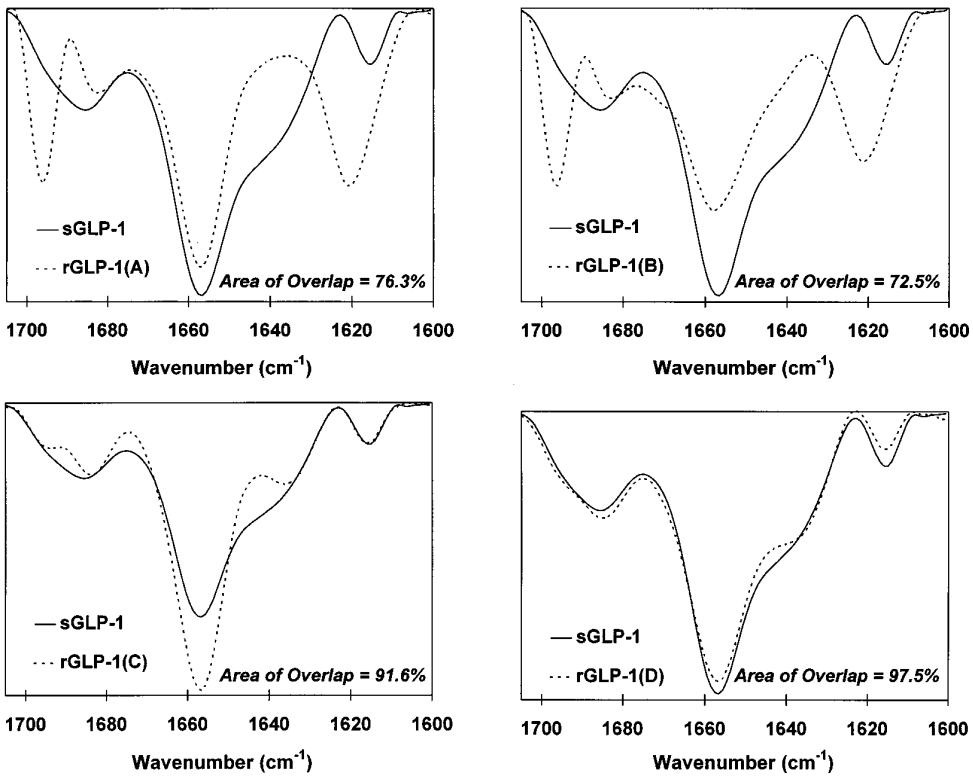

Figure 3—Second-derivative infrared spectra in the amide I region, comparing each rGLP-1 variant to sGLP-1. Corresponding secondary structural compositions are shown in Table 1.

Table 1—Secondary Structural Composition of sGLP-1 and rGLP-1 Variants

	GLP-1 preparation				
structure	sGLP-1	rGLP-1(A)	rGLP-1(B)	rGLP-1(C)	rGLP-1(D)
α-helix	46.2	35.0	26.5	64.3	50.2
β-sheet	25.6	8.0	20.0	15.5	20.9
turn	20.9	18.0	17.4	13.3	22.9
intermolecular β-sheet	7.3	39.0	36.1	6.9	6.0

region compared to sGLP-1 (Figure 3A; Table 1). Significant bands were found at 1620 and 1696 cm^{-1} for rGLP-1(A), which were not present in sGLP-1. These bands are associated with the formation of intermolecular β-sheet structures.[14,15] A corresponding decrease in α-helical structures compared to sGLP-1 is reflected by a reduction in the peak at 1657 cm^{-1}. These differences suggest that in-process treatment of rGLP-1(A) induced loss of disordered elements and formation of nonnative protein aggregates in the dried solid. Interestingly, Kim et al. did not observe bands in the 1610−1620 cm^{-1} region for GLP-1 precipitates induced by shaking or exposure to phenol, suggesting the transitions induced during rGLP-1(A) processing are different.[11] Although in both cases, variants were produced with decreased solubility. The fact that the in vitro bioactivities of sGLP-1 and acid solubilized rGLP-1(A) were equivalent suggests that the structural transitions do not alter the GLP-1 receptor binding interactions or the peptide refolds upon acid solubilization and subsequent dilution into the working concentration range of the bioassay (0.01−100 ng/mL).

Portions of rGLP-1(A) were reprocessed to determine if the structural changes (along with the resultant insolubility in neutral phosphate buffers) were reversible and whether they were due to chemical pertubations (presence of acetate contaminant), in-process pH-induced precipitation, or dehydration. The resultant FTIR spectra are shown in Figure 3B−D, and the corresponding estimates of secondary structural compositions are shown in Table 1. Preparation of rGLP-1(B), by washing (to remove the acetate contaminant) and lyophilizing as a slurry, did not dramatically alter the major secondary structural elements or the resultant solubility. In fact, rGLP-1(B) exhibited less α-helix (reduced 1657 cm^{-1} band) and increased β-sheet (increased 1647 cm^{-1} band) compared to rGLP-1(A). Preparation of rGLP-1(C) by washing, resolubilizing in 0.05 M ammonium hydroxide, and lyophilizing as a solution induced structural changes resulting in a spectra more consistent with sGLP-1 (area of overlap = 91.6%). The bands at 1620 and 1696 cm^{-1} were reduced or eliminated and the band at 1657 cm^{-1} was increased. These transitions are associated with formation of additional α-helix and less β-sheet compared with sGLP-1. However, rGLP-1(C) still exhibited reduced solubility in neutral phosphate buffer (<1 mg/mL), suggesting that α-helial content alone is not a determinant of or a prerequisite for solubility. rGLP-1(D) was prepared by resolubilizing (unfolding) in 6 M urea/1% acetic acid, followed by reverse phase chromatography and cation exchange chromatography (eluting directly into 0.05 M ammonium hydroxide, thereby eliminating the pH-induced precipitation step), and lyophilizing as a solution. The resultant FTIR spectra for rGLP-1(D) was almost identical with those of sGLP-1 (area of overlap = 97.5%), and rGLP-1(D) was soluble in neutral phosphate buffer (>10 mg/mL). This suggests that the in-process structural alterations were caused by pH-induced precipitation. Elimination of this step allowed for the preparation of excipient-free rGLP-1 powder having conformational and solubility properties equivalent to those of sGLP-1.

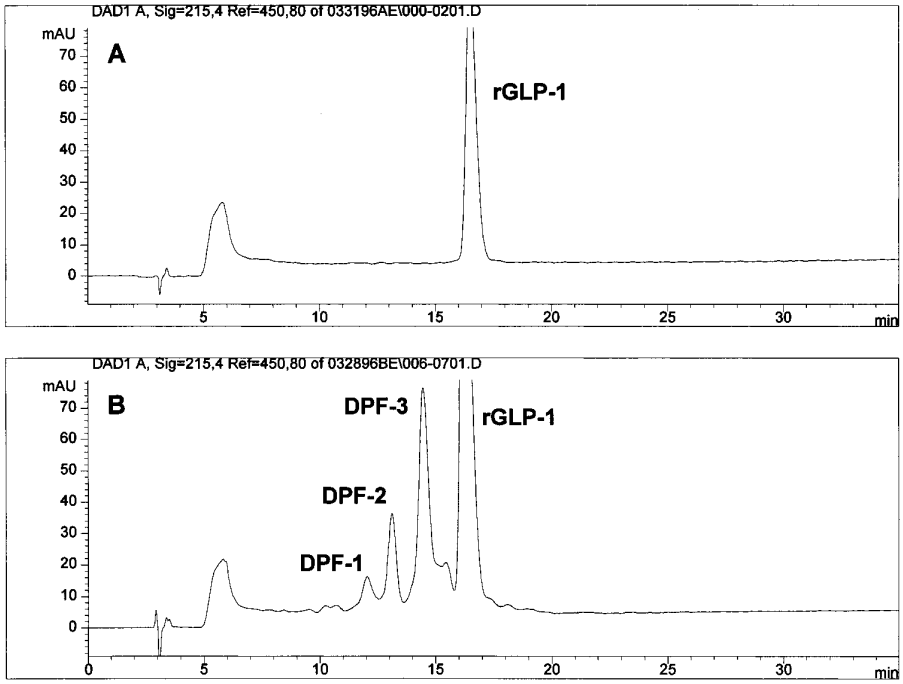

Figure 4—Analytical HPLC chromatograms indicating base-catalyzed degradation. Key: (A) rGLP-1 standard and (B) partially degraded rGLP-1 sample (5 mg/mL; 0.10 M NH_4OH; 37 °C for 1 day).

Purification of rGLP-1 by this scheme requires the peptide to be exposed to 0.05 M ammonium hydroxide (pH 10.7). Although the resulting preparation was highly pure, preliminary HPLC studies indicated that GLP-1 can undergo base-catalyzed degradation to three primary (and several minor) degradation peak fractions (Figure 4). This can potentially lead to peptide impurities in the final product. Therefore, we conducted studies to identify the base-catalyzed degradation products, determine their kinetic dependencies, and evaluate their in vitro bioactivity.

The base-catalyzed degradation peak fractions, identified in Figure 4 as DPF-1, DPF-2, and DPF-3, were isolated by HPLC. Electrospray ionization mass spectrometry (ESI-MS) indicated that the peptides present in each degradation peak fraction had equivalent molecular weight to rGLP-1. These findings ruled out hydrolysis, deamidation, oxidation, diketopiperazine formation, and succinimide formation as possible degradation pathways. However, amino acid racemization can occur in proteins and peptides following exposure to heat and alkali.[16] Peptide variants containing racemized amino acids could not be identified by ESI-MS since there are no mass differences in the intact peptides. A combination of enzymatic digestion using LAP and MALDI-MS was used to determine whether rGLP-1 variants containing racemized amino acids were present in the degradation peak fractions. LAP cleaves N-terminal L-amino acids at a higher kinetic rate than D-amino acids. Although the kinetic rate of cleavage varies for the L-amino acids, a peptide variant containing a D-amino acid can be identified by comparing its MALDI-MS spectra following LAP digestion with that of the control peptide. For instance, peaks corresponding to peptide fragments containing an N-terminal amino acid which was inefficiently cleaved would be evident in the MALDI-MS spectra. Therefore, MALDI-MS of a peptide containing a single D-amino acid following LAP digestion would indicate a peak corresponding to the mass of the peptide fragment contining the N-terminal D-amino acid. Theoretically, the digestion would terminate at this point in the peptide sequence. In our case (Figure 5), this is evident by comparing the spectra following LAP digestion of s(D-Ser^8)GLP-1 with those of sGLP-1 and rGLP-1. The spectra of sGLP-1 and rGLP-1 are equivalent and indicate intense peaks corresponding to inefficient cleavage of glutamic acid (Glu^3, Glu^{15}) and glycine (Gly^4, Gly^{16}) N-terminal amino acids. Minor peaks are also evident corresponding to peptide fragments containing N-terminal threonine, aspartic acid, serine, and alanine. However, the resultant spectra for s(D-Ser^8)GLP-1 indicates that the LAP digestion essentially terminates at the N-terminal D-Ser^8 peptide fragment. Similar analysis of rGLP-1 degradation peak fractions (DPF-1, DPF-2, DPF-3) indicate that racemized product(s) is present in each isolate, since in each case the LAP digestion does not proceed to the extent of the sGLP-1 and rGLP-1 controls. Analysis of DPF-1 indicates significant peaks corresponding to peptide fragments containing N-terminal Thr^7, Ser^8, Val^{10}, Ser^{11}, and Ser^{12} amino acids. Likewise, analysis of DPF-3 indicates significant peaks corresponding to peptide fragments containing N-terminal Thr^7, Ser^8, Val^{10}, and Ser^{11} amino acids. This suggests that DPF-1 and DPF-3 each contain several racemized GLP-1 variants. The method is not capable of identifying more than one racemized amino acid in a given peptide, since the LAP digestion will terminate at the first N-terminal D-amino acid. Therefore, spectra showing multiple peptide fragments which are not consistent with the controls are indicative of several different racemized variants in the preparation. Analysis of DPF-2 suggests that this isolate is composed of a GLP-1 racemized variant(s) containing D-Ser^{11}, since the LAP digestion essentially terminates at a peptide fragment containing an N-terminal Ser^{11}. Our results indicate serine residues in GLP-1 are most sensitive to base-catalyzed racemization.

As shown in Figure 6, the degradation peak fractions containing racemized GLP-1 variants were less bioactive than GLP-1 in the luciferase bioassay, and bioanalytical stability data were consistent with reverse phase HPLC. This indicates that the analytical methods were stability

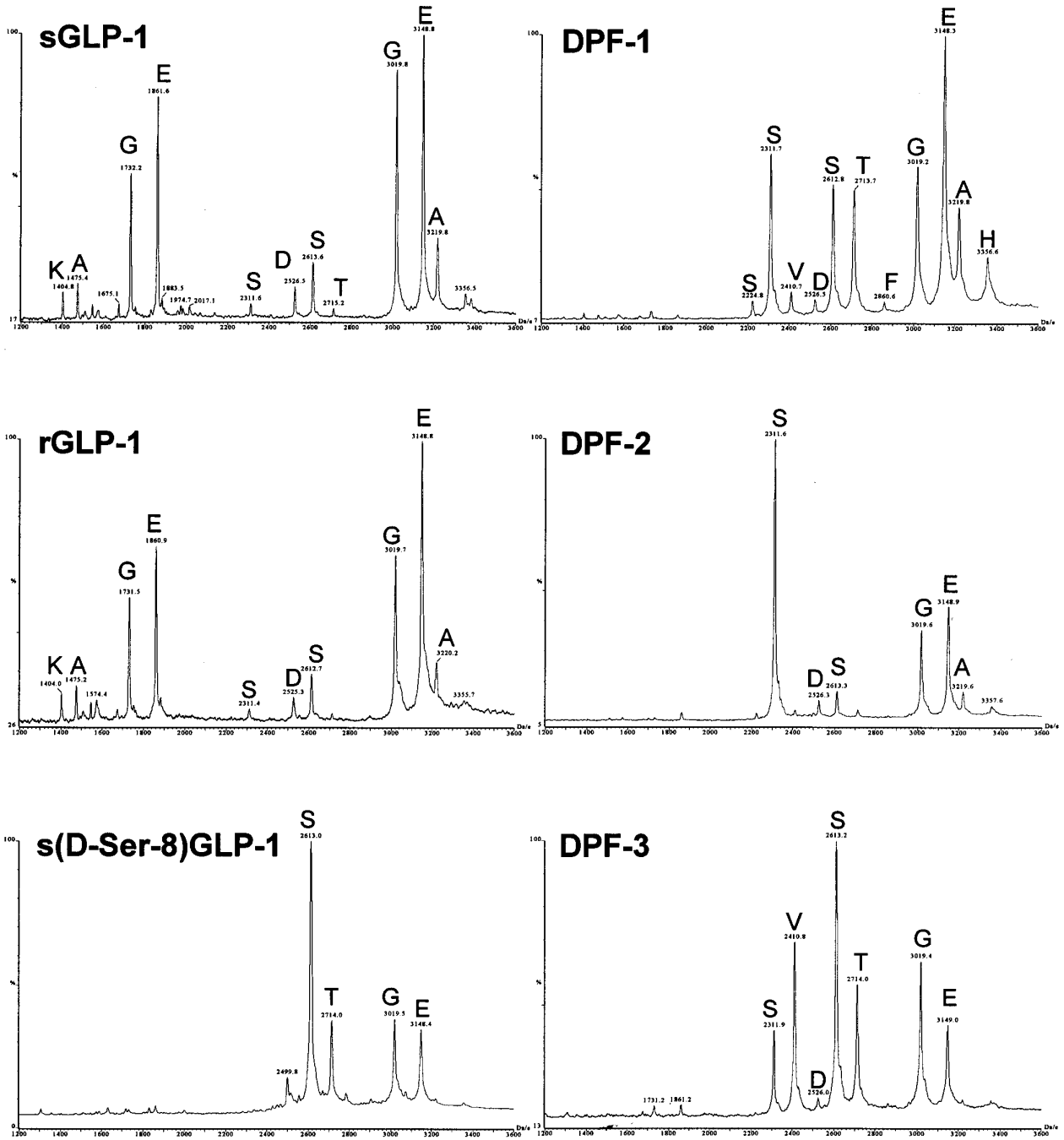

Figure 5—MALDI-MS spectra of GLP-1 variants following LAP digestion. Each peak corresponds to a peptide fragment containing an N-terminal amino acid (indicated) which was inefficiently cleaved.

indicating. Kinetic studies were carried out as a function of temperature, peptide concentration, and ammonium hydroxide concentration. No differences in racemization behavior were observed between sGLP-1 and rGLP-1 variants. As shown in Figure 7, the degradation rate increases with increasing temperature and decreasing peptide concentration. The mechanism of amino acid racemization involves the removal of the α-proton of an amino acid, resulting in the formation of a planar carbanion.[17] Factors which determine the relative racemization rates of amino acids in proteins and peptides are the electron-withdrawing capacities of the various R-substituents, neighboring group effects, inherent structure-related effects, and matrix effects.[17,18] GLP-1 is known to undergo self-association, leading to an oligomer with a higher helical content than the monomer.[11,18] Increases in solution viscosity were observed as peptide concentrations were increased from 5 mg/mL (nonviscous solution) to 25 mg/mL (gel-like solution), suggesting that self-association increased as a function of peptide concentration, as expected. The decrease in the racemization rate with increasing peptide concentration may be related to changes in intramolecular interactions of neighboring residues and/or steric constraints resulting from self-association. The degradation rate also increases as a function of ammonium hydroxide concentration (Figure 8). This was

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