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Chromatographic separation and mass spectrometric identification of positional isomers of polyethylene glycol-modified growth hormone-releasing factor (1-29)

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Abstract

A one-step chromatographic method capable of separating all isomers of polyethylene glycol (PEG)-growth hormone-releasing factor (GRF) (1-29) conjugates was developed. The unmodified GRF (1-29) and seven different isomers of PEG-GRF (1-29) conjugates were separated by using a simple reversed-phase HPLC method depending on the differences of hydrophobicity due to the number and site of PEG attachment. The PEGylation sites of all isomers of PEG-GRF (1-29) conjugates were identified by determining the molecular masses of the Lys-C digested fragments with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. This study is a first report for the separation of all PEG-conjugate isomers and would be useful for further studies to find the promising conjugate by evaluating biological activity and stability of each isomer.

Keywords: PEGylation; Growth hormone-releasing factor; Isomer separation; Reversed-phase HPLC; Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

1. Introduction

Growth hormone-releasing factor (GRF) (1-29) is a fully active and the shortest fragment of GRF (1-44), which promotes the release of growth hormone (GH) from the anterior pituitary [1,2]. Despite its therapeutic value for the treatment of certain GH-related disorders, the GRF (1-29) still has a major limitation in clinical use due to the short biological half-life [3]. The GRF (1-29) is indeed sensitive to enzymatic degradations by dipeptidylpeptidase IV (DPP IV) and trypsin-like enzymes in plasma [4,5]. As a strategy to overcome the clinical problems, the GRF has been modified by polyethylene glycol (PEG) [6–8]. The covalent attachment of PEG, PEGylation, is a technique widely used to provide functional bioconjugates with improved therapeutic properties, including a better resistance to proteolytic degradation, longer circulating half-life and reduced immunogenicity [9–11].

In analytical science, the PEGylated molecules are among the most challenging products because they are composed of heterogeneous molecules depending on the number and position of the attached PEG molecules. In particular, the separation and characterization of positional isomers in a heterogeneous mixture are procedures of growing importance because only one or a few species among the PEGylated molecules substantially exhibit the optimal biological properties. Although several attempts to analyze PEGylated peptides and proteins with high-resolution techniques have been recently reported [12–16], the literature for the separation and characterization of each positional isomer in PEGylated molecules was relatively very few. Monkarsh et al. separated 11 species of mono-PEGylated interferon α-2a using ampholyte-free chromatofocussing-

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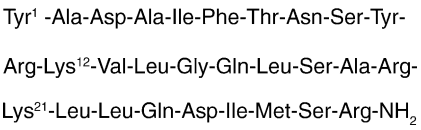

Fig. 1. Primary structure of GRF (1-29). Possible PEGylated sites are Tyr^1, Lys^{12} and Lys^{21}.

like cation-exchange HPLC method [17]. The positional isomers of mono-PEGylated salmon calcitonin were separated by reversed-phase high-performance liquid chromatography (RP-HPLC) and different enzymatic stability according to the PEGylation site was found [18,19]. As shown in Fig. 1, GRF (1-29) has three primary amino groups (N-terminus, Lys^{12}, and Lys^{21}) and the PEGylated GRF (1-29) showed different biological activity depending on the PEGylation site [6,7]. The PEGylation at Lys^{21} did not significantly affect the biological activity relative to unmodified GRF (1-29) regardless of the attached PEG size, whereas the PEGylation at N-terminus and Lys^{12} decreased the biological activity and such a decrease was dependent on the PEG size. The difference of activity preservation was significant as the high molecular mass of PEG was attached. Piquet et al. reported the scale-up of the purification of PEG (M_r: 5000)-conjugated GRF (1-29) species by cation-exchange chromatography and their separation into two mono-PEGylated (positional isomers PEGylated at Lys^{12} and Lys^{21}, respectively) and one di-PEGylated GRF (1-29) by RP-HPLC [20].

In this study, the separation of seven PEG (M_r: 1000)-conjugated GRF (1-29) (three mono-, three di-, and tri-PEGylated GRF (1-29)) by RP-HPLC in a single run is described. The PEGylation sites of each PEGylated GRF (1-29) were identified by proteolytic digestion followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

2. Experimental

2.1. Materials

GRF (1-29) was supplied from A&PEP Inc. (Wolsan, South Korea). Succinimidyl propionate monomethoxy-PEG (mPEG-SPA, M_r: 1000) was obtained from Nektar Therapeutics (Huntsville, AL, USA). Acetonitrile (HPLC grade) were purchased from J.T. Baker (Philipsburg, NJ, USA). Endoproteinase Lys-C (from Lysobacter enzymogenes, sequencing grade), trifluoroacetic acid (TFA), and α-cyano-4-hydroxycinnamic acid (α-CHCA) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and were obtained commercially.

2.2. PEGylation of GRF (1-29)

PEGylation of GRF (1-29) was performed to generate all possible isomers of PEG-GRF (1-29)s based on our previous report on the production of positional isomers of mono-PEGylated salmon calcitonin depending on the reaction pHs with a slight modification [19]. In brief, three milligrams (as a solid form) of mPEG-SPA (M_r: 1000) were added to 5 mL of GRF (1-29) solution (1 mg/mL in 50 mM phosphate buffer, pH 7.5) (molar ratio of GRF (1-29) to $mPEG_{1000}$-SPA = 1:2) at room temperature. The PEGylation mixture was shaken gently at room temperature for 60 min and the PEGylation reaction was quenched by addition of 0.2 mL of 1 M glycine solution. To see the size-based composition of mono-, di-, and tri-PEG-GRF (1-29), the PEG-GRF (1-29) mixture was subjected to size-exclusion chromatography (SEC) using Biosep SEC-S2000 (300 mm × 7.8 mm, Phenomenex, Torrance, CA, USA) equilibrated with 10 mM phosphate-buffered saline (PBS) (pH 7.4) at a flow rate of 0.45 mL/min. The fluorescence was monitored at excitation and emission wavelength of 280 and 315 nm, respectively.

2.3. Separation of PEG-GRF (1-29) isomers by RP-HPLC

The PEG-GRF (1-29) mixture was applied to a LiChrospher 100 RP-8 column (250 mm × 4.0 mm, 5 μm, Merck, Germany) at ambient temperature. Gradient elution was carried out at a flow-rate of 1.0 mL/min with solvent A (0.2% TFA in water) and solvent B (0.2% TFA in acetonitrile). The following gradient profile was chosen: 34–37% B over 8 min, 37–41% B over 14 min, 41–48% B over 8 min. The HPLC fractions corresponding to respective peaks were collected separately, flushed off with nitrogen, and stored at 4 °C.

2.4. Identification of PEGylation sites

A proteolytic digestion was performed by adding 10 μL of endoproteinase Lys-C (100 μg/mL in water) to 100 μL of 50 mM Tris-HCl buffer solution (pH 8.5) containing the respective isomers of PEG-GRF (1-29)s (approximately 100 μg/mL) at 37 °C for 120 min. The respective Lys-C digested samples were directly applied to MALDI-TOF-MS. MALDI-TOF-MS was carried out using a Voyager Biospectrometry Workstation (PerSeptive Biosystems, MA, USA). Samples were prepared by mixing 5 μL of aliquot with 10 μL of the matrix solution, a saturated solution of α-CHCA in 50% of water in acetonitrile with 0.1% trifluoroacetic acid. One μL of the sample mixture was spotted into a well of the sample plate and dried by vacuum evaporation prior to mass spectrometry. Data for 2 ns pulses of the 337 nm nitrogen lasers were averaged for each spectrum in a linear mode, and a positive ion TOF detection was performed using an accelerating voltage of 25 kV. Spectra were smoothened with a 19 point Savitzky–Golay filter and the external calibration was performed using Mass Standard Kit 1 (PerSeptive Biosystems), a mixture of angiotensin I, ACTH (clip 1–17), ACTH (clip 18–39), ACTH (clip 7–38), and bovine insulin.

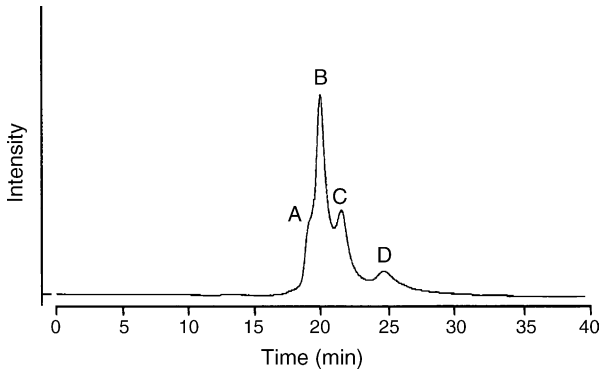

Fig. 2. HPSEC chromatogram of PEG-GRF (1-29) mixture. The mixture was separated into tri-PEG-GRF (1-29) (A), di-PEG-GRF (1-29) (B), mono-PEG-GRF (1-29) (C), and unmodified GRF (D). The fluorescence was monitored at excitation and emission wavelength of 280 and 315 nm, respectively.

3. Results and discussion

PEGylation of peptide and protein having several primary amino groups, which are highly active to PEGylation reaction, inevitably produces the various isomers depending on the number and site of PEG attachment. As GRF (1-29) has three primary amino groups (N-terminus, Lys^{12}, and Lys^{21}) (Fig. 1), the covalent attachment of PEG produces a heterogeneous mixture of seven possible isomers of PEG-GRF (1-29), i.e., three mono-PEG-GRF (1-29)s (N-terminus-; Lys^{12}-; Lys^{21}-), three di-PEG-GRF (1-29)s (N-terminus- and Lys^{12}-; N-terminus- and Lys^{21}-; Lys^{12}- and Lys^{21}-) and one tri-PEG-GRF (1-29). Size-exclusion HPLC (HPSEC) has been used to separate PEGylated peptides with insufficient resolving power [10]. As shown in Fig. 2, when the PEG-GRF (1-29) mixture was applied to the HPSEC, three PEG-GRF (1-29) species with unmodified GRF (1-29) were observed in a size-dependent manner. Each peak was collected from HPSEC and subjected to the MALDI-TOF-MS. Fig. 3 shows the MALDI-TOF-MS spectra of each peak fraction corresponding to mono- (number-averaged molecular mass (M_n) 4420), di- (M_n: 5505) and tri-PEG-GRF (1-29) (M_n: 6580) based on the PEG number from unmodified GRF (1-29) (m/z 3358). As both mono- and di-PEG-GRF (1-29) are still heterogeneous mixture containing three positional isomers, the sophisticated analytical method is necessary to separate each isomer.

Based on the previous study of separation of three positional isomers present in mono-PEGylated salmon calcitonin [18], a RP-HPLC method using gradient elution was applied to separate each isomers present in mono- and di-PEG-GRF (1-29). Fig. 4A shows the chromatogram of PEGylation mixture separated by RP-HPLC, exhibiting 8 peaks (a–h): a retention time of 11.97 min (a), 15.10 min (b), 16.92 min (c), 19.90 min (d), 20.65 min (e), 23.96 min (f), 25.98 min (g), and 28.07 min (h), respectively. These eight peaks appear to correspond to those of one unmodified GRF (1-29) and seven different isomers of PEG-GRF (1-29)s. The first peak (a) was identified to be unmodified GRF (1-29) as shown

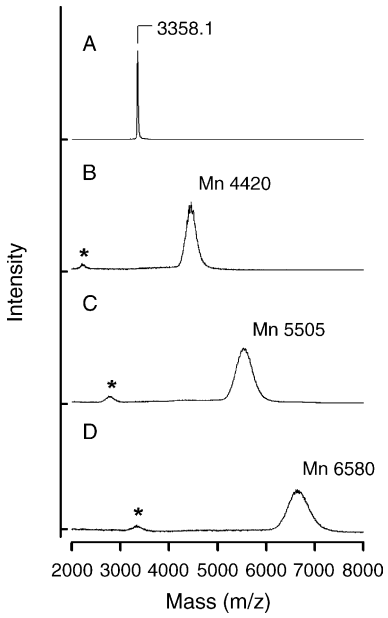

Fig. 3. MALDI-TOF-MS spectra of (A) unmodified GRF (1-29), (B) mono-PEG-GRF (1-29), (C) di-PEG-GRF (1-29), and (D) tri-PEG-GRF (* represents the $[M+2H]^{2+}$ ion peak of each peak) collected from HPSEC.

in Fig. 4B. When the mono-, di- and tri-PEG-GRF (1-29) collected from HPSEC were subjected to the RP-HPLC under the same chromatographic condition, the RP-HPLC chromatograms of mono- and di-PEG-GRF (1-29)s showed three distinct peaks, respectively, whereas that of tri-PEG-GRF (1-29) represented single peak corresponding to the last peak (h) in Fig. 4A (Fig. 4C–E). Each three peaks shown in chro-

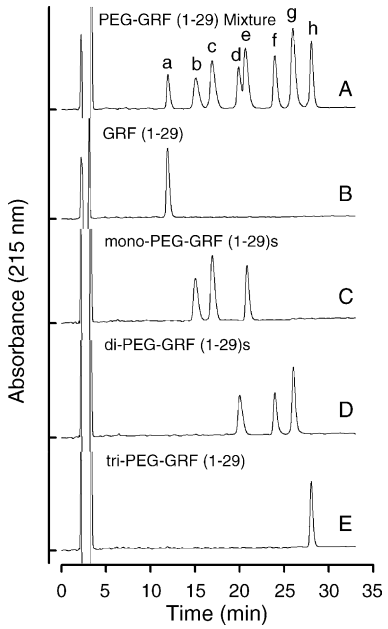

Fig. 4. Separation of PEG-GRF (1-29) isomers by RP-HPLC. Chromatograms of (A) PEG-GRF (1-29) reaction mixture, (B) unmodified GRF (1-29), (C) mono-PEG-GRF (1-29)s, (D) di-PEG-GRF (1-29)s, and (E) tri-PEGylated GRF.

Table 1
Identification of PEGylation sites of PEG-GRF (1-29) isomers by MALDI-TOF-MS of Lys-C digested PEG-GRF (1-29)s

PEG-GRF (1-29)s	PEGylation site	Expected mass of Lys C-treated PEG-GRF (1-29)	Peaks separated by RP-HPLC[a]	Observed mass of Lys C-treated PEG-GRF (1-29)[b]	Peak identity
Mono-PEG-GRF (1-29)s	N-terminus	2529	b	3008	Mono-PEG-Lys^{21}-GRF (1-29)
	Lys^{12}	3482	c	3482	Mono-PEG-Lys^{12}-GRF (1-29)
	Lys^{21}	3007	d	5520	Di-PEG-$Lys^{12,21}$-GRF (1-29)
Di-PEG-GRF (1-29)s	N-terminus, Lys^{12}	4562	e	2514	Mono-PEG-Tyr^1-GRF (1-29)
	N-terminus, Lys^{21}	2529, 3007	f	2533, 3004	Di-PEG-Tyr^1, Lys^{21}-GRF (1-29)
	Lys^{12}, Lys^{21}	5518	g	4574	Di-PEG-Tyr^1, Lys^{12}-GRF (1-29)
Tri-PEG-GRF (1-29)	N-terminus, Lys^{12}, Lys^{21}	6598	h	6598	Tri-PEG-GRF (1-29)

[a] Peak fractions were collected from chromatogram in Fig. 4A.
[b] Mass was determined by MALDI-TOF-MS.

matograms of mono- and di-PEG-GRF (1-29) may indicate separation of three possible positional isomers from each PEG-GRF (1-29). Each peak represented in Fig. 4A was identified as follows: the peaks of b, c and e are mono-PEG-GRF (1-29)s; the peaks of d, f and g are di-PEG-GRF (1-29)s; the peak of h are tri-PEG-GRF (1-29).

To identify the PEGylation sites of isomers of PEG-GRF (1-29)s, the enzymatic digestion of PEGylated peptide followed by comparison of the peptide mapping pattern with that obtained for the parent peptide using MALDI-TOF-MS was used [21,22]. The seven peaks (b–h) corresponding to PEG-GRF (1-29) were collected and the fractions were subjected to endoproteinase Lys-C digestion (Table 1). The endoproteinase Lys-C exclusively cleaves only the carboxy-terminal side of the lysine residues. The Lys-C digestion of unmodified GRF (1-29) results in three fragments of Tyr^1–Lys^{12} (calculated mass; 1448.6), Val^{13}–Lys^{21} (calculated mass; 971.2), and Leu^{22}–Arg^{29} (calculated mass; 974.2). As demonstrated before, PEG attachment appears to increase the resistance of the peptide to proteolytic degradation, probably resulting from the steric hindrance of the PEG strands [21,22]. Due to differences in the number and the site of PEG attachment, Lys-C digested isomers of PEG-GRF (1-29)s resulted in different mass spectrometric profiles of PEGylated fragments. Table 1 represents the expected and observed molecular mass of Lys C-digested PEG-GRF (1-29) isomers by MALDI-TOF-MS. The MALDI-TOF-MS of Lys C-digested fractions of peak b, c and e showed peaks of M_n: 3008, 3482 and 2514, respectively, which correspond to mono-PEG attachment at Lys^{21}, Lys^{12} and N-terminus (Tyr^1), respectively. In the MALDI-TOF-MS of di-PEG-GRF (1-29) fractions, the Lys C-digested peak d and g showed M_n: 5520 and 4574 corresponding to di-PEG- $Lys^{21,12}$- and di-PEG-Tyr^1, Lys^{12}-GRF (1-29), respectively, whereas the peak f produced two peaks of PEG-GRF (1-29) fragments of M_n: 2533 and 3004, which means that two PEG molecules was conjugated to Tyr^1 and Lys^{21}. The M_n of Lys C-digested peak h was determined to be 6598 corresponding to that of tri-PEG-GRF (1-29) itself, which has no cleavable sites for Lys-C due to the PEG-modification of all lysine residues of GRF (1-29). These findings demonstrate that seven peaks of PEG-GRF (1-29) found in RP-HPLC chromatogram are isomers having different numbers of PEG at different amine sites.

In conclusion, all isomers of PEG-GRF (1-29)s were successfully separated by a one-step RP-HPLC method with high-resolution, and their attached PEG numbers and sites were unambiguously assigned by MALDI-TOF-MS analysis following proteolytic digestion. The procedures of chromatographic separation and mass spectrometric identification used in this study would be a valuable tool for the rational design of specific PEGylated peptides with optimal bioactivity and in vivo behavior in a future study.

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