ELSEVIER

Journal of Chromatography B, 754 (2001) 259–263

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Short communication

Identification of the modifying sites of mono-PEGylated salmon calcitonins by capillary electrophoresis and MALDI–TOF mass spectrometry

Dong Hee Na[a], Myung Ok Park[a], So Yoen Choi[a], Yong Suk Kim[a], Seung Seok Lee[a], Sun Dong Yoo[b], Hye Suk Lee[c], Kang Choon Lee[a,*]

[a]*Drug Targeting Laboratory, College of Pharmacy, SungKyunKwan University, Suwon 440-746, South Korea*
[b]*Pharmacokinetics Laboratory, College of Pharmacy, SungKyunKwan University, Suwon 440-746, South Korea*
[c]*College of Pharmacy, Wonkwang University, Iksan 570-749, South Korea*

Received 19 June 2000; received in revised form 13 November 2000; accepted 14 November 2000

Abstract

A capillary electrophoretic method (CE) was developed for the determination of the PEG-modification sites of three positional isomers of mono-PEG modified salmon calcitonins (mono-PEG-sCTs). Resistance to proteolytic degradation on the PEG modification sites resulted in different patterns of CE electropherograms for the tryptic digested mono-PEG-sCTs isomers, and the PEG modification sites were assigned accordingly. The PEG-modification sites were also confirmed directly by determining the molecular masses of the tryptic digested PEG-modified fragments of respective mono-PEG-sCT by the matrix-assisted laser desorption ionization time-of-flight (MALDI–TOF) mass spectrometry.

Keywords: Calcitonins

1. Introduction

Chemical modification of therapeutic polypeptides with polyethylene glycol (PEG) has been utilized as a novel approach to obtaining functional bioconjugates possessing increased resistance to proteolytic degradation, increased solubility, decreased immunogenicity, and low toxicity [1,2]. Difficulty in the physicochemical characterization of PEGylated polypeptides, however, remains a major barrier to the clinical application, due to their heterogeneity with respect to the distribution of both the number and position of attached PEG molecules [3].

The degree of modification of lysine groups in PEGylated polypeptides has been previously estimated spectrophotometrically by measurement of the residual amino groups using trinitrobenzene sulfonic acid [4], and by determining the number of PEG molecules attached to polypeptide by SDS–PAGE, or HPLC followed by MALDI–TOF mass spectrometry [5,6]. Recently, modification sites of mono-PEGylated peptides have been determined by tryptic digestion followed by HPLC and amino acid mapping [7,8]. These methods, however, often require

*Corresponding author. Fax: +82-31-290-7724.
E-mail address: kclee@skku.ac.kr (K.C. Lee).

0378-4347/01/$ – see front matter
PII: S0378-4347(00)00599-5

complicated and time-consuming sample preparation steps.

Capillary electrophoresis (CE) is a technique particularly suitable for the resolution of peptides with slightly differing net charges, and is increasingly used for the separation of peptides and proteins, requiring as little as a few nanoliters of sample volumes [9–12]. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI–TOF-MS) has been introduced in the biochemical research for the analysis of both small and large biomolecules of relevance to the biotechnology industry as a rapid and simple method of obtaining molecular mass information at pico- to femto-molar sensitivity [13–16]. The development of analytical techniques such as MALDI–TOF-MS and CE, and the combined use of two such powerful methods play an increasingly important role in the molecular analysis of bioconjugates of peptides and proteins [17].

Salmon calcitonin (sCT) is a single polypeptide chain calcitropic hormone consisting of 32 amino acids (3432 Da) with an N-terminal disulfide bridge between positions 1 and 7, and a C-terminal proline amide residue (Fig. 1). We have previously reported the modification of sCT with succinimidyl carbonate mono-methoxy polyethylene glycol (SC-mPEG) that has reactivity on the terminal amino and ε-amino group of lysine [5]. Modification of sCT with PEG results in a mixture of isomers of mono-, di-, and tri-PEG-sCTs. We have separated mono-PEG-sCT into three different positional isomers by reversed-phase HPLC [8]. In this report, we describe a rapid CE method that can be used to identify the modification sites of three positional isomers of mono-PEG-sCTs. A direct confirmation of the modification sites is also reported after tryptic digestion followed by MALDI–TOF-MS.

Cys^{1}-Ser-Asn-Leu-Ser-Thr-Cys-Val-Leu-Gly-**Lys^{11}**-
Leu-Ser-Gln-Glu-Leu-His-**Lys^{18}**-Leu-Gln-Thr-Tyr-
Pro-**Arg^{24}**-Thr-Asn-Thr-Gly-Ser-Gly-Thr-Pro-NH_2

Fig. 1. Primary structure of salmon calcitonin. Possible PEGylation sites are Cys^{1}, Lys^{11}, and Lys^{18} and trypsin cleaves sCT at three points, i.e. Lys^{11}, Lys^{18}, and Arg^{24}.

2. Experimental

2.1. Materials

Salmon calcitonin (synthetic cyclic sCT) and succinimidyl carbonate mono-methoxy polyethylene glycol (SC-mPEG, MW 5000 Da) were purchased from Bachem (Torrance, CA) and Shearwater Polymers (Huntsville, AL), respectively. Pentafluoropropionic acid (PFPA), trypsin, and α-cyano-4-hydroxycinnamic acid (α-CHCA) were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade and were obtained commercially.

2.2. Preparation and separation of positional isomers of mono-PEG-sCTs

PEGylation of sCT with SC-mPEG and separation of the resulting positional isomers of mono-PEG-sCTs were performed as described previously [8]. Briefly, 4.38 mg of SC-mPEG 5000 was added to 0.2 ml of sCT (5 mg/ml) in 0.1 *M* phosphate buffer saline solution (PBS, pH 8.0) (molar ratio of sCT to SC-mPEG=1 to 3). The mixture was incubated at 25°C with stirring for 30 min. The reaction was stopped by addition of 5 μl of 1 *M* glycine. The mono-PEG-sCT mixture was isolated and purified from the reaction mixture by size-exclusion chromatography using Superose 12 HR 10/30 (Pharmacia LKB, Sweden), eluted with PBS buffer solution (pH 7.0) at a flow-rate of 0.4 ml/min. The isolated mono-PEG-sCT mixture was applied to a LiChrospher 100 RP-18 column (4.0×125 mm, 5 μm, Merck), and three positional isomers were separated by a linear gradient elution using solvent A (0.1% PFPA in water) and solvent B (0.1% PFPA in acetonitrile) at a flow-rate of 1.0 ml/min, with the gradient condition from 36% B to 42% B over 20 min.

2.3. Tryptic digestion

Tryptic digestion was performed as described previously [18]. Briefly, 20 μl of trypsin (5 mg/ml in water) was added to each of three mono-PEG-sCT isomers at a concentration of approximately 20 μg/ml in 100 μl of 50 m*M* bicarbonate buffer (pH 8.0). A proteolytic digestion was carried out at 40°C for 1

h. Unmodified sCT was treated under the same conditions as a control. The tryptic digested samples were directly applied to CE and MALDI–TOF-MS.

2.4. Capillary electrophoresis

Separation of each fragment was performed in 100 m*M* phosphate buffer (pH. 2.5) as the electrolyte for 10 min, and the UV absorption was measured at 200 nm. The CE used was a Bio-Rad BioFocus 3000 CE system with a polyacrylamide-coated capillary, 50 μm I.D., 24 cm total length and 19.5 cm to the detector (Bio-Rad, Hercules, CA). The capillary was rinsed with distilled water and buffer solution for 120 and 180 s, respectively, prior to each injection. Samples were loaded by applying a nitrogen pressure, and the voltage across the ends of the capillary was set at 10 kV. The temperature of the capillary and samples was maintained at 20°C by a liquid cooling system.

2.5. MALDI–TOF-MS

MALDI–TOF-MS was performed using a Voyager Biospectrometry Workstation (PerSeptive Biosystem, MA). Samples were prepared by mixing 5 μl of aliquot with 5 μl of the matrix solution, a saturated solution of α-CHCA in 50% of water/acetonitrile with 0.3% 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 laser 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, MA), a mixture of ACTH (18–39), ACTH (7–38), and bovine insulin.

3. Results and discussion

Three different positional isomers of the N-terminus, Lys^{18}- and Lys^{11}-residue modified mono-PEGylated sCTs were prepared and separated as described previously [8]. Fractions corresponding to each of three isomers of mono-PEG-sCTs were subjected to tryptic digestion for CE and MALDI–TOF-MS analysis.

The endoproteinase trypsin generally cleaves only the carboxy-terminal side of the lysine and arginine residues. Tryptic digestion of native sCT results in four fragments, Cys^{1}-Lys^{11}, Leu^{12}-Lys^{18}, Leu^{19}-Arg^{24}, and Thr^{25}-Pro^{32}, and the CE electropherogram of tryptic digested native sCT is shown in Fig. 2.

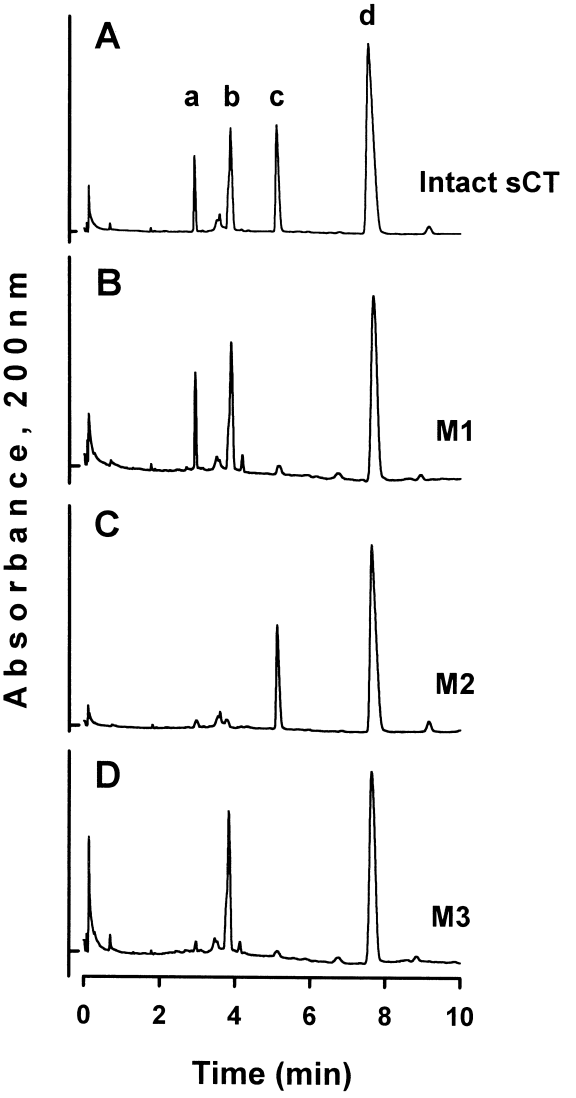

Fig. 2. Tryptic maps of native sCT and three positional isomers of mono-PEG-sCTs by capillary electrophoresis. M1; N-terminus–, M2; Lys^{18}–, M3; Lys^{11}–residue modified mono-PEGylated sCT.

The identity of the tryptic digested fragments of sCT has been assigned as peak **a** (Leu^{12}–Lys^{18}), **b** (Leu^{19}–Arg^{24}), **c** (Cys^{1}–Lys^{11}), and **d** (Thr^{25}–Pro^{32}), respectively, according to the net charge differences as reported by Camilleri et al. [11].

The electropherograms of the tryptic digested positional isomers of mono-PEG-sCTs are shown in Fig. 2 (M1–M3). PEG attachment appears to increase the resistance of protein to proteolytic degradation, resulting in significant differences in the tryptic digestion patterns of the isomers of mono-PEGylated sCTs. The increased resistance presumably reflects the steric hindrance of the PEG strands and acts as a direct barrier to cleavage. In Fig. 2, peaks **a**, **b** and **d** of M1 were comparable to those of native sCT but peak **c** was reduced. This indicates that the peak **c** fragment participated in PEGylation, i.e., the N-terminus amino group of sCT was conjugated with the PEG molecule. Whereas, peaks **c** and **d** of M2 appeared normal but peaks **a** and **b** were reduced, indicating that the peak **a** and **b** fragments participated in PEGylation, i.e., the Lys^{18} residue of sCT was conjugated with PEG. Similarly, peaks **b** and **d** of M3 appeared normal but peaks **a** and **c** were reduced, indicating that peaks **a** and **c** fragments participated in PEGylation, i.e. the Lys^{11} residue of sCT was conjugated. Under condition employed, tryptic digested PEG-modified fragments of each isomer showed broad distribution due to the polydispersity of PEG molecule and the slower electrophoretic mobility (migration time ranged from 30 to 40 min), and then were not useful for identifying the site of PEGylation. Therefore, the running time was limited to 10 min and the rapid analysis is the one of advantages over other methods.

To confirm the site of PEGylation of the tryptic digested fragments of 3 different positional isomers of mono-PEGylated sCTs that were not shown on CE electropherograms, the tryptic digested mixture of each isomer was further subjected to MALDI–TOF-MS. Fig. 3 shows the molecular distribution of the PEG containing fragments of the tryptic digested isomers of mono-PEGylated sCTs determined by MALDI–TOF-MS. The molecular masses of the tryptic digested fragments were determined to be 6028 Da corresponding to the N-terminus–PEG-sCT fragment (Cys^{1}–Lys^{11}, calc.; 5996), 6522 Da corresponding to Lys^{18}–PEG-sCT fragment (Leu^{12}–Arg^{24}, calc.; 6486), and 6869 Da corresponding to Lys^{11}–PEG-sCT fragment (Cys^{1}–Lys^{18}, calc.; 6830), respectively. Although only the midpoint values of the molecular mass range were determined by MALDI–TOF-MS due to the inherent polydispersity of PEG, these molecular masses were within a range of $<1\%$ deviation compared to the calculated values estimated from the molecular mass of intact SC-mPEG.

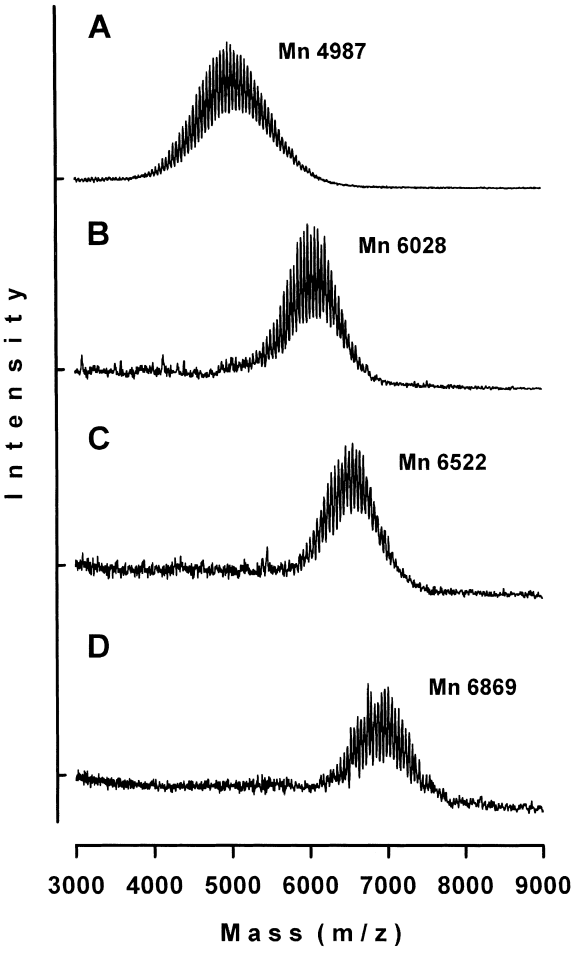

Fig. 3. MALDI–TOF mass spectra of PEGylated fragments of tryptic digested three positional isomers of mono-PEG-sCTs (**A**); SC-mPEG, (**B**); N-terminus-PEG-sCT fragment (Cys^{1}–Lys^{11}), (**C**); Lys^{18}–PEG-sCT fragment (Leu^{12}–Arg^{24}), and (**D**); Lys^{11}–PEG-sCT fragment (Cys^{1}–Lys^{18}).

In summary, PEGylation sites were determined for three mono-PEG-sCT isomers by comparing the differences in the CE electropherogram patterns after

tryptic digestion and were further confirmed by MALDI–TOF-MS. Our observation that PEGylation sites could be identified with nanoliters of sample volume, without amino acid analysis and/or amino acid sequencing steps, is important in the characterization of PEGylated therapeutic peptides.

Acknowledgements

This work was supported by grant No. 2000-2-21700-001-3 from the Basic Research Program of the Korea Science & Engineering Foundation, and partially by the Brain Korea 21 Program.

References

[1] Y. Kodera, A. Matsushima, M. Hiroto, H. Nishimura, A. Ishii, T. Ueno, Y. Inada, Prog. Polym. Sci. 23 (1998) 1233.
[2] P. Bailon, W. Berthold, Pharmaceutical Science and Technology Today 1 (1998) 352.
[3] S.R. Byrn, J.G. Stowell, J. Drug Target. 3 (1995) 239.
[4] P. McGoff, A.C. Baziotis, R. Maskiewicz, Chem. Pharm. Bull. 36 (1988) 3079.
[5] K.C. Lee, K.K. Tak, M.O. Park, J.T. Lee, B.H. Woo, S.D. Yoo, H.S. Lee, P.P. DeLuca, Pharm. Dev. Tech. 4 (1999) 269.
[6] S.P. Monkarsh, Y. Ma, A. Aglione, P. Bailon, D. Ciolek, B. DeBarbier, M.C. Graves, K. Hollfelder, H. Michel, A. Palleroni, J.E. Porter, E. Russoman, S. Roy, Y.-C.E. Pan, Anal. Biochem. 247 (1997) 434.
[7] O.B. Kinstler, D.N. Brems, S.L. Lauren, A.G. Paige, J.B. Hamburger, M.J. Treuheit, Pharm. Res. 13 (1996) 996.
[8] K.C. Lee, S.C. Moon, M.O. Park, J.T. Lee, D.H. Na, S.D. Yoo, H.S. Lee, P.P. DeLuca, Pharm. Res. 16 (1999) 818.
[9] P.D. Grossman, K.J. Wilson, G. Petrie, H.H. Lauer, Anal. Biochem. 173 (1988) 265.
[10] M.A. Moseley, L.J. Deterding, K.B. Tomer, J.W. Jorgenson, Anal. Chem. 63 (1991) 109.
[11] P. Camilleri, G.N. Okafo, C. Southan, R. Brown, Anal. Biochem. 198 (1991) 36.
[12] F. Tagliaro, M. Moffa, M. Gentile, G. Clavenna, R. Valentini, S. Ghielmi, M. Marigo, J. Chromatogr. B. 656 (1994) 107.
[13] R. Kaufmann, J. Biotechnol. 41 (1995) 155.
[14] C. Fenselau, Anal. Chem. 69 (1997) 661A.
[15] K. Wu, R.W. Odom, Anal. Chem. 70 (1998) 456A.
[16] M. Mann, G. Talbo, Curr. Opin. Biotech. 7 (1996) 11.
[17] J. Bullock, S. Chowdhury, D. Johnston, Anal. Chem. 68 (1996) 3258.
[18] K.C. Lee, T.S. Kang, B.H. Woo, J.T. Lee, H.S. Lee, P.P. DeLuca, J. Chromatogr. B. 694 (1997) 31.