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Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 533–539
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Application of HPLC–ESI–ITMS in the quality control of carboxyterminal sequence confirmation for the recombinant DNA product Hirudin Variant 3

Xiao D. Huang [a], Wu W. Tong [a,*], Xia L. Fan [b], Zhong H. Li [b]

[a] School of Life Science and Technology, China Pharmaceutical University, Tongjia-Xiang 24#, Nanjing 210009, China
[b] Jiangsu Institute for the Control of the Pharmaceutical Products, Nanjing, China

Received 1 April 2006; received in revised form 2 August 2006; accepted 8 August 2006
Available online 1 December 2006

Abstract

In the present study, HPLC–ESI–IT (ion-trap) MS was used for carboxyterminal (C-terminal) amino acid sequence confirmation of intact recombinant Hirudin Variant 3 (HV3) and alkylated HV3. The C-terminal amino acid sequence of HV3 was determined by the use of carboxypeptidase P (CPP), and by the combined use of carboxypeptidase P and carboxypeptidase Y (CPY). The C-terminal amino acid sequence of alkylated HV3 with 1,4-dithiothreitol (DTT) reduction was also confirmed by the combined use of CPP and CPY (abbreviated to CPP-CPY). Up to 19 amino acid residues were confirmed in the nanomolar concentration range by analyzing the molecular weights of the truncated peptides of HV3. Another five amino acids were confirmed in the nanomolar concentration range of alkylated HV3 with DTT reduction. For sequencing alkylated HV3 with DTT reduction, HV3 reduced with DTT followed by alkylation with iodoacetamide. The reaction mixture, which included alkylated HV3, DTT, and iodoacetamide, was then directly sequenced without any further pre-treatment. The reaction was designed in a time-, and concentration-dependent manner to obtain the maximum sequence information. The results showed that HPLC–ESI–ITMS cannot only determine the C-terminal amino acid sequence of HV3, but also gives important information about the enzymatic degradation and subsequent release of the C-terminal amino acids of HV3.
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Keywords: HPLC–ESI–ITMS; C-terminal sequencing; Carboxypeptidases P and Y

1. Introduction

MALDI-MS and ESI-MS are considered essential tools in biochemical research for quick and accurate determination of the molecular weights of proteins, nucleic acids, and other polymers. Sequence information on C-terminal amino acids is particularly important in the investigation of N-terminal blocked peptide/protein, in designing oligonucleotide probes, and in the quality control of recombinant DNA products.

C-terminal sequencing via chemical methods remains difficult even after many years of development [1–10]. An alternative approach to chemical sequencing, enzymatic sequencing by MALDI-MS combined with CPP and CPY, was reported to be successful in the analysis of truncated peptide fragments in a generated peptide ladder for C-terminal sequencing [11–22]. Due to the differences in the cleavage rates of amino acids by CPP and CPY, combined use of the two enzymes should be advocated. These two enzymes have relatively broad substrate specificity and complement each other, resulting in cleavages between almost all amino acid residues.

In the present study, we attempted to monitor the truncated peptide fragments of recombinant DNA products or natural proteins using HPLC–ESI–ITMS. ESI-MS of proteins or peptides was performed by producing a multiple charge state during ionization. Smith [23] reported that ES-MS was an effective tool for the analysis of multiply charged ions representing consecutive losses of C-terminal amino acids. The C-terminals of HV3 and alkylated HV3 with DTT reduction were previously successfully confirmed in our laboratory by combining HPLC–ESI–ITMS with CPP and CPY. Owing to the multiple and consecutive (±1) charge states of proteins or peptides during ionization, we were able to distinguish the truncated peptide fragments more easily. HPLC was successfully employed to separate peptide fragments and reduce interference by removing impurities.

* Corresponding author.
E-mail address: wuwutong@cpu.edu.cn (W.W. Tong).

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doi:10.1016/j.jpba.2006.08.009

HPLC–ESI–ITMS is expected to be more widely used for quality control in confirming the C-terminal amino acid sequence of recombinant DNA products or natural proteins.

2. Experimental

2.1. Reagents and chemicals

Recombinant HV3 was prepared in our laboratory by recombinant DNA technology, expression, and multiple-steps purification [24]. The sequence of the protein, deduced from its corresponding DNA sequence is: ITYTDCTESGQ-NLCLCEGSNVCGKGNKCILGSQGKDNQCVTGEGTPKP-QSHNQGDFEPIPEDAYDE [24].

DTT was purchased from BBI in Beijing, China. Iodoacetamide was purchased from Acros in Beijing, China. CPP and CPY were purchased from Sigma–Aldrich, USA.

2.2. High-performance liquid chromatography/mass spectrometry

The HPLC part of the analytical system consisted of an Agilent Series 1100 LC system comprising a degasser, a binary pump, an autosampler, and a thermostat-controlled column compartment. Chromatographic separation took place on a reversed-phase column (Zorbax Prosphere C18, 300 A, 5 μm, 2.1 mm i.d. × 150 mm length).

Mobile phase A consisted of 0.02% TFA in 98% ultra-pure water and 2% acetonitrile. Mobile phase B consisted of 0.02% TFA in 98% acetonitrile and 2% ultra-pure water. The flow rate was 0.195 ml/min. The solution used for post-column fix consisted of propionic and isopropyl alcohol (20:80, v/v). The flow rate for the post-column fix for ESI analysis was 0.005 ml/min. The separation was performed with an increasing gradient of B as shown below. The column temperature was 50 °C.

Time	%B
0.0	0
5.0	20
15.0	50
25.0	95

The analytes were detected by an Agilent 1100 series LC/MSD Trap ion-trap mass spectrometer equipped with an electrospray ionization (ESI) source operated in the positive mode. MS data were acquired over a scan range of 200–2200 amu. Scan resolution was 13000 *m/z*/s.

2.3. Methods

2.3.1. Molecular weights of intact HV3, reduced HV3, alkylated HV3 without DTT reduction, and alkylated HV3 with DTT reduction

HV3 was dissolved in pH 2.0 formic acid to prepare a solution of 0.5 μg/μl. One microliter of the above solution was introduced into the HPLC–ESI–ITMS system for the determination of the molecular weight of intact HV3.

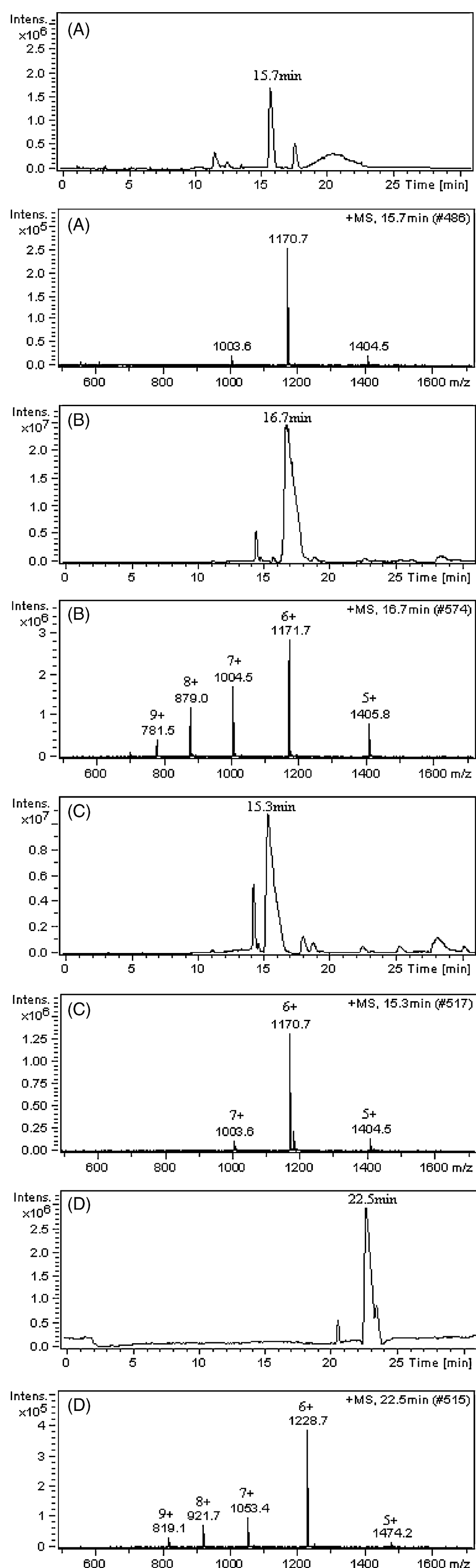

Fig. 1. Both the base peak chromatogram and the ESI-MS spectra of intact recombinant HV3 (A), reduced HV3 (B), alkylated HV3 without DTT reduction (C), and alkylate d HV3 with DTT reduction (D).

HV3 was dissolved in 30 mM NH_4HCO_3 (pH 7.8) to prepare a solution of 3 μg/μl. Sixty microliters of this solution was then reduced with 5 μl of 100 mM DTT for 15 min at 50 °C under nitrogen. This mixture was later alkylated by treating it with 5 μl of 100 mM iodoacetamide for 2 h at 37 °C, under nitrogen in the dark. Ten microliters of the reaction mixture was dissolved in 90 μl pH 2.0 formic acid. Then, 5 μl of the resultant solution was introduced into the HPLC–ESI–ITMS system for the determination of the molecular weight of alkylated HV3 with DTT reduction.

HV3 was dissolved in 30 mM NH_4HCO_3 (pH 7.8) to prepare a solution of 3 μg/μl. Sixty microliters of the solution was alkylated in 30 mM NH_4HCO_3 (pH 7.8), by treatment under nitrogen in the dark with 5 μl 100 mM iodoacetamide for 2 h at 37 °C. Ten microliters of the reaction mixture was dissolved in 90 μl pH 2.0 formic acid. Then, 5 μl of the resultant solution was introduced into the HPLC–ESI–ITMS system for the determination of the molecular weight of alkylated HV3 without DTT reduction.

HV3 was dissolved in 30 mM NH_4HCO_3 (pH 7.8) to prepare a solution of 3 μg/μl. Sixty microliters of the solution was reduced in 30 mM NH_4HCO_3 (pH 7.8), by treatment under nitrogen in the dark with 5 μl 100 mM DTT for 15 min at 50 °C. Ten microliters of the reaction mixture was dissolved in 90 μl pH 2.0 formic acid. Then, 5 μl of the resultant solution was introduced into the HPLC–ESI–ITMS system for the determination of the molecular weight of reduced HV3.

2.3.2. C-terminal sequence confirmation of HV3 using CPP or CPP-CPY

Lyophilized HV3 was dissolved in a buffer of 15 mM ammonium acetate and the pH was adjusted to 4.0 with formic acid. The solution of HV3 was added to the solution of CPP (at enzyme:peptide ratios of 1:8, 1:16, 1:24, 1:32, and 1:64 by weight, respectively) or CPP-CPY (at enzyme:peptide ratios of 1:8, 1:16, 1:24, 1:32, 1:48, and 1:64 by weight, respectively), briefly vortexed, and then incubated at ambient temperature. Fractions of appropriate volume were removed after 1 min, 3 min, 7 min, 10 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 20 h, 24 h, 30 h, 32 h, 48 h, 56 h, and 72 h incubation, acidified with the same amount of 1% TFA to stop the digestion, and then briefly vortexed for HPLC–ESI–ITMS analysis. The approximate quantity at each time point was 100 pmol.

2.3.3. C-terminal sequence confirmation of alkylated HV3 with DTT reduction using CPP-CPY

HV3 was dissolved in 30 mM NH_4HCO_3 (pH 7.8) to prepare a solution of 3 μg/μl. Sixty microliters of this solution was then reduced with 5 μl of 100 mM DTT for 15 min at 50 °C under nitrogen. This mixture was later alkylated by treating it with 5 μl of 100 mM iodoacetamide for 2 h at 37 °C under nitrogen in the dark. Eight hundred and thirty microliters of 15 mM ammonium acetate was added to the reaction mixture and the pH was adjusted to 4.0 with formic acid to prepare a solution of about 900 μl. One hundred microliters of the resultant solution was added to 20 μl of CPP-CPY solution (containing CPP and CPY 0.5 μg), briefly vortexed, and then incubated at room temperature. Fractions were removed after specified time intervals (i.e. 30 s, 1 min, 2 min, 5 min, and 10 min) followed by acidification with the same amount of 1% TFA to stop the digestion. All five fractions were mixed and then briefly vortexed for HPLC–ESI–ITMS analysis.

2.4. Statistical mass assignments

The statistical protocol presented here uses the equation for the two-tailed *t*-test:

$$t_{\text{calcd}} = \frac{|\bar{x} - \mu|\sqrt{n}}{s}$$

Table 1
The mean value of *m*/*z* of the multiply charged peptide fragments after CPP-CPY digestion of HV3

Sequence of the digested peptide fragments	Symbol of the fragments	*m*/*z* of $(M+4H^+)^{4+}$	*m*/*z* of $(M+5H^+)^{5+}$	*m*/*z* of $(M+6H^+)^{6+}$	*m*/*z* of $(M+7H^+)^{7+}$
IT... TPKPQSHNQGDFEPIPEDAYDE	C_0		1404.50 ± 0.00	1170.74 ± 0.05	1003.60 ± 0.10
IT... TPKPQSHNQGDFEPIPEDAYD	C_1		1378.72 ± 0.04	1149.24 ± 0.05	985.06 ± 0.05
IT... TPKPQSHNQGDFEPIPEDAY	C_2		1355.70 ± 0.00	1130.08 ± 0.04	968.60 ± 0.07
IT... TPKPQSHNQGDFEPIPEDA	C_3		1323.06 ± 0.21	1102.86 ± 0.05	945.20 ± 0.20
IT... TPKPQSHNQGDFEPIPED	C_4		1308.98 ± 0.04	1091.04 ± 0.05	934.93 ± 0.12
IT... TPKPQSHNQGDFEPIPE	C_5		1285.96 ± 0.07	1071.95 ± 0.07	918.53 ± 0.15
IT... TPKPQSHNQGDFEPIP	C_6		1260.22 ± 0.04	1050.46 ± 0.05	
IT... TPKPQSHNQGDFEPI	C_7		1240.84 ± 0.05	1034.22 ± 0.04	
IT... TPKPQSHNQGDFEP	C_8		1217.70 ± 0.28	1015.6 ± 0.12	
IT... TPKPQSHNQGDFE	C_9		1198.82 ± 0.11	999.12 ± 0.22	
IT...TPKPQSHNQGDF	C_{10}		1173.04 ± 0.09	977.52 ± 0.18	
IT...TPKPQSHNQGD	C_{11}		1143.50 ± 0.07	952.98 ± 0.04	
IT...TPKPQSHNQG	C_{12}		1120.46 ± 0.05	933.7 ± 0.12	
IT...TPKPQSHNQ	C_{13}		1109.06 ± 0.05	924.24 ± 0.05	
IT...TPKPQSHN	C_{14}		1083.48 ± 0.04	902.86 ± 0.09	
IT...TPKPQSH	C_{15}		1060.64 ± 0.11	883.84 ± 0.05	
IT...TPKPQS	C_{16}	1290.86 ± 0.34	1033.13 ± 0.19		
IT...TPKPQ	C_{17}	1269.32 ± 0.08	1015.74 ± 0.05		
IT...TPKP	C_{18}	1237.44 ± 0.24	990.06 ± 0.11		
IT...TPK	C_{19}	1212.92 ± 0.16	970.62 ± 0.08		

C_0: Intact HV3. C_n (n = 1–19): peptide fragments that have lost n amino acid residues from the C-terminal of HV3.

where $\bar{x}$ is the average experimental mean of amino acid residue, μ the asserted mean of amino acid residue, n the number of replicates, and s is the experimental standard deviation. For the assignment of amino acid residues, t_{calcd} is compared with t_{table} for a given confidence interval ($P = 0.95$, $\alpha = 0.05$). $t_{calcd} > t_{table}$ indicates that the experimental weights come from a population with a mean different from the asserted weights at the given confidence level.

3. Results and discussion

3.1. Molecular weights of intact HV3, reduced HV3, alkylated HV3 without DTT reduction, and alkylated HV3 with DTT reduction

From the base peak chromatogram, we obtained the ESI mass spectra of intact HV3 (Fig. 1A), reduced HV3 (Fig. 1B), alkylated HV3 without DTT reduction (Fig. 1C), and alkylated HV3 with DTT reduction (Fig. 1D). The molecular weights of intact HV3, reduced HV3, alkylated HV3 without DTT reduction, and alkylated HV3 with DTT reduction are 7018, 7024, 7018, and 7366, respectively. The molecular weight of alkylated HV3 without DTT reduction is the same as intact HV3, indicating that there are no free thiols. The molecular weight of reduced HV3 indicates that there are three disulfides. We can further confirm that there are three inner molecular disulfides by determining the molecular weight of alkylated HV3 with DTT reduction. The difference of molecular weight between intact HV3 and alkylated HV3 with DTT reduction is 348, from which we can deduce that, after DTT reduction and iodoacetamide alkylation, there are six acetamides taking the place of six hydrogens in intact HV3. From these results, we can conclude that there are three disulfides and no free thiols in HV3.

3.2. C-terminal confirmation of HV3 using CPP or CPP-CPY

HV3 was digested with CPP-CPY at enzyme:substrate ratios of 1:8, 1:24, 1:32, 1:48, and 1:64, and digested with CPP at enzyme:substrate ratios of 1:8, 1:24, 1:32, and 1:64. As shown in Table 1, the peptide fragments are called C_0 and C_n ($n = 1$–19). C_0 represents intact HV3. C_n ($n = 1$–19) represents truncated peptide fragments that have lost n amino acid residues from the C-terminal of HV3.

These results led us to the conclusion that CPP or CPP-CPY can be used in the C-terminal confirmation of HV3. The truncated peptide fragments C_0–C_{19}, with the exception of C_8–C_{10}, as shown in Fig. 2, were found in the fractions at all enzyme:substrate ratios with CPP or CPP-CPY. C_8–C_{10} were obtained only in the fractions at enzyme:substrate ratios from 1:24 to 1:64 with CPP, or from 1:24 to 1:48 with CPP-CPY. Furthermore, C_0–C_7 and C_{11}–C_{15} were observed at appropriate intensities, whereas C_8–C_{10} always occurred at a relatively weak intensity in the fractions from CPP or CPP-CPY digestion. Among C_{16}–C_{19}, C_{16} and C_{18} had weaker intensities than the other two peptide fragments.

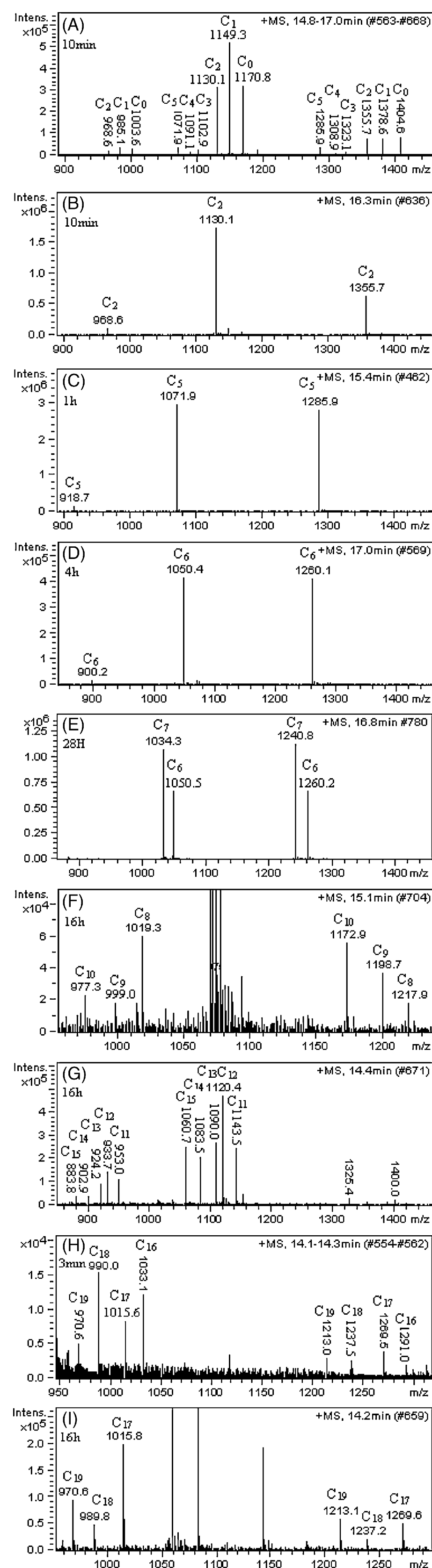

Fig. 2. The ESI spectra for the multiply charged peptide fragments of the fractions at 3 min, 10 min, 30 min. 1 h, 4 h, 10 h, 16 h, 28 h, and 48 h after CPP-CPY digestion of HV3 at an enzyme:substrate ratio of 1:24, which included C_0, C_1, C_2, C_3, C_4, C_5, C_6, C_7, C_8, C_9, C_{10}, C_{11}, C_{12}, C_{13}, C_{14}, C_{15}, C_{16}, C_{17}, C_{18}, C_{19}.

Table 2
Comparison of the actual weights and experimentally derived weights of the released amino acid residues from the C-terminal of HV3

Amino acid residues (symbol, position)	Experimental weights (4^+) ± S.D. (t_{calcd}, n)	Experimental weights (5^+) ± S.D. (t_{calcd}, n)	Experimental weights (6^+) ± S.D. (t_{calcd}, n)	Experimental weights (7^+) ± S.D. (t_{calcd}, n)	Average weights	Actual weights	Δm
Glu (E, 1)		128.90 ± 0.22 (2.20, 5)	129.00 ± 0.60 (0.45, 5)	129.68 ± 0.88 (1.26, 4)	129.19	129.12	0.07
Asp (D, 2)		115.10 ± 0.22 (0.10, 5)	114.96 ± 0.54 (0.54, 5)	115.22 ± 0.38 (0.76, 5)	115.09	115.09	0.00
Tyr (Y, 3)		163.20 ± 1.04 (0.04, 5)	163.32 ± 0.27 (1.17, 5)	163.80 ± 1.40 (0.77, 3)	163.44	163.18	0.26
Ala (A, 4)		70.40 ± 1.14 (1.33, 5)	70.92 ± 0.50 (0.71, 5)	71.87 ± 2.14 (0.64, 3)	71.06	71.08	0.02
Asp (D, 5)		115.30 ± 0.27 (1.71, 5)	114.84 ± 0.68 (0.82, 5)	114.8 ± 1.21 (0.41, 3)	114.98	115.09	0.11
Glu (E, 6)		128.90 ± 0.22 (2.20, 5)	129.24 ± 0.33 (0.82, 5)		129.07	129.12	0.05
Pro (P, 7)		96.90 ± 0.42 (1.18, 5)	97.44 ± 0.33 (2.18, 5)		97.17	97.12	0.05
Ile (I, 8)		115.50 ± 1.41 (2.34, 2)	111.72 ± 0.78 (4.12*, 5)		113.61	113.16	0.45
Pro (P, 9)		95.00 ± 1.41 (2.12, 2)	98.88 ± 1.07 (3.67*, 5)		96.94	97.12	0.18
Glu (E, 10)		128.90 ± 0.22 (2.20, 5)	129.60 ± 2.20 (0.49, 5)		129.25	129.12	0.13
Phe (F, 11)		147.70 ± 0.45 (2.60, 5)	147.24 ± 1.09 (0.12, 5)		147.47	147.18	0.29
Asp (D, 12)		115.20 ± 0.27 (0.90, 5)	115.68 ± 0.78 (1.69, 5)		115.44	115.09	0.35
Gly (G, 13)		57.00 ± 0.50 (0.22, 5)	56.76 ± 1.00 (0.65, 5)		56.88	57.05	0.17
Gln (Q, 14)		127.90 ± 0.42 (1.23, 5)	128.28 ± 0.50 (0.67, 5)		128.09	128.13	0.04
Asn (N, 15)		114.20 ± 0.45 (0.50, 5)	114.12 ± 0.50 (0.09, 5)		114.16	114.1	0.06
His (H, 16)		137.38 ± 0.75 (0.70, 5)			137.38	137.14	0.24
Ser (S, 17)	87.08 ± 1.59 (1.29, 5)	86.88 ± 0.75 (0.61, 5)			86.98	87.08	0.10
Gln (Q, 18)	128.13 ± 1.04 (1.32, 5)	128.40 ± 0.42 (1.44, 5)			128.26	128.13	0.13
Pro (P, 19)	97.12 ± 1.15 (1.87, 5)	97.20 ± 0.67 (0.27, 5)			97.16	97.12	0.05

t_{calcd}^*: $t_{calcd} > t_{table}$; $t_{table} = 2.776$ ($n = 5$) ($P = 0.95$, $\alpha = 0.05$); $t_{table} = 12.706$ ($n = 2$) ($P = 0.95$, $\alpha = 0.05$); Δm: the difference between the average weights and actual weights.

The mean value of *m*/*z* of the multiply charged peptide fragments after CPP-CPY digestion is shown in Table 1. A comparison of the actual weights and experimentally derived weights of the released amino acid residues from the C-terminal of HV3 is shown in Table 2. From the data listed in Table 2, we can see that there is only a small difference (Δm) between the average weights and the actual weights. The maximum and minimum differences (Δm) between the average and actual weights are 0.45 (I, 8) and 0.00 (D, 2) respectively. As shown in Table 2, all of the 19 amino acid residues at the C-terminal, except for Ile (I, 8) and Pro (P, 9), could be confirmed accurately by the multiply charged (4^+–7^+) peptide fragments. C_8 (Ile), C_9 (Pro), and C_{10} (Glu) always had a relatively weak intensity. C_8 (Ile) and C_9 (Pro) could not be confirmed accurately, but we attempted to confirm these two from the average weights of the multiply charged (5^+ and 6^+) peptide fragments, since the average weights were similar to the actual weights.

Although, signal suppression by TFA occurred, HPLC–ESI–ITMS is advantageous for its separating function and for confirmation of the C-terminal sequence of proteins or peptides using multiply charged peptide fragments. The truncated peptide populations could be separated from impurities by HPLC to reduce interference, though the weak intensity of peptide fragments such as C_8 (Ile) and C_9 (Pro) may make them difficult to distinguish. There were ambiguities, however, as shown in Table 2, with the analysis of the isomeric pair leucine/isoleucine (e.g. Ile (I, 8)) and lysine/glutamine (e.g. Gln (Q, 14) and Gln (Q, 18)).

3.3. C-terminal sequence confirmation of alkylated HV3 with DTT reduction using CPP-CPY

The results shown in Fig. 3 indicate the presence of the first six truncated peptide fragments, suggesting the loss of the first to fifth C-terminal amino acid residues of alkylated HV3 with DTT reduction. The mean value of *m*/*z* of the multiply charged peptide fragments is shown in Table 3. A comparison of the actual weights and the experimentally derived weights of the released amino acid residues of alkylated HV3 with DTT reduction is shown in Table 4.

After reduction and alkylation, the reaction mixture, including alkylated HV3 with DTT reduction, DTT and iodoacetamide, was diluted up to 13 times with 15 mM ammonium

Table 3
The mean value of *m*/*z* of the multiply charged peptide fragments after CPP-CPY digestion of alkylated HV3 with DTT reduction

Sequence of the digested peptide fragments	Symbol of the fragments	*m*/*z* of $(M+5H^+)^{5+}$	*m*/*z* of $(M+6H^+)^{6+}$	*m*/*z* of $(M+7H^+)^{7+}$
IT…TPKPQSHNQGDFEPIPEDAYDE	C_0^*	1474.08 ± 0.17	1228.58 ± 0.09	1053.3 ± 0.11
IT…TPKPQSHNQGDFEPIPEDAYD	C_1^*	1448.25 ± 0.27	1207.10 ± 0.14	1034.8 ± 0.12
IT…TPKPQSHNQGDFEPIPEDAY	C_2^*	1425.26 ± 0.18	1187.94 ± 0.11	1018.39 ± 0.10
IT…TPKPQSHNQGDFEPIPEDA	C_3^*	1392.5 ± 0.10	1160.72 ± 0.15	995.1 ± 0.14
IT…TPKPQSHNQGDFEPIPED	C_4^*	1378.4 ± 0.29	1148.79 ± 0.22	984.95 ± 0.07
IT…TPKPQSHNQGDFEPIPE	C_5^*	1355.36 ± 0.09	1129.67 ± 0.11	968.5 ± 0.16

C_0^*: Alkylated HV3 with DTT reduction; C_n^* ($n = 1$–5): peptide fragments that have lost *n* amino acid residues from the C-terminal of alkylated HV3 with DTT reduction.

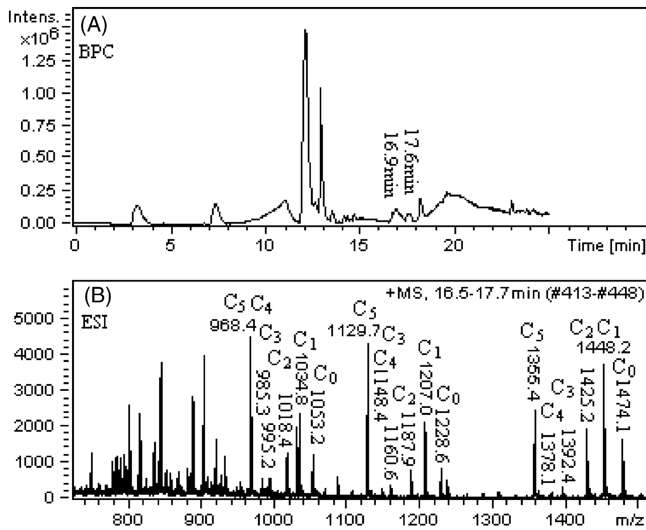

Fig. 3. The base peak chromatogram (A) and the ESI spectra (B) of the truncated peptide fragments after CPP-CPY digestion of alkylated HV3 with DTT reduction. C_0, C_1, C_3, C_4 and C_5 were included in the base peak at RT = 16.9 min. C_2 was included in the base peak at RT = 17.6 min.

acetate and the pH was adjusted to 4.0 with formic acid to prepare the reaction mixture. A portion of the reaction mixture was then taken and reacted with CPP-CPY without further pre-treatment. By diluting with 15 mM ammonium acetate and adjusting the pH with formic acid, we were able to determine the C-terminal amino acid sequence of alkylated HV3 with DTT reduction. As shown in Tables 3 and 4, by identifying the multiply charged (5^+–7^+) digested peptide fragments, we were able to confirm the first five amino acids.

In this study, we successfully determined alkylated HV3 with DTT reduction without further separation from DTT and iodoacetamide.

3.4. Degradation of the C-terminal of HV3 using CPP-CPY

The degradation of the C-terminal of HV3 was studied using a combination of HPLC–ESI–ITMS with CPP-CPY. The reaction was designed in a time- and concentration-dependent manner to obtain the maximum sequence information.

We were able to easily obtain important qualitative information. To illustrate the degradation process of HV3, as shown in Fig. 4, fractions at 3 min, 10 min, 30 min, 1 h, 4 h, 10 h, 16 h, 28 h, and 48 h after CPP-CPY digestion at an enzyme:substrate ratio of 1:24 were taken into account. As shown in Fig. 4, in the 3 min to 1 h fractions, C_0, C_1, C_3, C_4, C_5 were included in the base peak at RT = 15.5 min, 15.5 min, 15.6 min, and 15.4 min, and C_2 was included in the base peak at RT = 16.6 min, 16.3 min, 16.3 min, and 16.5 min. In the 4–48 h fractions, C_6, C_7 were included in the base peak at RT = 17.0 min, 16.6 min, 16.3 min, 16.7 min, and 16.6 min. C_8, C_9, C_{10} were included in the base peak at RT = 15.1 min in the 16 h fraction. C_{11}, C_{12}, C_{13}, C_{14}, C_{15} were included in the base peak at RT = 14.3 min, 14.3 min, 14.2 min, 14.3 min, 14.3 min, 14.3 min, 14.2 min, 14.7 min, and 14.4 min in the 3 min to 48 h fractions. C_{16}, C_{17}, C_{18}, C_{19} were

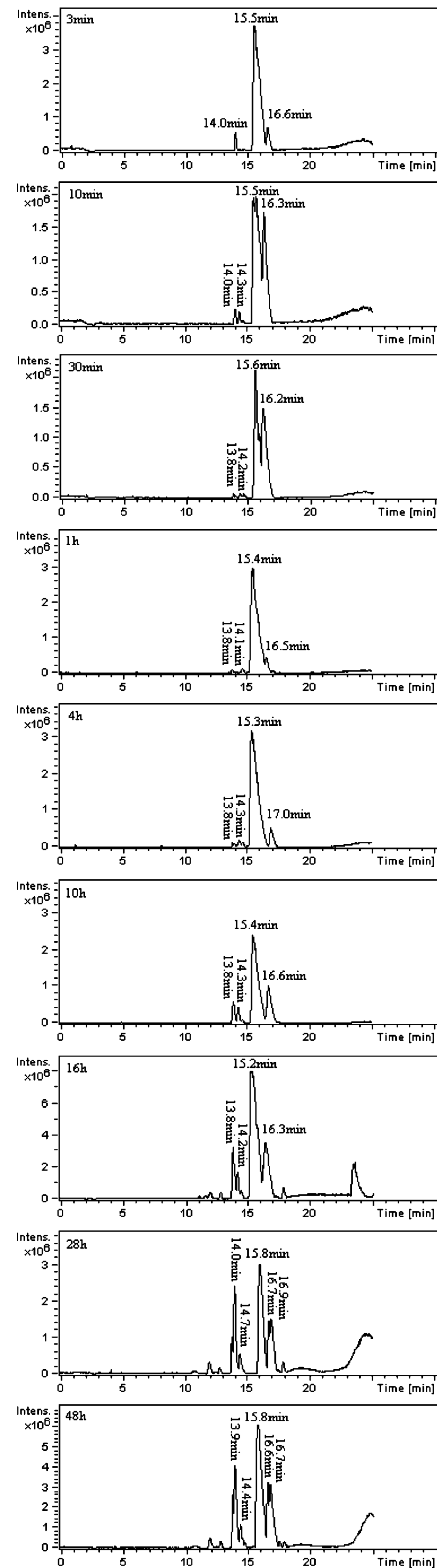

Fig. 4. The base peak chromatogram of the fractions at 3 min, 10 min, 30 min, 1 h, 4 h, 10 h, 16 h, 28 h, and 48 h after CPP-CPY digestion of HV3 at an enzyme:substrate ratio of 1:24.

Table 4
comparison of the actual weights and experimentally derived weights of the released amino acid residues from the C-terminal of alkylated HV3 with DTT reduction

Amino acid residues (symbol, position)	Experimental weights (5^+) ± S.D.	Experimental weights (6^+) ± S.D.	Experimental weights (7^+) ± S.D.	Average weights	Actual weights
Glu (E, 1)	129.35 ± 1.11	128.88 ± 0.84	129.97 ± 0.99	129.35 ± 0.54	129.12
Asp (D, 2)	115.25 ± 0.50	115.04 ± 0.66	114.80 ± 1.04	114.98 ± 0.31	115.09
Tyr (Y, 3)	163.50 ± 1.00	163.32 ± 0.97	163.45 ± 1.48	163.38 ± 0.39	163.18
Ala (A, 4)	70.50 ± 1.32	71.50 ± 1.73	71.05 ± 0.49	71.02 ± 0.50	71.08
Asp (D, 5)	115.13 ± 1.75	114.8 ± 1.21	115.15 ± 0.49	115.16 ± 0.34	115.09

included in the base peak at RT = 14.0 min, 14.0 min, 13.8 min, 13.8 min, 13.8 min, 13.8 min, 13.8 min, 14.0 min, and 13.9 min in the 3 min to 48 h fractions.

From the above results, we can qualitatively extrapolate the degradation process and the corresponding ESI-MS spectra.

4. Conclusions

The results of this study show that HPLC–ESI–ITMS can be used as a tool to confirm the C-terminal sequence of recombinant HV3. C_0–C_7 and C_{10}–C_{19} were confirmed accurately. Due to the low intensity, we were unable to confirm C_8 and C_9 accurately. Up to 19 amino acids were confirmed in our study. Ambiguities exist, however, for the isomeric pair leucine/isoleucine (e.g. Ile (I, 8)) and for lysine/glutamine (e.g. Gln (Q, 14) and Gln (Q, 18)). Simultaneously, we confirmed the C-terminal sequence of alkylated HV3 with DTT reduction, which also included a mixture of alkylated HV3 with DTT reduction, DTT, and iodoacetamide without further pre-treatment. We were able to qualitatively index the enzymatic degradation and release of HV3. Despite signal suppression by TFA, the interference was markedly reduced by the use of HPLC, and the multiply charged fragments were confirmed. Efforts to improve the sensitivity of ESI-MS by the use of other ion pair reagents such as formic acid instead of TFA in the mobile phase are now being made in our laboratory. HPLC–ESI–ITMS is much more widely used than MALDI-MS, especially for quality control of pharmaceuticals in China, and is expected to be more widely used in the quality control of recombinant DNA products.

Acknowledgement

We are very grateful to Jiangsu Institute for Drug Control for their technical support.

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