JOURNAL OF MASS SPECTROMETRY, VOL. 32, 1140–1142 (1997)

JMS Letters

Dear Sir,

Analysis of Vero Toxins 1 and 2 by High-performance Liquid Chromatography/Electrospray Ionization Mass Spectrometry

During the past 10 years, disease outbreaks of hemorrhagic colitis (HC) and hemolytic–uremic syndrome (HUS) in humans have been increasing worldwide owing to the ingestion of beef and dairy products contaminated with Vero toxin-producing strains of *Escherichia coli* (VTEC), in particular serotype O157:H7.[1] In 1996, VTEC caused an extraordinarily huge-scale outbreak in Japan, involving more than 9000 patients; 11 cases were accompanied by HUS and resulted in death.[2] *E. coli* O157:H7 and several other serotypes produce two cytotoxins to Vero cells, of which Vero toxin 1 (VT1, Shiga-like toxin 1) has been reported to be immunologically, physicochemically and biologically identical to Shiga toxin, whereas Vero toxin 2 (VT2, Shiga-like toxin 2) is immunologically distinct from VT1 and shares 56% amino acid identity with VT1.[3] Vero toxins are subunit toxins comprising an A subunit, which inhibits intracellular protein synthesis via a specific RNA *N*-glycosidase activity, and five non-covalently associated receptor-binding B subunits, which facilitate the entry of the A subunit into susceptible cells.[3]

Current methods for the determination of Vero toxins produced by VTEC are classified into two groups: one that

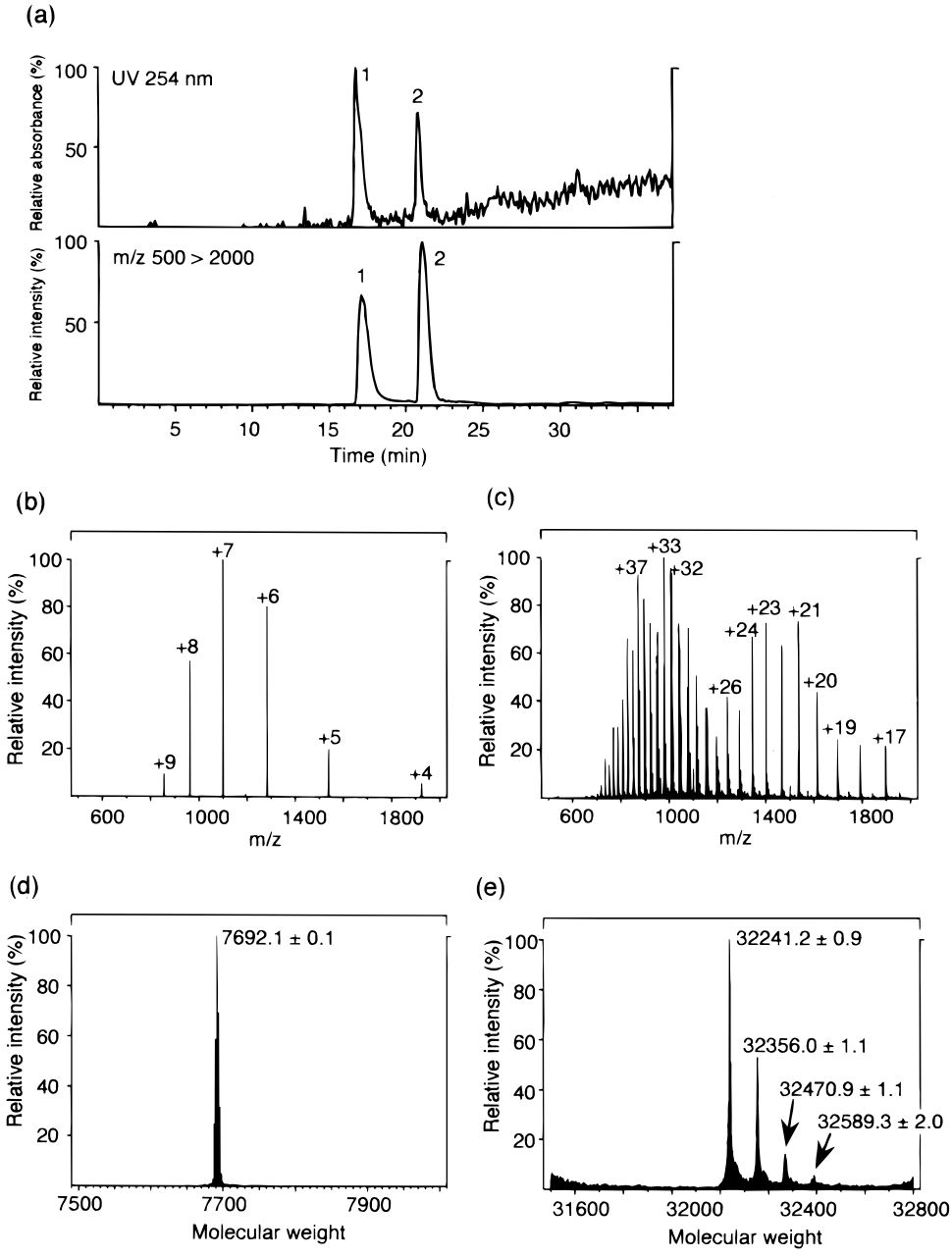

Figure 1. Microcapillary HPLC/ESI-MS analysis of VT1: (a) UV chromatogram monitored at 254 nm and selected ion chromatogram reconstructed from total ion scanning; (b, c) ESI mass spectra of peaks 1 and 2 respectively; (d, e) deconvoluted spectra of peaks 1 and 2 respectively. The values in (d) and (e) are the average molecular mass and standard deviation of a mass spectral plot for each multiply charged ion, calculated by using the BIOMASS deconvolution program installed in the Finnigan TSQ-7000 instrument.

CCC 1076–5174/97/101140–03 $17.50

Received 6 May 1997
Accepted 2 July 1997

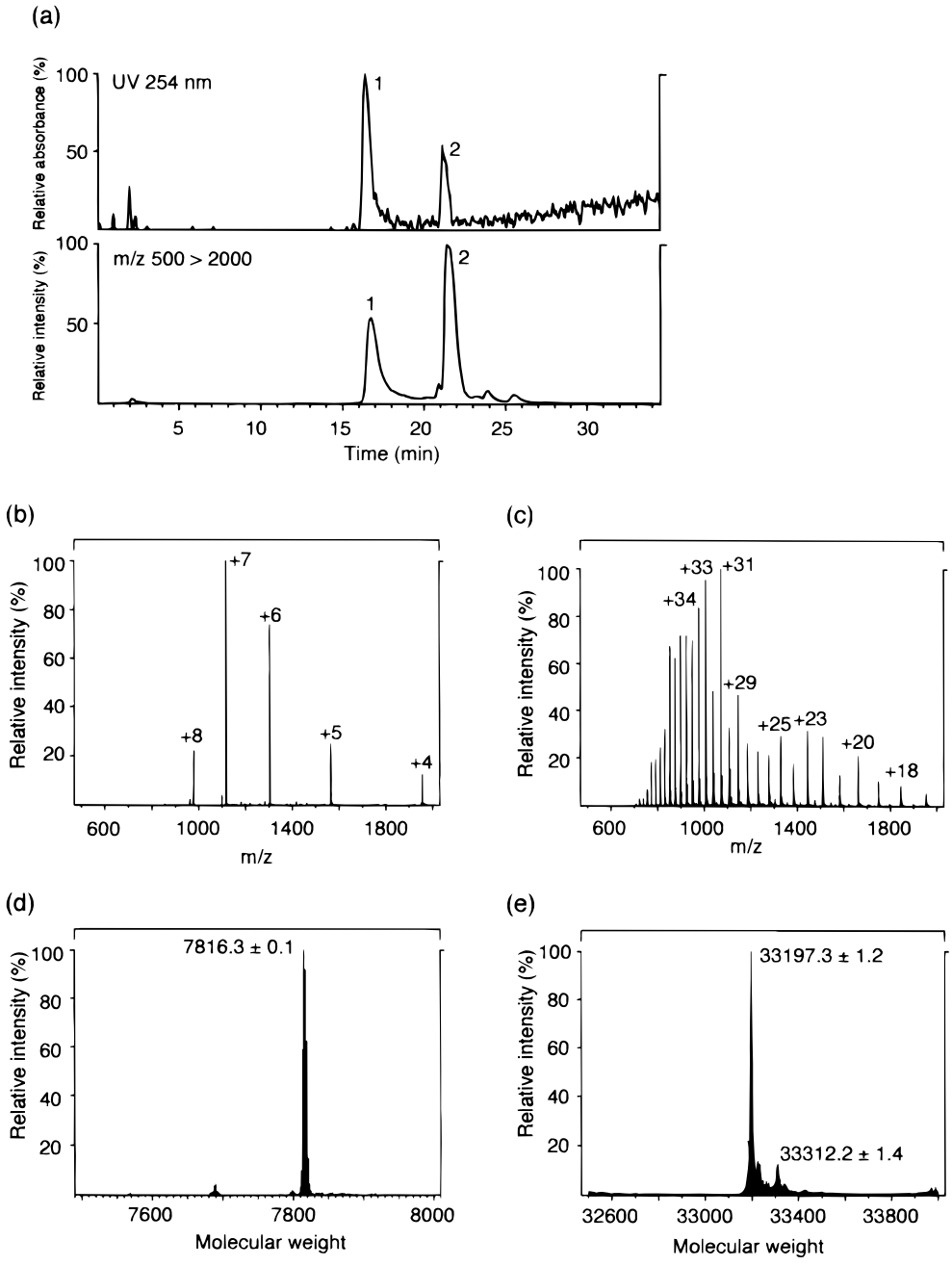

Figure 2. As Fig. 1 but for VT2.

detects Vero toxins by bioassay using culture cells, reversed passive latex agglutination assay and enzyme-linked immunosorbent assay, and the other that detects Vero toxin-coding genes by DNA colony hybridization tests and the polymerase chain reaction method.[4] Although these methods are widely used, a physicochemical method that can detect intact Vero toxins is highly desired. Mass spectrometry (MS) is a powerful tool for obtaining molecular weight information on various compounds. There has been only one report about mass spectrometric identification, in which tryptic digests of VT1 were analyzed by fast atom bombardment MS.[5] However, it required complex and time-consuming procedures, such as separation of A and B subunits, followed by fragmentation and peptide separation. Electrospray ionization (ESI)-MS with high-performance liquid chromatography (HPLC) has recently been used for the determination of intact proteins at the low picomole level without cleavage to peptides.[6] Application of this technique might enable a reliable identification of Vero toxins and would be a promising means of chemical diagnosis of HC and HUS. In the present study we describe the analysis of VT1 and VT2 using HPLC/ESI-MS.

VT1 and VT2 were expressed in recombinant *E. coli* using the pUC 118 and pBR 322 vector respectively and purified by the methods previously described.[7,8] The purity of VT1 and VT2 was estimated by HPLC analysis using a TSK-gel G2000SW column (Tosoh, Tokyo, Japan), which provided each single peak. Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis (SDS–PAGE) in 15% gels of VT1 and VT2 showed respectively two Coomassie blue-stained bands, corresponding to the A and B subunits as previously described.[7,8] The purified VT1 and VT2 showed cytotoxicity to Vero cells, killing about 50% of the cells at 1 and 0.5 pg respectively. These values are well consistent with those previously reported.

Now that the purified VT1 and VT2 could be obtained, they were analyzed by HPLC/ESI-MS. ESI mass spectra were obtained on a TSQ-7000 triple-stage quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with an electrospray ion source and a polymeric reversed phase microcapillary HPLC column (PLRP-S, 1 mm × 150 mm, 1000 Å, 8 μm; Michrom Bioresources, Inc., Pleasanton, CA, USA). The solvent system for HPLC was a linear gradient of solvent A mixed with solvent B from 5% B to 90% B in 40 min. Solvent A was acetonitrile–water (2:98)–2% acetic acid and solvent B was acetonitrile–water (90:10)–2% acetic acid. Figure 1 shows the results of the microcapillary HPLC/ESI-MS analysis of VT1. The two major peaks are well separated and detected in the UV chromatogram monitored at 254 nm and the selected ion chromatogram reconstructed from total ion scanning [Fig. 1(a)]. The ESI mass spectra of peaks 1 and 2 shown in Figs 1(b) and 1(c) are the sums of the scans in the range marked by a horizontal bar. Figure 1(d) shows the deconvoluted mass spectrum of peak 1. The observed average molecular weight 7692.1 ± 0.1 is slightly (2 u) greater than the theoretical value of the B subunit of VT1 (7690.3) which is calculated from the nucleotide sequence of the gene encoding Vero toxins.[4] The deconvoluted mass spectrum of peak 2 shown in Fig. 1(e) gave four ion signals. The molecular mass 32241.4 ± 1.2 is slightly (16 u) greater than the theoretical value of the A subunit of VT1 (32 225.7).[4] The discrepancy (16 u) between the observed and theoretical masses of the A subunit of VT1 might be derived from the molecular form nicked between two cysteine residues, which is greater (18 u) than the original one.[4,5] The other three ion signals observed in the deconvoluted mass spectrum of peak 2 show the molecular masses 32 356.0 ± 1.1, 32 470.9 ± 1.1 and 32 589.3 ± 2.0, and the differences in the molecular masses of the four peaks are approximately the same values at 115. These results suggest that post-translational polyaspartylation might be one of the conceivable candidates, similar to the case of tubulin which undergoes a post-translational addition of varying numbers of glutamyl or glycyl units.[9,10] Although VT1 showed the same cytotoxicity as that previously reported, further investigation of its biological activities should be carried out because of its significance from a toxicological point of view.

Microcapillary HPLC/ESI-MS analysis of VT2 provided the molecular mass of the A subunit (33 197.3 ± 1.2) and B subunit (7816.3 ± 0.1) (Fig. 2). The observed average molecular weight is slightly (5 u) greater than the theoretical value of the A subunit of VT2 (33 192.6),[4] and the difference corresponded to 0.01% of this molecular mass; that of the B subunit is completely consistent with the theoretical value.[4] These results show that the structures of the A and B subunits of VT2 used in the present study are identical to those previously reported. Additionally, a small ion signal having the molecular mass 33 312.2 ± 1.4 is also observed in the deconvoluted mass spectrum of peak 2 [Fig. 2(e)], whose molecular mass is 115 u larger than that of the major ion signal (33 197.3 ± 1.2).

In the present study, VT1 and VT2 were analyzed by microcapillary HPLC/ESI-MS, demonstrating accurate molecular masses. This MS method opens up the possibility of screening and diagnostic applications. Additionally, the identification of the molecular structure of VT1 and VT2 using ESI-MS-MS combined with enzymatic digestion is in progress.

We thank Drs. Y. Takeda, S. Yamasaki and M. Ohmura (Research Institute, International Medical Center of Japan) for providing recombinant VTEC, Tosoh Co. for the gift of the HPLC column and Dr. K. Masuda (Faculty of Pharmacy, Meijo University) for useful discussions.

Yours,

FUMIO KONDO,[1]* SHINICHI KOBAYASHI,[1] MASAKADO MATSUMOTO,[1] SEIJI YAMADA,[1] MAKOTO SAITO,[1] YASUMOTO SUZUKI,[1] NAOHISA ISHIKAWA,[1] TOYOFUMI NAKANISHI[2] and AKIRA SHIMIZU[2]

[1] Aichi Prefectural Institute of Public Health,
Tsuji-machi, Kita-ku,
Nagoya 462,
Japan

[2] Department of Clinical Pathology,
Osaka Medical College,
Takatsuki,
Osaka 569,
Japan

* *Correspondence to*: F. Kondo, Aichi Prefectural Institute of Public Health, Tsuji-machi, Kita-ku, Nagoya 462, Japan

References

1. B. P. Bell, M. Goldoft, P. M. Griffin, M. A. Davis, D. C. Gordon, P. I. Tarr, C. A. Bartleson, J. H. Lewis, T. J. Barrett and J. G. Wells, *JAMA* **272**, 1349 (1994).
2. Infectious Agents Surveillance Center, *Infectious Agents Surveillance Report 17*, 1996, p. 180.
3. C. A. Lingwood, *Adv. Lipid Res.* **25**, 189 (1993).
4. Y. Takeda, H. Kurazono and S. Yamasaki, *Microbiol. Immunol.* **37**, 591 (1993).
5. T. Takao, T. Tanabe, Y.-M. Hong, Y. Shimonishi, H. Kurazono, T. Yutsudo, C. Sasakawa, M. Yoshikawa and Y. Takeda, *Microb. Pathogen.* **5**, 357 (1988).
6. T. Nakanishi, A. Shimizu, N. Okamoto, A. Ingendoh and M. Kanai, *J. Am. Soc. Mass Spectrom.* **6**, 854 (1995).
7. M. Noda, T. Yutsudo, N. Nakabayashi, T. Hirayama and Y. Takeda, *Microb. Pathogen.* **2**, 339 (1987).
8. T. Yutsudo, N. Nakabayashi, T. Hirayama and Y. Takeda, *Microb. Pathogen.* **3**, 21 (1987).
9. J. Mary, V. Redeker, J.-P. L. Caer, J.-C. Prome and J. Rossier, *FEBS Lett.* **353**, 89 (1994).
10. V. Redeker, N. Levilliers, J.-M. Schmitter, J.-P. L. Caer, J. Rossier, A. Adoutte and M.-H. Bre, *Science* **266**, 1688 (1994).