Hsiao-Chun Tseng[1]
Yin-Chang Liu[1,2]

[1]Department of Life Science and
[2]Institute of Molecular Medicine,
National Tsing-Hua University,
Hsin-Chu 30043, Taiwan

Immobilized betulinic acid column and its interactions with phospholipase A2 and snake venom proteins

Betulinic acid (BA) is a plant-derived pentacyclic triterpenoid. Although BA has been found to have diverse pharmacological effects, including anti-tumor and anti-inflammatory actions and potential as inhibitor of phospholipase A2 (PLA2), its cellular targets remain unclear. In this study, BA was immobilized onto an acrylamide matrix. The immobilized-BA column could retain the purified PLA2 of bovine pancreas or the PLA2 of snake venom from *Naja nigricollis*. The bound PLA2 were not eluted by high salt concentrations but were eluted by either acid or calcium free buffer. Besides the PLA2, a group of basic proteins of snake venom with molecular weights of about 7 kDa were also strongly bound by immobilized BA. One of these proteins was identified as γ-cardiotoxin. The usefulness of immobilized BA for exploring the cellular targets of BA is discussed.

Key Words: Betulinic acid; Affinity chromatography; Phospholipase A2

Received: Febuary 4, 2004; revised: June 15, 2004; accepted: August 2, 2004

DOI 10.1002/jssc.200401752

1 Introduction

Betulinic acid (3-β-hydroxy-lup-20(29)-en-28-oic acid) is a pentacyclic triterpenoid. The compound can be found in various plants including the bark of white birch, *Betula alba* [1, 2]. Betulinic acid (BA) has potential anti-HIV [3], anti-malarial [4], anti-tumor [5], and anti-inflammatory properties [6]. BA selectively induces apoptosis in human melanoma [7], and reduces ultraviolet-C-induced DNA breakage in congenital melanocytic naeval cells [8]. In an in-vitro study, BA was shown to inhibit the enzymatic activity of bovine pancreatic phospholipase A2 [9]. As indicated above, BA possesses an array of pharmacological activities; it seems likely that BA may have more than one molecular target in cells besides the possible candidate phospholipase A2 (PLA2). Being interested in the molecular targets of BA, we describe, in this report, the production of an immobilized-BA column. We found that immobilized BA exhibited strong interactions with purified PLA2 of bovine pancreas or the PLA2 in snake venom of *Naja nigricollis*. In addition to PLA2, other proteins in snake venom such as cardiotoxin γ were also found to bind strongly with the immobilized BA.

Correspondence: Yin-Chang Liu, Department of Life Science and Institute of Molecular Medicine, National Tsing-Hua University, Hsin-Chu 30043, Taiwan. Phone: +886 3 574 2757.
Fax: +886 3 571 5934. E-mail: ycliu@life.nthu.edu.tw.

Abbreviations: BA, betulinic acid; PLA2, phospholipase A2; bp-PLA2 bovine pancreas phospholipase A2; PBS, phosphate buffered saline.

2 Materials and methods

2.1 Materials

Betulinic acid (BA), bovine pancreatic phospholipase A2, and crude snake venom from *Naja nigricollis* were obtained from the Sigma Company. Beads of bis-acrylamide/azlactone (UltralLink™ Biosupport Medium) was purchased from the Pierce Company. Antiserum to PLA2 of snake venom (*Naja naja atra*) and purified PLA2 of *Naja naja atra* were kindly provided by Dr. Chang, L.S. (Institute of Biomedical Science, National Sun Yat-Sen University, Kaohsiung).

2.2 Preparation of immobilized-BA affinity column

For this purpose, the UltralLink™ Immobilization Kit (Pierce) was used and the manufacturer's instructions followed. Briefly, 0.25 g of the resin of bis-acrylamide/azlactone copolymer (Pierce) was suspended in 6 mL BupH™ citrate-carbonate buffer (0.1 M bicarbonate, 0.6 M sodium citrate, pH 9) and allowed to swell to form a gel. After initial washes, the gel was collected and BA solution (30 mg in 6 mL DMSO) was added in dropwise manner. After a 1-h incubation at room temperature for the coupling reaction, the gel was washed with 10 gel-bed volumes of 1 X PBS to remove the excess of BA. Then, the gel was incubated with 6 mL quenching solution (3.0 M ethanolamine, pH 9.0) for 2.5 h at room temperature to quench the unreacted azlactone groups. Following this step, the gel was washed extensively with 10 bed-volumes of PBS and 10 bed-volumes of wash solution (1.0 M NaCl) to remove quenching reagent. Finally, the gel was suspended in 3

Original Paper

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

bed-volumes of storage buffer (0.05% sodium azide in PBS).

2.3 PLA2 enzymatic activity assay

PLA2 activity was determined by a titrimetric method described in the Worthington Manual (Worthington Co.) with slight modifications. Briefly, the substrate, a 2% lecithin emulsion, was prepared as follows: To 4.0 g of reagent grade soybean lecithin (Sigma Co.), 30 mL of 1.0 M sodium chloride, 10 mL of 0.1 M calcium chloride, and 100 mL of reagent grade water were added. The mixture was stirred overnight and was sonicated for 10 min before being diluted with reagent grade water to a final volume of 200 mL. Each enzymatic assay was performed at 25°C in a reaction vessel containing10 mL of 2% lecithin emulsion; the pH of the emulsion was adjusted to 8; then a sample with appropriately diluted enzyme was added to the mixture, and the volume of 0.01 N NaOH required to maintain the pH at 8 for 4–5 min was recorded. The actual enzyme activity in a sample was obtained after subtraction of the rate without enzyme.

2.4 SDS-PAGE and Western blotting

The SDS-PAGE and Western blotting were performed according to the standard procedures as described previously [10].

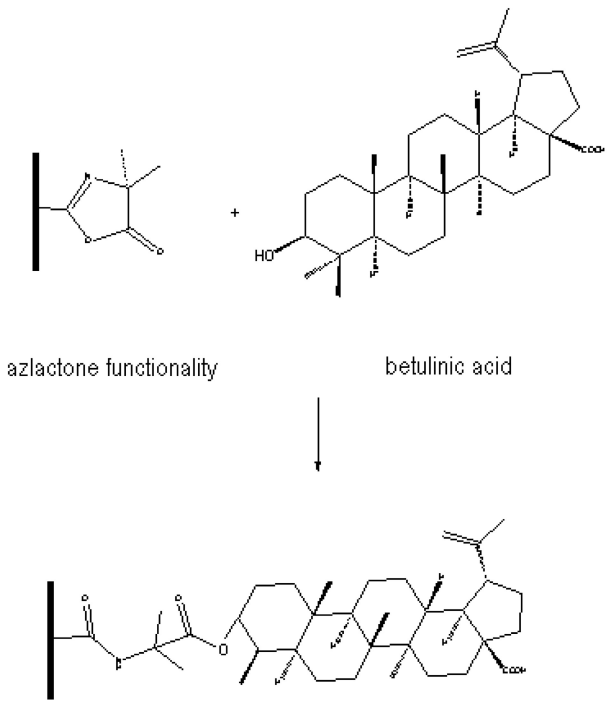

Figure 1. The reaction of betulinic acid for immobilization to matrix composed of a bis-acryamide/azlactone copolymer (UltraLink™ Biosupport Medium, Pierce).

2.5 HPLC and ESI-MS

The procedures of HPLC and ESI-MS were similar to those described previously in [11]. In brief, protein samples were applied to a reverse-phase HPLC column (C18, 4.6 × 250 mm of Vercopak Co.) with a 50-min linear gradient of 0 to 50% acetonitrile in 0.1% trifluoroacetic acid. Fractions were collected and detected by the System Gold 166 detector (Beckman Coulter Co.). Protein samples obtained from HPLC were prepared at ≈10 pmol/μL in H_2O and samples with volume 2–5 μL were injected into the source of the mass spectrometer at a rate of 10 μL/min, utilizing a carrier solvent of H_2O:ACN (1:1, *v/v*). Electrospray ionization mass spectra were obtained in the positive ion modes with a Micromass Quattro Ultima mass spectrometer. Protein data were acquired over a wide scan mass range of *m/z* ca. 500–1600 with 16 points/*m/z* or over a narrow scan mass range of *m/z* 800–1000 at 64 data points/*m/z*, with a scan duration of 15–25 s. The cone voltage was set at 60 V. Nitrogen was used as the nebulizing and drying gas with flow rates of 0.8 and 8.3 L/min, respectively. Ten to thirty three spectra were averaged, smoothed, baseline subtracted, and transformed using the MaxEnt1 software.

3 Results

3.1 Immobilization of betulinic acid onto beads

The molecular structure of betulinic acid (BA, see **Figure 1**), containing a single carboxylic group and a hydroxyl group, offers clues to explain how it could form a covalent bond with an active site residue of the matrix. The carboxylic group has been found to be essential for biological activity [9, 12] and was therefore excluded in considerations of immobilization. Thus, the nucleophilic hydroxyl group was selected for reacting with suitable matrix. For the matrix, we chose UltraLink™ Biosupport Medium (Pierce Co., and see Figure 1) because it contains an azlactone functionality that could react with the hydroxyl group of BA and would result in a covalent linkage with a 4-atom spacer arm. Due to the unique azlactone chemistry, there is no leaving group or toxic chemical by-product as a result of the coupling reaction. The coupling reaction and the preparation of the immobilized-BA affinity column were performed according to the manufacturer's guidlines (see Materials and Methods). To test the utility of the immobilized-BA column, we chromatographed bovine pancreatic phospholipase A2 (PLA2) on the immobilized-BA column because it has been shown that BA could inhibit the activity of PLA2 of bovine pancreas [9].

3.2 Interaction between immobilized betulinic acid and bp-PLA2

In a typical chromatographic run, an aliquot of bovine pancreatic-PLA2 solution (about 1.5 mg of PLA2) was loaded

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

onto the immobilized-BA column (2 mL in bed volume) pre-equilibrated with buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 5 mM $CaCl_2$. An initial wash to remove the excess of PLA2 was performed. Then, in the subsequent washes with salt-gradient (0.2–1.5 M NaCl), no significant PLA2 was found in the eluate. However, a considerable fraction of PLA2 (≈50%) was detected when 0.1 N hydrochloric acid was applied (see **Figure 2**), suggesting that the binding of PLA2 with the immobilized

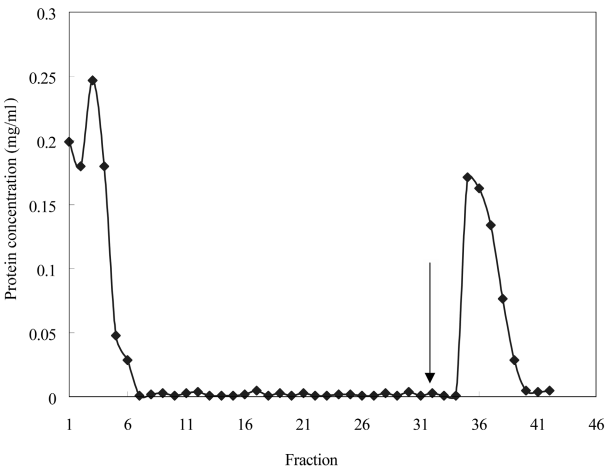

Figure 2. Elution profile of bovine pancreas-PLA2 on the immobilized-BA column. About 1.5 mg bp-PLA2 was loaded onto the immobilized-BA column (2 mL) which had been equilibrated in buffer consisting of Tris-HCl (50 mM), $CaCl_2$ (5 mM), NaCl (100 mM), pH 7.4. Arrow indicates the beginning of elution with acidic solvent (0.1 N HCl). Fractions 1–2: flow-through; fractions 3–32: wash with salt concentration increased from 200 mM to 1.5 M; fractions 33–42: acid eluate (the eluate was collected in tubes which already contained 0.1 mL of 1 M Tris-Cl, pH 8.0 to maintain the proteins in active conformation.). *Y*-axis shows the protein concentration determined by Coomassie blue dye-binding and *X*-axis shows the numbers of fractions collected (each fraction contained 1 mL eluate).

BA is very tight. This binding is specific since it did not occur with uncoupled matrix, and the irrelevant proteins such as bovine serum albumin were not retained by the column (data not shown). Moreover, the interaction between PLA2 and immobilized BA binding was Ca^{2+} dependent since the retention of PLA2 was not found under calcium-free conditions (data not shown), suggesting an alternative method to elute the bound PLA2 from the column.

3.3 Interactions between immobilized betulinic acid and proteins from snake venom

To test if the immobilized BA column could bind the PLA2 found in snake venom, we performed the experiment with

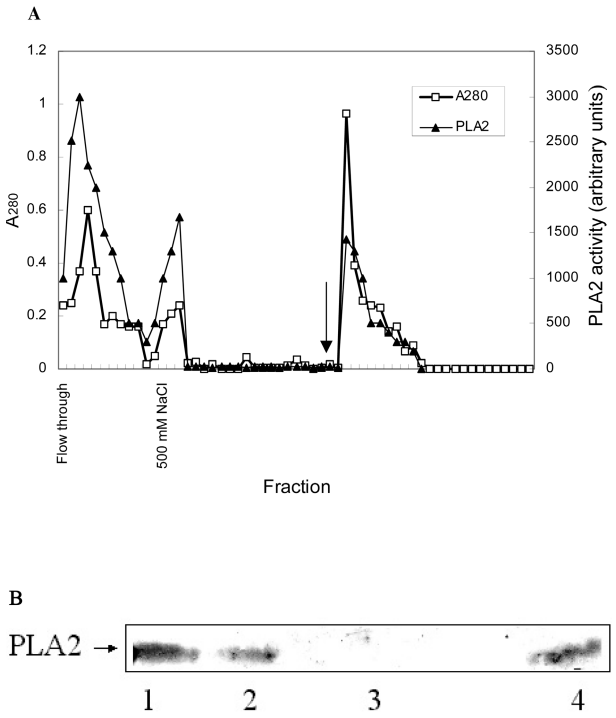

Figure 3. Affinity chromatography of snake venom proteins of *Naja nigricollis* on the immobilized-BA column. (A) Elution profiles of protein concentration (A_{280}, –□–) and PLA2 activity (–▲–). About 2 mg of snake venom proteins was applied to the immobilized-BA column (2 mL) which had been equilibrated with the buffer as described in Figure 2 and chromatography was performed as described in Figure 2. The arrow indicates the acid eluate. (B) Western blotting analysis of PLA2 for crude venom and samples from selected fractions: 1. crude venom; 2. flow-through; 3. high-salt wash; 4. acid elute.

snake venom proteins from *Naja nigricollis*. As in the previous experiment, the excess of proteins was removed by an initial wash and the bound proteins were eluted stepwise by high salt concentration and finally by acid. As in the elution profile shown in **Figure 3**, the PLA2 activity of snake venom was only eluted after acid washing. However, the PLA2 was not the only protein of snake venom that bound to the immobilized BA and was eluted by acid. Examination with SDS-PAGE revealed that the bound proteins consisted of two protein bands, a predominant one with a molecular weight of about 7 kDa, and a minor one of about 14 kDa (**Figure 4**). Further investigation by Western analysis indicated that PLA2 was the 14 kDa peptide (Figure 4). The 7 kDa protein band was actually a mixture of peptides according to subsequent separation by reverse phase HPLC (**Figure 5**) and ESI-Mass spectrometry (**Figure 6**). One of these peptides was identified as γ-cardiotoxin.

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

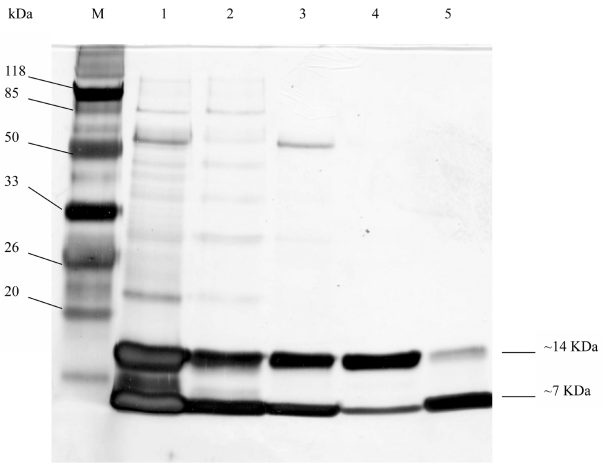

Figure 4. Protein analysis of snake venom and fractions collected during the affinity chromatography on immobilized-BA column. Proteins were separated on 12.5% SDS-PAGE and stained by silver staining procedure. Lane M, marker proteins; lane 1, crude venom of *Naja nigricollis* (20 μg of protein); lane 2, flow-through; lane 3, mid-high salt wash (0.5 M NaCl); lane 4, high salt wash (1.5 M NaCl); lane 5, acid elute.

The protein of interest did not come out in flow-through mode but was eluted by mild salt. Taken together, these results support our idea that BA has diverse cellular targets in conjunction with its diverse pharmacological effects. While we were preparing the manuscript we became aware that in addition to PLA2, DNA polymerase β [13] and topoisomerase I [12] have been reported to be inhibited by BA. These findings prompt us to undertake future studies of the interactions between the immobilized-BA and the two enzymes just mentioned.

In this report, we did not address the coupling efficiency of BA with the matrix (UltraLink™ Biosupport Medium from Pierce Co.) since the exact amount of BA immobilized onto the matrix has not been worked out. At the moment, we can only say that there were at most 28 mg of BA coupled to 2 mL of the matrix. A solution to this problem might be forthcoming on adopting a better method for assaying the inhibition of PLA2 by BA or a direct way of measuring the BA in solution.

In conclusion, BA was immobilized onto a matrix of acrylamide and the immobilized BA was shown to exhibit strong interactions with proteins including PLA2 of bovine pancreas or of snake venom from *Naja nigricollis*. The immobilized-BA column is useful for protein purification and for studying the cellular targets of BA.

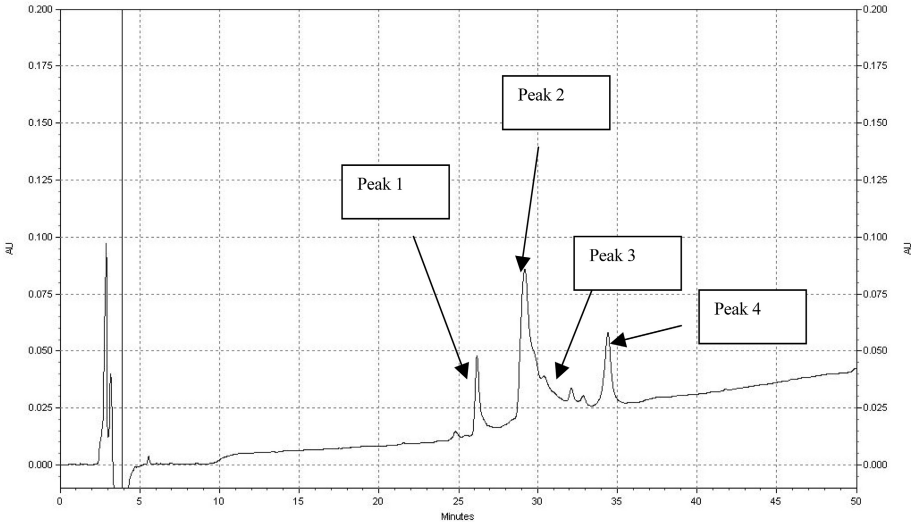

Figure 5. Reverse-phase HPLC of snake venom proteins (*Naja nigricollis*) which had been acid-eluted from the immobilized-BA column. Sample of acid-eluted fractions of snake venom protein following BA-column affinity chromatography was applied to a reverse-phase HPLC column (C18, 4.6 × 250 mm) with a 50-min linear gradient of 0 to 50% acetonitrile in 0.1% trifluoroacetic acid. Fractions of HPLC were collected and detected by the System Gold 166 detector (Beckman Coulter). The Y axis shows the absorbance at 230 nm. The peaks 1–4 were further analyzed by ESI-MS as described in Figure 6.

4 Discussion

In this report, we described the production of an immobilized-BA column and showed the interactions of the immobilized-BA with PLA2 of bovine pancreas or snake venom. The strong interactions between the immobilized-BA and PLA2 and the specificity of the interactions suggest that immobilized BA is suitable for studying the protein targets of BA. In addition to PLA2, immobilized BA was able to bind strongly with some proteins of snake venom. One of these proteins was identified as γ-cardiotoxin. In fact, besides these strong binding proteins, there were other proteins less strongly bound with BA, such as the significant band between 50 and 85 kD on lane 3 of Figure 4.

Acknowledgements

We thank the lab fellows of Dr. W.-G. Wu of this institute for providing valuable technical assistances in activity assay of PLA2 and identification of cardiotoxins. We also thank Dr. L.-S. Chang of National Sun Yat-Sen University for the gift of the antibody to PLA2. This work was supported by grants from the National Science Council of Taiwan (NSC 92-2311-B-007-013) and National Research

© 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

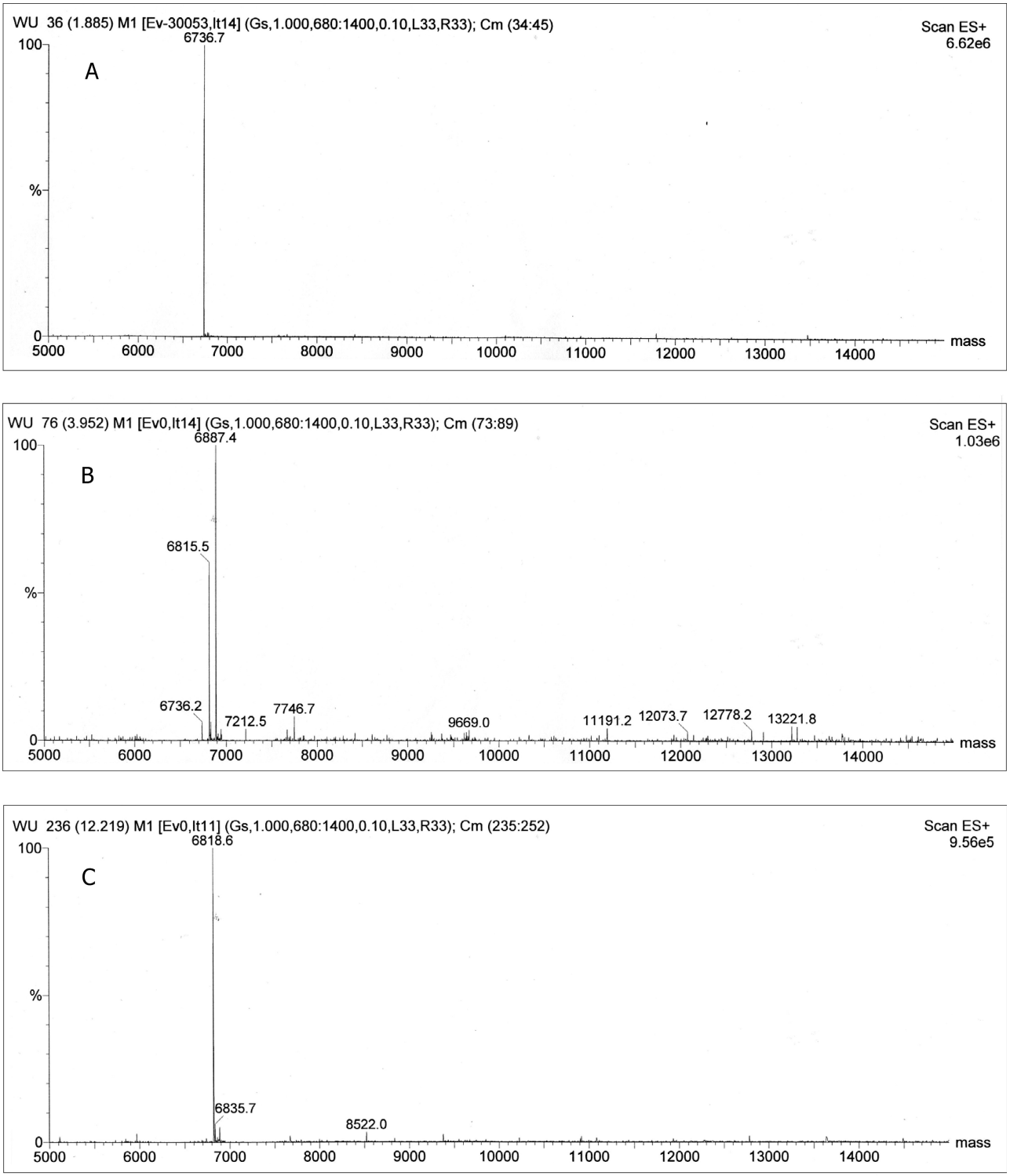

Figure 6. ESI-MS analysis of snake venom proteins. Sample of snake venom proteins collected from reverse phase HPLC column designated as peaks 1–4 described in Figure 5 were analyzed using Micromass Quattro Ultima with electron spray ionization (ESI-MS) method and the spectra were interpreted with MaxEnt1 as described in the Materials and Methods. ESI-MS analysis gave sample of peaks 1, 2, and 4 of HPLC profile, the mass patterns 6A, 6B, and 6C, respectively. Analysis of peak 3 was unsuccessful. The patterns of 6A (peak 1), 6B (peak 2) and 6C (peak 4) show molecular weight of 6736.7, 6887.4, 6818.6 Da, respectively. This data suggested that peak 4 is cardiotoxin γ. This was confirmed with pure cardiotoxin γ.

 © 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Program for Genomic Medicine project (NSC 92-3112-B-007-004).

References

[1] S. Fulda, I. Jeremias, H.H. Steiner, T. Pietsch, K.M. Debatin, *Int. J. Cancer* **1999**, *82*, 435–441.

[2] I. Baglin, A.C. Mitaine-Offer, M. Nour, K. Tan, C. Cave, M.A. Lacaille-Dubois, *Mini Rev. Med. Chem.* **2003**, *3*, 525–539.

[3] T. Fujioka, Y. Kashiwada, R.E. Kilkuskie, L.M. Cosentino, L.M. Ballas, J.B. Jiang, W.P. Janzen, I.S. Chen, K.H. Lee, *J. Nat. Prod.* **1994**, *57*, 243–247.

[4] G. Bringmann, W. Saeb, L.A. Assi, G. Francois, A.S. Sankara Narayanan, K. Peters, E.M. Peters, *Planta Med.* **1997**, *63*, 255–257.

[5] K. Yasukawa, M. Takido, T. Matsumoto, M. Takeuchi, S. Nakagawa, *Oncology* **1991**, *48*, 72–76.

[6] M.C. Recio, R.M. Giner, S. Manez, J. Gueho, H.R. Julien, K. Hostettmann, J.L. Rios, *Planta Med.* **1995**, *61*, 9–12.

[7] E. Pisha, H. Chai, I.S. Lee, T.E. Chagwedera, N.R. Farnsworth, G.A. Cordell, C.W. Beecher, H.H. Fong, A.D. Kinghorn, D.M. Brown, et al., *Nat. Med.* **1995**, *1*, 1046–1051.

[8] G.I. Salti, J.V. Kichina, T.K. Das Gupta, S. Uddin, L. Bratescu, J.M. Pezzuto, R.G. Mehta, A.I. Constantinou, *Melanoma Res.* **2001**, *11*, 99–104.

[9] P. Bernard, T. Scior, B. Didier, M. Hibert, J.Y. Berthon, *Phytochemistry* **2001**, *58*, 865–874.

[10] B.S. Tzang, Y.C. Lai, M. Hsu, H.W. Chang, C.C. Chang, P.C. Huang, Y.C. Liu, *DNA Cell Biol.* **1999**, *18*, 315–321.

[11] N. Gong, A. Armugam, P. Mirtschin, K. Jeyaseelan, *Biochem. J.* **2001**, *358,* 647–56.

[12] A.R. Chowdhury, S. Mandal, B. Mittra, S. Sharma, S. Mukhopadhyay, H.K. Majumder, *Med. Sci. Monit.* **2002**, *8*, BR254–265.

[13] J. Ma, S.R. Starck, S.M. Hecht, *J. Nat. Prod.* **1999**, *62*, 1660–1663.

 © 2004 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim