Nanoliter-Volume Selective Sampling of Peptides Based on Isoelectric Points for MALDI-MS

ARTICLE *in* ANALYTICAL CHEMISTRY · NOVEMBER 2004

Impact Factor: 5.64 · DOI: 10.1021/ac049230c · Source: PubMed

2 AUTHORS, INCLUDING:

Haixia Zhang
National Research Council of Canada, Sakat…

27 PUBLICATIONS **639** CITATIONS

SEE PROFILE

Anal. Chem. **2004,** *76,* 6814−6818

Nanoliter-Volume Selective Sampling of Peptides Based on Isoelectric Points for MALDI-MS

Haixia Zhang and Ken K.-C. Yeung*

Departments of Chemistry and Biochemistry, The University of Western Ontario, London, Ontario, Canada N6A 5B7

A simple way to selectively isolate peptides based on their isoelectric points (pI) for MALDI mass spectral analysis is described. An applied voltage was used to electromigrate peptides into a capillary. The capillary was modified with a zwitterionic surfactant, 1,2-dilauroyl-*sn*-phosphatidylcholine (DLPC), to suppress the electroosmotic flow (EOF) during injection. Hence, either the cationic or the anionic peptides were introduced, depending on the voltage polarity. By controlling the pH, selective loading of peptides was performed to isolate trace components from a mixture. The injected sample plugs were subsequently spotted in nanoliter volumes for MALDI-MS analysis. No significant sample losses resulting from selective sampling were detected. Low attomole-level detection of peptides (adrenocorticotropic hormone fragment 18−39, pI 4.25) was achieved from a mixture containing other peptides (angiotensin I, pI 6.92, and bradykinin, pI 12.00) at 100 000-fold higher concentrations.

Current state-of-the-art mass spectrometry (MS) is capable of detecting trace proteins and peptides at zeptomole levels.[1,2] Nevertheless, the trace components must be isolated from all abundant components to avoid ionization suppression.[3] When dealing with highly complex protein mixtures in proteomes, multidimensional separation techniques, such as two-dimensional gel electrophoresis[4] and multidimensional liquid chromatography (LC),[5−7] are necessary to resolve the sample components prior to MS analysis. However, they are very time-consuming and labor-intensive. In cases in which analysis time is a priority, researchers have to sacrifice resolution for sample throughput by choosing single dimensional chromatography[8] or other fast sample purification methods, such as surface-enhanced laser desorption/ionization (SELDI).[9−12]

An alternative approach for fast, simple sample selection based on charge/pI is described in this work. An applied electric field is used to selectively introduce either cationic or anionic peptides into a capillary. The selectively injected peptides can be subsequently spotted for MALDI-MS analysis.[1] On the basis of the differences in pIs, this technique can be used to isolate trace peptides from a mixture without performing any preseparations.

Sampling based on the electromigration of ions is commonly used in capillary electrophoresis (CE) and is referred to as electrokinetic injection. In conventional electrokinetic injection using unmodified silica capillaries, electroosmosis occurs and causes a bulk flow of solution toward the cathode. The electroosmotic mobility ($3-7 \times 10^{-4}$ cm^2/Vs) is usually higher than the mobilities of peptides and proteins, and thus, the electroosmotic flow (EOF) will nonselectively sweep both cationic and anionic protein/peptide molecules into the capillary during electrokinetic injection.

In recent reports by McLaren and Chen,[13,14] a controlled pressure was applied to counterbalance the EOF, thus enabling the selective introduction of ions. Alternatively, the EOF can be suppressed by coating the capillary inner walls with reagents, such as polymers[15−19] or surfactants.[20−23] One of the most effective methods was the use of zwitterionic surfactants.[22,24] An EOF as low as 2×10^{-5} cm/Vs was achieved in capillaries pretreated with

* To whom correspondence should be addressed. Fax: 519 661 3022. E-mail: kyeung@uwo.ca.

(1) Keller, B. O.; Li, L. *J. Am. Soc. Mass Spectrom.* **2001**, *12*, 1055−1063.
(2) Belov, M. E.; Gorshkov, M. V.; Udseth, H. R.; Anderson, G. A.; Smith, R. D. *Anal. Chem.* **2000**, *72*, 2271−2279.
(3) Zhang, B.; McDonald, C.; Li, L. *Anal. Chem.* **2004**, *76*, 992−1001.
(4) *Proteome research: two-dimensional gel electrophoresis and identification methods*; Rabilloud, T., Ed.; Springer: Berlin; New York, 2000.
(5) Lubman, D. M.; Kachman, M. T.; Wang, H.; Gong, S.; Yan, F.; Hamler, R. L.; O'Neil, K. A.; Zhu, K.; Buchanan, N. S.; Barder, T. J. *J. Chromatogr., B* **2002**, *782*, 183−196.
(6) Wall, D. B.; Parus, S. J.; Lubman, D. M. *J. Chromatogr., B* **2002**, *774*, 53−58.
(7) Washburn, M. P.; Wolters, D.; Yates, J. R., III *Nat. Biotechnol.* **2001**, *19*, 242−247.
(8) Shen, Y.; Tolic, N.; Masselon, C.; Pasa-Tolic, L.; Camp, D. G., II; Hixson, K. K.; Zhao, R.; Anderson, G. A.; Smith, R. D. *Anal. Chem.* **2004**, *76*, 144−154.
(9) von Neuhoff, N.; Kaiser, T.; Wittke, S.; Krebs, R.; Pitt, A.; Burchard, A.; Sundmacher, A.; Schlegelberger, B.; Kolch, W.; Mischak, H. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 149−156.
(10) Merchant, M.; Weinberger, S. R. *Electrophoresis* **2000**, *21*, 1164−1177.
(11) Issaq, H. J.; Conrads, T. P.; Prieto, D. A.; Tirumalai, R.; Veenstra, T. D. *Anal. Chem.* **2003**, *75*, 149A−155A.
(12) Tang, N.; Tornatore, P.; Weinberger, S. R. *Mass Spectrom. Rev.* **2004**, *23*, 34−44.
(13) McLaren, D. G.; Chen, D. D. Y. *Anal. Chem.* **2004**, *76*, 2298−2305.
(14) McLaren, D. G.; Chen, D. D. Y. *Electrophoresis* **2003**, *24*, 2887−2895.
(15) Horvath, J.; Dolnik, V. *Electrophoresis* **2001**, *22*, 644−655.
(16) Catai, J. R.; Somsen, G. W.; de Jong, G. J. *Electrophoresis* **2004**, *25*, 817−824.
(17) Gonzalez, N.; Elvira, C.; Roman, J. S.; Cifuentes, A. *J. Chromatogr., A* **2003**, *1012*, 95−101.
(18) Johannesson, N.; Wetterhall, M.; Markides, K. E.; Bergquist, J. *Electrophoresis* **2004**, *25*, 809−816.
(19) Zhang, J.; Horvath, C. *Electrophoresis* **2003**, *24*, 115−120.
(20) Righetti, P. G.; Gelfi, C.; Verzola, B.; Castelletti, L. *Electrophoresis* **2001**, *22*, 603−611.
(21) Hautala, J. T.; Linden, M. V.; Wiedmer, S. K.; Ryhanen, S. J.; Saily, M. J.; Kinnunen, P. K. J.; Riekkola, M.-L. *J. Chromatogr., A* **2003**, *1004*, 81−90.
(22) Cunliffe, J. M.; Baryla, N. E.; Lucy, C. A. *Anal. Chem.* **2002**, *74*, 776−783.
(23) Wang, C.; Lucy, C. A. *Electrophoresis* **2004**, *25*, 825−832.
(24) Yeung, K. K.-C.; Lucy, C. A. *Anal. Chem.* **1997**, *69*, 3435−3441.

10.1021/ac049230c CCC: $27.50
Published on Web 10/09/2004

1,2-dilauroyl-*sn*-phosphatidylcholine (DLPC).[22] In addition, DLPC is a surfactant with two hydrocarbon tails (C12) that is known to form semipermanent bilayers. The use of similar two-tailed surfactants in CE−MALDI-MS without significant interferences from the surfactant has been reported.[25]

EXPERMIMENTAL SECTION

Chemicals. 1,2-Dilauroyl-*sn*-phosphatidylcholine (DLPC, Avanti Polar Lipids, Inc., Alabaster, AL) was prepared as a 0.1 mM solution in 20 mM calcium chloride and 20 mM Tris acetate (pH 7.2).[22] Reagent grade Tris, acetic acid, hydrochloric acid, and sodium hydroxide were purchased from EM Science (Gibbstown, NJ). Mesityl oxide and calcium chloride were obtained from Sigma-Aldrich (St. Louis, MO). Deionized water (Millipore, Billerica, MA) was used in preparation of all solutions.

α-Cyano-4-hydroxycinnamic acid (CHCA) was obtained from Sigma-Aldrich. It was purified in 50 °C ethanol (95%) and recrystallized before use.[1] HPLC grade acetone and methanol were from Fisher Scientific Ltd. (Hampton, NH). Adrenocorticotropic hormone fragment 18−39 (ACTH, MW 2465.7, pI 4.25), angiotensin I (MW 1296.5, pI 6.92), and bradykinin (MW 1060.2, pI 12.00) were from Sigma-Aldrich and were used as received. The isoelectric points were computed using online pI calculator provided by ExPASy.[26]

Mobility Measurement and Selective Sampling with Optical Detection. An Agilent ^{3D}CE (Palo Alto, CA) capillary electrophoresis instrument was used in all experiments. Detection was performed at 254 nm for mesityl oxide (EOF marker) and 200 nm for peptides. The silica capillaries (Polymicro Technologies, Phoenix, AZ) had a total length of 30.5 cm (22.0 cm to the detector window), an inner diameter of 50 μm, and outer diameter of 360 μm. Each new capillary was preconditioned with high pressure rinsing (1 bar) in the following sequence: 0.1 M HCl for 5 min; water for 2 min; 0.1 M sodium hydroxide for 5 min; water again for 2 min; and finally, DLPC for 20 min. Prior to each run, the capillary was recoated with the DLPC solution for 10 min, followed by a 2-min rinse with the buffer (10 mM Tris, pH 6.00, adjusted with acetic acid). New capillaries and new DLPC solutions were prepared every week to ensure best reproducibility in the EOF.

The electrophoretic mobilities (μ) of the peptides and the EOF were determined from the migration times (t) at a constant applied field (V), using the equation

$$\mu = Ll/tV \quad (1)$$

where L and l are the total length and length to detector of the capillary, respectively. The sample concentrations were 5 mM for mesityl oxide and 0.1 mg/mL for the peptides. Injections of samples were performed hydrodynamically (5 mbar) in five replicates.

When selective sampling was performed for optical detection, the peptide solution (200 μL, prepared in buffer) was placed at the detector end of the capillary (8.5 cm to detector), which was grounded. The buffer was placed at the other end (22 cm to detector), where +10 kV was applied (2 min) for the injection of ACTH or −10 kV was used (2 min) for the injection of angiotensin I and bradykinin. The sample concentrations were: 0.2, 0.1 and 0.15 mg/mL respectively for ACTH, angiotensin I and bradykinin. Following the sample injection, a vacuum (−20 mbar) was applied to suction the injected sample plug past the capillary window for detection.

Selective Sampling with MALDI-MS Analysis. To facilitate MALDI-MS analysis following selective sampling, a CE/MS special capillary cassette (Agilent) was used. This cassette extended one end of the capillary outside the instrument, which was grounded by a platinum electrode connected to the instrument. The total capillary length was 39.0 cm. To perform selective injection, the peptide solution was placed at the external end of the capillary while the internal end was in buffer. The typical sample volume was 200 μL, which was sufficient to prevent significant pH changes due to electrolysis and evaporation within a day of experiments. Nevertheless, smaller volumes as low as 20 μL had been used, without significant differences in the MS data. A positive potential (+26 kV) was applied when injecting ACTH, and a negative potential (−26 kV) was used when injecting angiotensin I and bradykinin, for 2 min in each case. Immediately after the injection, the capillary tip was immersed in 0.5% (v/v) HCl for 2 s to remove any excess sample solution. The plug was then pushed out of the capillary at 50 mbar and deposited onto a stainless steel plate precoated with CHCA matrix. Unless otherwise stated, the eluted sample solution was spotted as 30-nL droplets at 15-s intervals. A three-layer method was employed for matrix deposition.[1] Briefly, 1 μL of 5 mg/mL CHCA in acetone/methanol (3:2 v/v) solution was first deposited onto the target, followed by 0.15 μL of a saturated CHCA solution in methanol/water/HCI (60:40:1, v/v/v). After drying, the sample solution was spotted as the third layer.

MALDI-Mass Spectrometry and Data Processing. A Bruker Reflex IV MALDI time-of-flight mass spectrometer (Bremen/Leipzig, Germany) was used to perform all MS analyses. The instrument was equipped with a 3-ns 337-nm nitrogen laser, and a laser energy of 25% was selected. The ion extraction delay time was 200 ns. Reflector and positive ion mode were used. All voltage settings were left at the default values preset by Bruker. Mass spectra were recorded as sums of 20 individual shots under video monitoring. Igor Pro (WaveMetrics, Lake Oswego, OR) was used for data processing and presentation.

RESULTS AND DISCUSSION

Electroosmotic Mobility. Previously, DLPC had been reported to significantly suppress the EOF over a wide pH range: 3×10^{-5} cm^2/Vs at pH 7, 1×10^{-4} cm^2/Vs at pH 10, and 1×10^{-4} cm^2/Vs at pH 3.[22] In this report, a pH 6.00 buffer was used, and the measured EOF ranged from 1 to 3×10^{-5} cm^2/Vs for up to 1 week after the initial preconditioning. Such an EOF was much lower in magnitude, as compared to the mobilities of the three selected peptides measured at the same pH, -1.53×10^{-4} cm^2/Vs for ACTH, 9.58×10^{-5} cm^2/Vs for angiotensin I, and 1.85×10^{-4} cm^2/Vs for bradykinin (electropherograms not shown), and therefore was suitable for selective sampling.

Evaluation of Selective Sampling with Optical Detection. The effectiveness of selective sampling was first evaluated by optical detection. Experiments were performed individually for each peptide because they cannot be distinguished by UV

(25) Yeung, K. K.-C.; Kiceniuk, A. G.; Li, L. *J. Chromatogr., A* **2001**, *931*, 153−162.
(26) http://ca.expasy.org/tools/pi_tool.html.

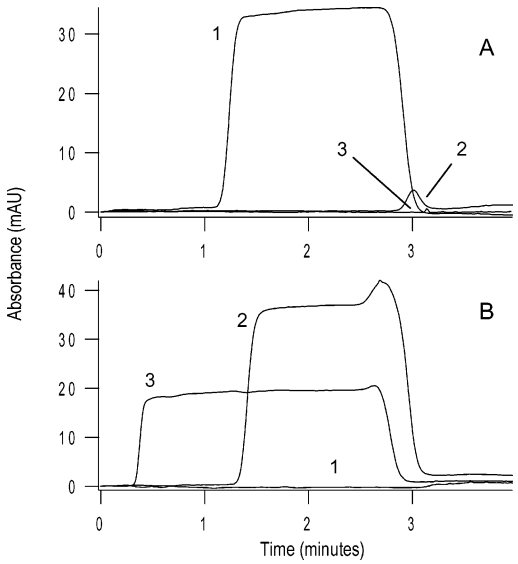

Figure 1. UV absorption signals of the selectively injected sample plugs: (A) sample at cathode and (B) sample at anode. Peptides: ACTH (1), angiotensin I (2), and bradykinin (3).

absorption. First, the sample was placed at the cathode to selectively migrate anions into the capillary, that is, ACTH. The direction of the residual EOF was backward (into the sample vial) in this case, which could keep the neutral and cationic molecules from entering the capillary. Figure 1A shows the signals of the electrokinetically injected plugs from the three peptides. As expected, a strong signal was observed only for ACTH. The height of the signal was very similar to that observed when the sample was injected hydrodynamically (~34 mAU), suggesting little or no sample losses. For bradykinin and angiotensin I, only very small peaks were observed at ~3 min. It was speculated that a small amount of these peptides entered the capillary after the voltage was switched off.

To illustrate the reversible nature of selective sampling, the electrode polarity was reversed (Figure 1B). In this case, strong signals were recorded for angiotensin I and bradykinin, but no signals were observed for ACTH. Even though the direction of the residual EOF was forward (into the capillary) in this case, introduction of ACTH was prevented by its electromigration. The peak heights for angiotensin I and bradykinin were also very similar to those observed when injected by pressure (~35 and 19 mAU, respectively). The difference in plug length between angiotensin I and bradykinin was attributed to their mobility difference.

Evaluation of Selective Sampling with MALDI-MS. A positive field was applied to selectively inject ACTH in the presence of angiotensin I and bradykinin at a concentration ratio of 1:300:300, respectively. On the basis of the data from optical detection, the sample plug length was estimated to be 9 cm in length with ~30 fmol of ACTH. Following the injection, the sample plug was pushed out in six fractions of ~30 nL (5 fmol each). MALDI spectra were obtained for all six fractions. Since the mass spectra from adjacent fractions were very similar, only the spectra from the first, third, and fifth fractions were presented (Figure 2A–C). In all three cases, the $(M + H)^+$ peaks from ACTH were clearly observed. No significant signals from angiotensin I were detected, and only very weak signals from bradykinin were

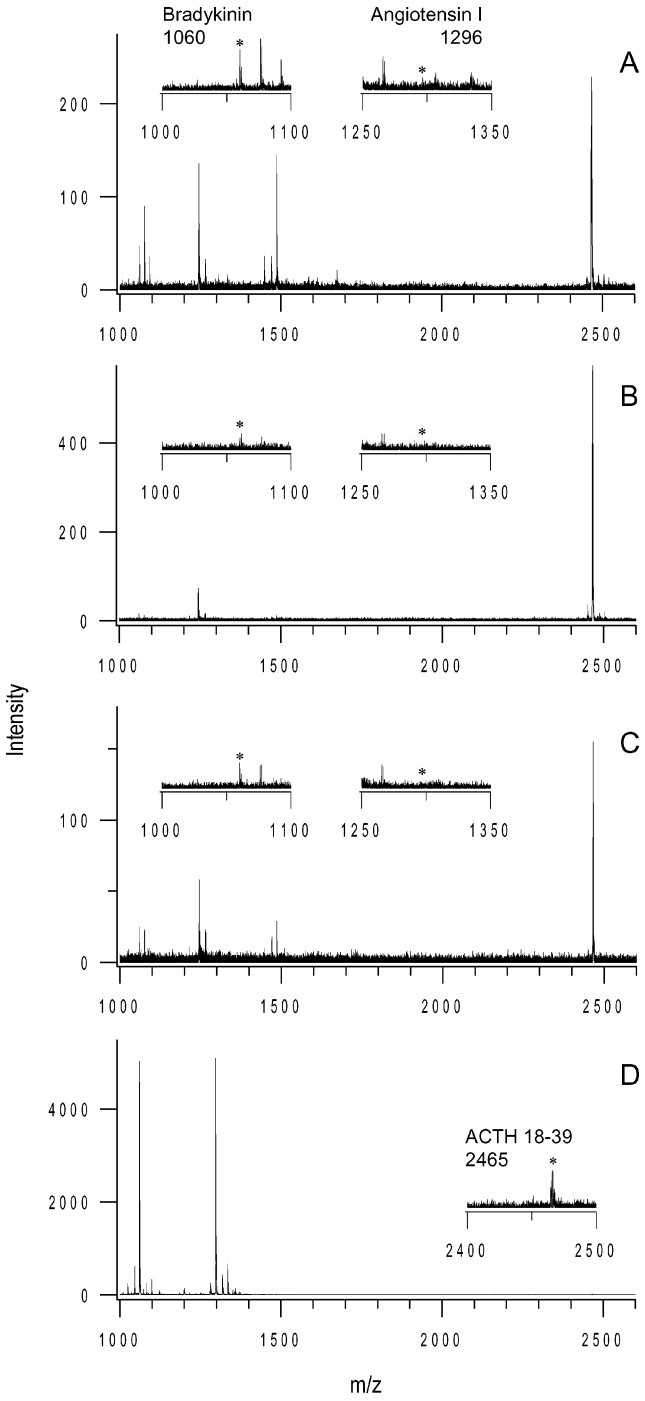

Figure 2. MALDI-MS spectra of fractions collected from the selectively sampled ACTH: (A) first fraction, (B) third fraction, and (C) fifth fraction. (D) Spectrum of the original sample mixture injected and spotted hydrodynamically. Conditions: sample placed at cathode; concentration ratio of ACTH, bradykinin, and angiotensin I, 1:300:300.

detected. For comparison, the spectrum of the original sample mixture, without selective injection, was included as Figure 2D. Extremely intense signals from angiotensin I and bradykinin were observed, and a very weak ACTH peak was recorded due to ionization suppression.

It should be noted that the ACTH intensities observed from the middle of the sample plug (fractions 2–4) were stronger than those from the start and end of the plug (fractions 1, 5, and 6).

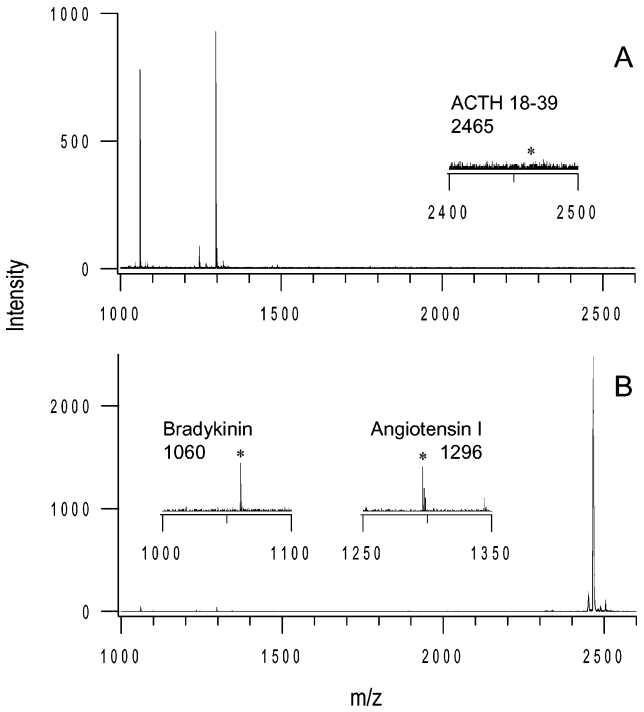

Figure 3. MALDI-MS spectra of ACTH, bradykinin, and angiotensin I at a concentration ratio of 300:1:1, respectively. Conditions: (A) selective sampling and (B) conventional sampling with pressure; sample placed at anode.

This was attributed to the dilution from the HCl rinse as well as laminar flow band broadening during sample spotting. Hence, fractions 2−4 were used in all other experiments.

In the next study, the electrode polarity was reversed to selectively inject angiotensin I and bradykinin while rejecting ACTH (in a concentration ratio of 1:1:300 respectively). As expected, intense $(M + H)^+$ peaks were observed from angiotensin I and bradykinin, and no signals were detected from ACTH (Figure 3A). The mass spectrum from the original sample was also included for comparison (Figure 3B).

Fractional Sampling at Higher Background-to-Sample Ratios. The effectiveness of isolating a trace peptide from higher concentrations of background peptides was investigated. Figure 4A−C shows the MS spectra for the selectively injected ACTH in the presence of angiotensin I and bradykinin at concentration ratios of 10^3, 10^4, and 10^5, respectively. The ACTH concentration was kept constant at 6.67×10^{-8} M, which theoretically resulted in ~2 fmol in a spot of 30 nL. In all three cases, ACTH signals were clearly detected, with only very weak signals from angiotensin I and bradykinin.

The overall signal-to-noise ratios of the ACTH peaks were lower than those in Figure 2A−C due to the reduced ACTH concentration. In particular, the intensity was much lower at the concentration ratio of 10^5 (Figure 4C), where the concentrations of angiotensin I and bradykinin were ~7 mM. In this case, the peptides' contribution in ionic strength became significant; thus, the electric field within the sample vial decreased significantly, which in turn hindered the injection of ACTH.

Sample Recovery and Detection Limit. The extent of sample loss during selective sampling was estimated by comparing the signal of selectively sampled ACTH to that of a pure ACTH solution spotted conventionally. In both cases, the ACTH concentration was 6.67×10^{-8} M, and the spot volume was 30 nL. The sample used for selective sampling contained angiotensin I and bradykinin at 10^3 times higher concentrations. At least seven replicates were performed under each condition. The average peak intensity was 208 (arbitrary unit) from the pure ACTH sample and 215 from the selectively injected ACTH, with a relative standard deviation (RSD) of 30% in both cases. Although the large RSD precludes any quantitative conclusions, the results point to a lack of significant sample losses.

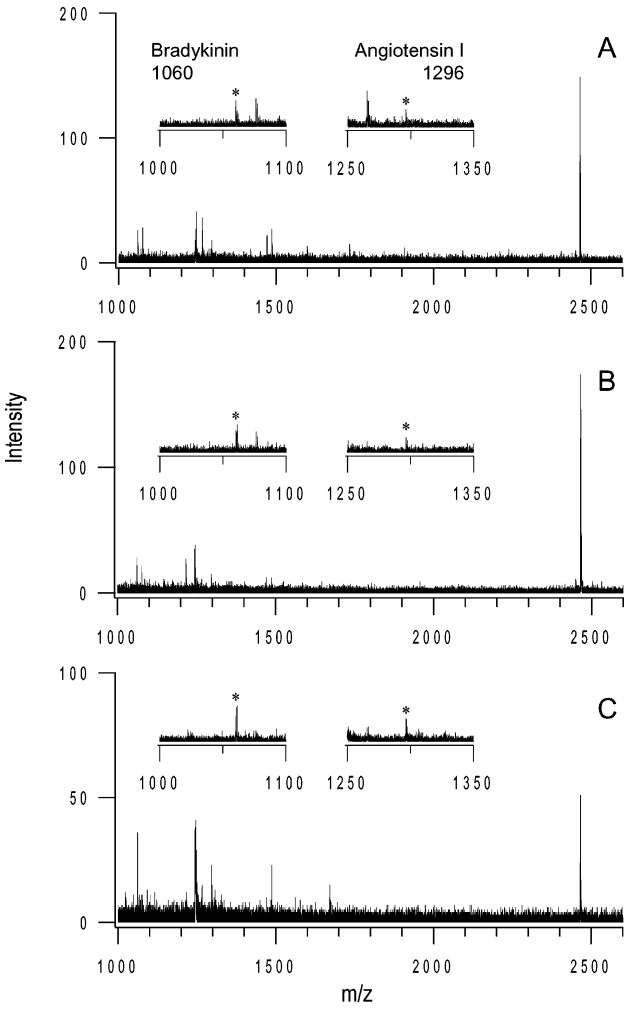

Figure 4. MALDI-MS spectra of selectively sampled ACTH. Bradykinin and angiotensin I concentrations were in excess by factors of (A) 10^3, (B) 10^4, and (C) 10^5.

Next, the detection limit for our selective sampling technique was determined. The amount of ACTH deposited in each spot was lowered successively by reducing the sample spot volume or sample concentration (the angiotensin I and bradykinin concentrations were kept at 1000 times higher). The lowest amount of ACTH that consistently resulted in detectable peaks was 100 amol. The average signal-to-noise ratio (S/N) was 10, based on seven replicates. It was obtained from a spot volume of 6 nL (3 s at 50 mbar) and an ACTH concentration of 1.67×10^{-8} M. Sample spot volumes smaller than 6 nL had been reported in the literature,[1] but it required the use of capillaries with smaller inner diameters, and was not performed in this work.

Detection of 50 amol of ACTH was attempted by lowering the concentration to 0.84×10^{-8} M while keeping the angiotensin I

and bradykinin concentrations at 1000 times higher. Detectable signals were only obtained in ~1/3 of the attempts, and the average S/N was around 3. Nevertheless, when conventional sampling of a pure ACTH solution was performed, very similar results were obtained; that is, an average S/N of ~3 and also not detectable in some attempts. This implied that the detectability was limited by the MS instrumentation and not the selective sampling procedure. Given that the sample recovery of our selective sampling method was, indeed, very high, our method should deliver subatto- or zeptomole level detection when applied to more sensitive MS instruments.

CONCLUSIONS

The presented sampling technique allows selective introduction of peptides on the basis of a difference in the pI. We were able to detect a trace peptide from abundant peptides that were up to 10^5 times more concentrated. Although our method cannot be used as a high-resolution separation technique for complex mixtures, it provides an effective way to isolate trace components from simple mixtures. Importantly, only a small sample volume is required, and no significant sample losses are evident; hence, the technique should be applicable to ultrahigh sensitive mass spectrometry. Examples of potential applications include the isolation of trace components in HPLC fractions and the selective detection of phosphorylated or sulfated peptides (pI lowered by the modification). The procedure is very simple and requires minimum instrumentation, such as a high-voltage power supply and a capillary. In addition, although only data from MALDI-MS is presented, the selectively injected samples can easily be infused directly into nanospray ionization MS.

ACKNOWLEDGMENT

The work is supported by the Natural Science and Engineering Research Council of Canada, the Canada Foundation for Innovation, the Ontario Innovation Trust, and the University of Western Ontario. Thanks to Dr. Nicole Baryla of Eli Lilly Canada, Dr. Charles Lucy of the University of Alberta, and Dr. Gilles Lajoie of the University of Western Ontario for their helpful comments and suggestions.

Received for review May 26, 2004. Accepted August 30, 2004.

AC049230C