Gradient Chromatofocusing-Mass Spectrometry: A New Technique in Protein Analysis

Lian Shan,* James A. Hribar, Xiang Zhou, and David J. Anderson

Department of Chemistry, Cleveland State University, Cleveland, Ohio, USA

A new analytical technique, gradient chromatofocusing-mass spectrometry (gCF-MS), was developed employing ion-exchange high-performance liquid chromatography (HPLC) interfaced to an electrospray-quadrupole mass spectrometer in the determination of proteins. There have been few reports, if any, of a HPLC-MS technique for proteins in which the ion-exchange column is directly interfaced to the mass spectrometer. The employment of a linear pH gradient elution scheme directly interfaced to mass spectrometry is also unique in the present work. The technique was demonstrated by the separation of six proteins (carbonic anhydrase II, enolase, β -lactoglobulin A, lactoglobulin B, soybean trypsin inhibitor, and amyloglucosidase) employing a descending linear pH gradient from pH 9 to 2.6 on a 50 mm $\times$ 2.1 mm DEAE HPLC column using volatile buffer components. A signal enhancement solution consisting of 8% formic acid in acetonitrile was pumped post-column and was mixed 1:1 with column effluent and then directed on-line into the mass spectrometer. Molecular masses of the proteins were determined within $\pm 0.010\%$ to 0.033% (± 100 to 330 ppm) with peak height total ion current detection limits of 4 to 78 pmol of injected amounts ($S/N = 3$). This technique is applicable to the analysis of proteins and other charged molecules. (J Am Soc Mass Spectrom 2008, 19, 1132–1137) © 2008 American Society for Mass Spectrometry

There is great interest in developing a mass spectrometry (MS)-compatible high-performance liquid chromatography (HPLC) technology which performs charge-based separations, a principal dimension in the 2D-gel electrophoresis technique, for application in proteomic and protein characterization studies. Development of such a HPLC technique would be progress towards addressing the limitations of the 2D-gel electrophoresis technique, which has been hampered by its design incompatibility for direct interfacing with MS (requiring excision of each protein band from the gel), limitation in quantitative dynamic range, inability of determining small proteins (5–8000 Da), high labor intensity, long sample run times, as well as other disadvantages [1, 2].

Development of charged-based HPLC techniques directly coupled to mass spectrometry (MS) has been a difficult challenge. This is because the mobile phase salts commonly used in ion-exchange chromatography, the most used charge-based HPLC technique, suppress the MS signal. The most utilized approach incorporating ion-exchange chromatography with mass spectrometry has been to use a 2D ion-exchange/reversed-phase HPLC technique, directing the ion-exchange fractions to a reversed-phase column for further separation and removal of salt before on-line mass spectrometry. This

has been applied to proteomic studies in the analysis of proteins [3] and protein digests [4, 5]. A “stair step” gradient is employed for the ion-exchange dimension. This first dimension thus serves as a crude initial separation step, and is not the stage that is directly interfaced to the mass spectrometer.

The application of two-dimensional chromatography using the combination of chromatofocusing and reversed-phase HPLC has also been reported [6–8]. In this application, chromatofocusing is employed as an off-line fractionation step. The fractions collected at 0.2 or 0.3 pH units are then applied to a reversed-phase HPLC, from which the eluent is directed into a mass spectrometer for analysis. However, in these studies, chromatofocusing is still used as a low resolution, first stage separation step.

Capillary isoelectric focusing has also been interfaced to an electrospray-mass spectrometer for protein and peptide analysis. It has been successfully employed in the characterization of the proteome of *E. Coli* with the detection of 400 to 1000 proteins [9, 10]. This technique requires the use of low concentrations, which compromises peak width, or complete removal of the carrier ampholytes with a microdialysis setup, to obtain an on-line MS signal [11, 12]. Problems with this technique are protein adsorption to the capillary walls, which lead to reproducibility problems [13] and the effect of protein concentration on the pH gradient [14].

A few reports have been published using volatile displacer ions/acids in the mobile phase, such as ammonium acetate/acetic acid or ammonium formate/

Address reprint requests to Dr. D. J. Anderson, Department of Chemistry, Cleveland State University, 2121 Euclid Avenue, Cleveland, Ohio 44115-2214, USA. E-mail: d.anderson@csuohio.edu

* Present address: Frantz BioMarkers LLC, 7740 Metric Drive, Mentor, Ohio 44060, USA.

formic acid, for ion-exchange chromatography techniques directly interfaced to an electrospray mass spectrometer in the analysis of drugs [15, 16], chlormequat [17], nucleoside triphosphates [18], and peptides [19]. However, these reports have not led to widespread use of ion-exchange chromatography interfaced to MS because the volatile ions have relatively weak displacing power [20, 21]. This necessitates the use of high concentrations of displacer ions in the mobile phase that not only suppress the MS signal but also limits the applications to smaller, more readily displaced analyte molecules. Higher concentration of displacer eluents are required for large molecules, such as proteins, due to the large molecule's multiple sites of interaction with chromatographic packing materials [22].

The present work demonstrates the direct interfacing of a high-resolution ion-exchange technique, gradient chromatofocusing [23–26], to MS employing electrospray ionization (ESI). This technique, gradient chromatofocusing–mass spectrometry (gCF-MS), is a general approach for interfacing ion-exchange chromatography to mass spectrometry, drawing upon a pH elution mechanism through employment of a linear pH gradient using volatile buffer components. This differs from the displacement mechanism of the usually employed salt gradients, and has better compatibility with MS through the use of volatile buffer species (such as lactic acid, acetic acid, ammonium bicarbonate and pyridine) and lower buffer concentrations. This allows ion-exchange MS to be applied to different types of ionic analytes. If the principal mechanism of elution of proteins is displacement, then high buffer concentrations are needed, which precludes on-line MS detection. However, high concentration buffers are not required in the pH-based gCF-MS technique, which permits protein analysis. The use of a pH gradient in this technique is also advantageous over salt gradient ion-exchange techniques in that the pH elution mechanism is a better indicator of the pI value of the protein. The present work employs the gCF-MS technique for the separation of proteins and the direct determination of their molecular mass.

Experimental

Proteins

Carbonic anhydrase II (CA II) from bovine erythrocytes (C-3640) (0.089 $\mu\text{g}/\mu\text{L}$), enolase from baker's yeast (E-6126) (0.22 $\mu\text{g}/\mu\text{L}$), β -lactoglobulin A and B (β -LA and β -LB) from bovine milk (L-7880 and L-8005, respectively) (0.18 $\mu\text{g}/\mu\text{L}$ and 0.089 $\mu\text{g}/\mu\text{L}$, respectively), trypsin inhibitor (TI) from *Glycine max* (T-9003) (0.244 $\mu\text{g}/\mu\text{L}$), and amyloglucosidase (AG) from *Aspergillus niger* (A-7420) (0.311 $\mu\text{g}/\mu\text{L}$) were obtained from Sigma (St. Louis, MO) and were made up with water to the concentrations given in the parentheses.

Instrumental Design

The instrumental design for gradient chromatofocusing interfaced via ESI to a quadrupole mass spectrometer is given in Figure 1.

Chromatography Conditions

The aqueous application Buffer A consisted of 25 mM ammonium bicarbonate and 25 mM pyridine adjusted to pH 9 with concentrated ammonium hydroxide. The aqueous elution Buffer B consisted of 25 mM acetic acid and 25 mM lactic acid (no pH adjustment, measured pH of 2.6). The buffer components were either ACS reagent or HPLC grades. An increasing gradient of Buffer B mixed with Buffer A was generated and pumped at 0.2 mL/min through a 50 mm $\times$ 2.1 mm HPLC column packed with Protein-Pak DEAE 8HR (8 μm diameter, 1000 Å pore size) weak anion-exchange packing material from Waters (Milford, MA) at 6.9×10^6 Pa using a HPLC packer from Alltech (Deerfield, IL). The program used to generate the outlet pH gradient (pH 9.0 to 2.6) plotted in Figure 2 was: 38% B for 4 min, 38% to 41% B linear gradient for 10 min, 41% to 51% B linear gradient for 4 min, 51% to 61% B linear gradient for 10 min, 61% to 95% B linear gradient for 14 min, and 95% to 100% B linear gradient for 8 min. A 10 μL sample (protein amounts given in Table 1) was injected. The pH gradient was determined from a blank run, without injection of sample, by collection and pH measurement of 0.4 mL fractions collected after the UV detector.

Mass Spectrometry Conditions and Data Analysis

Column effluent was mixed on-line 1:1 with 8% formic acid in acetonitrile, which enhanced the total ion current (TIC) mass spectrometric signal. The parameters for the mass spectrometer were as follows: detection mode was positive; source temperature was 90 °C; scanning of quadrupole was 800 to 2500 m/z in 3.5 s; cone voltage was ramped linearly from 40 to 90 V during the scan; capillary voltage was 3.5 kV; nitrogen nebulizer gas had a flow rate of 12 L/h; nitrogen drying gas had a flow rate of 300 L/h. Before the run, the mass spectrometer was calibrated by infusion experiments. This was done by following the procedure given by the manufacturer, using a mixture of sodium iodide (2 mg/mL) and cesium iodide (0.05 mg/mL) in 50:50 isopropanol and water.

Data were analyzed by MassLynx ver. 3.3 from Waters. Chromatograms were processed as follows: no background subtract; half-width of the smoothing window, ± 3 scans; number of smooths, 2; smoothing method, mean. For generating the mass spectra, continuum data scans were combined over the half-height portion of the peak. MaxEnt parameters were set at default values: uniform Gaussian algorithm at a width-at-half-height, 0.750 Da; mass range, 5000 to 100,000 Da;

Figure 1. Gradient chromatofocusing-mass spectrometry system. (a) Aqueous application Buffer A and (b) aqueous elution Buffer B are mixed and pumped at 0.2 mL/min by (c) a HP 1100 HPLC gradient system from Agilent (Palo Alto, CA) to form (e) an inlet pH gradient. When the inlet pH gradient is introduced onto (h) a weak anion-exchange HPLC column produces (f) a column pH gradient, which is important to focusing the peaks, and (g) an outlet pH gradient, which causes separation of the peaks [23, 24]. Sample is injected by (d) a HP 1100 Autosampler from Agilent. (i) Signal enhancement solution consisting of 8% formic acid in acetonitrile is pumped by (j) a LC-AD_{VP} HPLC pump from Shimadzu (Columbia, MD) at 0.2 mL/min and mixed with the column effluent in (k) a 10 μ L Visco-Jet Micro-Mixer from Lee Company (Westbrook, CT). The flow after the micro-mixer was split 1 to 7.9 with (l) a splitter, directing 355 μ L/min to (m) a HP 1100 UV detector from Agilent set to 280 nm and 45 μ L/min through an ESI source into (n) a Micromass Quattro II electrospray ionization-triple quadrupole mass spectrometer from Waters (Milford, MA) operated in the single quadrupole mode. (o) A Compaq Professional Workstation AP200 computer from Hewlett-Packard (Palo Alto, CA) computer controlled the HPLC, autosampler, MS, and UV detector, as well as being used for the collection and analysis of data.

resolution, 1.0 Da/channel; minimum intensity channels, 33% for both left and right.

Results and Discussion

Six proteins were separated and analyzed by gradient chromatofocusing directly interfaced to a quadrupole mass spectrometer with the total ion current (TIC) chromatogram given in Figure 2. The pH gradient was generated with volatile buffer components that are compatible with mass spectrometry.

Mass Spectrometry Aspects

The mass spectra for the protein peaks were generated, both the scanning m/z and the deconvoluted spectra. Example scanning m/z and deconvoluted spectra are given in Figure 3 for β -lactoglobulin B. Experimentally determined detection limits are summarized in Table 1. Experimental molecular masses matched theoretical values within ± 0.010 to 0.033% (± 100 to 330 ppm), which is within the reported accuracy of quadrupole techniques (0.01% to 0.04%) [1]. All protein peaks gave scanning m/z spectra that could be deconvoluted to

determine correct molecular masses. The exception was amyloglucosidase. The reasons for this are discussed below. No adducts were noted in the deconvoluted spectra, even in runs in which formic acid was not present in the signal enhancement solution.

The detection limits of the technique for the $50\text{ mm} \times 2.1\text{ mm}$ column were at the pmol level. It ranged from 4 to 78 pmol, using the data given in Table 1 and a criterion of a signal-to-noise (S/N) for the TIC chromatogram peak height equal to three. Coefficients of variation for duplicate runs of the five proteins given in Table 1 (all except amyloglucosidase, which did not yield a discernable scanning m/z spectrum) using the mass chromatogram peaks (summing the signal from the specific m/z signals for each protein) averaged 0.6% (range 0% to 1.4%) and 9.4% (range 3.4% to 15.2%) for the retention times and peak areas, respectively. There was no significant loss of performance after more than 48 h of continuous operation.

Proteins with subunit components (β -lactoglobulin A, β -lactoglobulin B, and enolase) were determined as their subunit molecular masses. The technique demonstrated the ability to differentiate protein isoforms co-eluting within one peak, with two forms of trypsin

Figure 2. The TIC chromatogram of the protein mixture (amounts given in Table 1) on a DEAE HPLC column with the pH gradient plotted. The peaks are (a) carbonic anhydrase II; (b) enolase; (c) β -lactoglobulin B; (d) β -lactoglobulin A; (e) soybean trypsin inhibitor; (f) amyloglucosidase (G2 form); and (g) amyloglucosidase (G1 form).

inhibitor identified in peak e of Figure 2, having measured molecular masses of 20,086 and 19,973. These peaks correspond to intact trypsin inhibitor and a fragment of trypsin inhibitor missing the C-terminus leucine (deduced from the mass difference), respectively. This capability of differentiating multiple co-eluting components, as well as the accurate determination of molecular mass, are important advantages of MS detection over UV.

There was no discernable scanning mass spectrum and only a weak TIC signal for amyloglucosidase, even with a tenfold increase in injection amount over the amount given in Table 1. Negative mode detection was also tried and did not yield a discernable mass spectrum. The low MS signal for this protein may be due to

Figure 3. (a) Scanning and (b) deconvoluted mass spectra of the β -lactoglobulin B peak in Figure 2. Largest signal is 1.8 e^5 cps and 3.5 e^5 cps for scanning and deconvoluted spectra, respectively. Scans were accumulated over the half-height portion of the peak. MaxEnt 1 from Waters was used to generate the deconvoluted mass spectra. Data manipulation parameters are given in the Experimental section.

one or more factors. It could be due to its high carbohydrate content (20% to 23% by weight [30] which is known to suppress electrospray response [31, 32]. In addition, loss of the specific scanning MS signal can occur in highly glycosylated proteins due to heterogeneity in the glycoprotein carbohydrate structure, which can either come from a fundamental heterogeneity in the native glycoprotein molecules or arise from heterogeneous fragmentation of carbohydrate group(s) in the ionization process [33]. From previous anion-exchange chromatography work [30] peaks f and g in Figure 2 are tentatively assigned as the G2 and G1 isoforms of amyloglucosidase, respectively.

Post-Column Signal Enhancement Solution

A signal enhancement solution was pumped post-column and mixed with the column effluent to augment the detection of proteins. This signal enhancement solution consisted of an organic component, acetonitrile, and a weak acid component, formic acid. The

Table 1. Total ion chromatography results

Protein	Total ion current		pI	Elution pH	Pk wt Hf- Ht (pH unit)
	Inj amt (pmol)	Pk ht S/N			
CA II	31	21	5.4 ^a	9.03	^b
Enolase	24	3.9	6.1 [27]	8.35	0.25
β -LB	24	5.7	5.23 [28]	4.72	0.23
β -LA	49	7.5	5.13 [28]	4.40	0.16
TI	122	4.7	4.61 ^c	3.87	0.18
AG	38	^d		3.36	0.21
			3.50 [29]	2.84	^b

^aFrom manufacturer's technical information.

^bNot calculated since protein partially eluted in the plateau region of the pH gradient.

^cCalculated by PeptideMass program of the Expert Protein Analysis System (ExPASy) of the Swiss Institute of Bioinformatics (<http://us.expasy.org>).

^dNot given because percentage of different isoforms not known.

acetonitrile promotes optimal spraying that is critical to the ESI process. It does this by lowering the surface tension of the mobile phase to produce a stable spray [32]. In addition, the organic component may augment the MS signal by promoting more efficient desolvation of the analyte in the ESI process due to an increase in the percentage of the more volatile solvent [32]. The formic acid component of the signal enhancement solution promotes positive charging of the protein through its acid characteristics [32].

In general, the optimal composition of the signal enhancement solution was found to be 8% formic acid in 75% to 100% acetonitrile in water for the various proteins, increasing the TIC signal by a factor of 6 to 18 for most of the proteins (for carbonic anhydrase II there was no TIC peak noted without the signal enhancement solution). It should be noted, however, that the optimum acetonitrile concentration determined in this study is dependent on instrumental parameters [32]. Thus, the optimum organic content needs to be determined for the specific mass spectrometer system and parameters used.

Another consideration for determining the optimum formic acid concentration is noise. It was noted that TIC noise level increased by a factor of 1.6 to 4.4 in different regions of the chromatogram when a signal enhancement solution of 8% formic acid in acetonitrile was used compared to the noise level when acetonitrile without formic acid was used. However, the protein S/N did improve by a factor of 2 to 3 for the 8% formic acid in acetonitrile compared with that of acetonitrile alone. It is possible, however, that further gains in S/N may be realized at lower formic acid concentrations, in which signal enhancement is maintained but noise level is reduced.

Chromatofocusing Aspects

Chromatofocusing is the only chromatographic technique that separates proteins using a linear pH gradient. One recent chromatofocusing technique performed an initial off-line fractionation of proteins by collecting 0.2 to 0.3 pH range fractions and then subjecting these fractions to a reversed-phase-LC-MS analysis [6–8]. In contrast, the gCF-MS technique reported here was completely on-line, maintaining the high-resolution chromatofocusing separation through its direct interfacing to the mass spectrometer via ESI. The improved resolution of gradient chromatofocusing compared with the commonly used salt gradient ion-exchange HPLC arises from its enhanced focusing capabilities [26]. Use of pH gradient elution at relatively low buffer concentrations allows for direct analysis of proteins separated by ion-exchange HPLC by mass spectrometry.

Chromatofocusing techniques provide general pI-based separations of proteins. Table 1 shows that the order of elution, for the most part, follows the predicted pI order (the exception is carbonic anhydrase II). However, it is noted that elution does not occur at the

theoretical pI. There are several reasons for this. One reason is that ion-exchange chromatography is a surface-based technique, meaning that the adsorption of the protein on the ion exchanger occurs through a particular region of the protein molecule. Thus, the chromatographic behavior is not necessarily governed by an overall property of the protein, such as pI. There are also other phenomena occurring in the chromatofocusing technique that contribute to the retention mechanism, including Donnan potential [34, 35], hydrophobic interaction [36], and buffer concentration [25, 26]. It should be noted that the buffer concentration can greatly enhance the separating capability of the technique. For example, a 3-fold or greater increase in resolution has been noted when the buffer concentration is manipulated in gradient chromatofocusing [26].

Of particular note is the discrepancy in the elution pH for carbonic anhydrase and enolase and their isoelectric points. This may be explained by the elution being affected by a displacement mechanism caused by the high buffer concentration of application buffer (25 mM each of ammonium bicarbonate and pyridine), as well as the effect of the other factors mentioned above. Also, for enolase, an alkaline pI species has been reported, which is thought to result from a dissociation of enolase [27]. This may at least partially explain enolase's high elution pH noted in the present work.

The choice of pH gradient to use will depend on the proteins determined. Theoretically, proteins will be retained on an anion-exchange column, which have pIs at least one unit lower than the pH of the application buffer and will be eluted at a pH equal to the pI of the protein. Thus, the presently reported gradient (pH 9.0 to pH 2.6) can theoretically determine proteins in the pI range 2.6 to 8. However, this theoretical chromatographic behavior is not strictly held to, as the present work demonstrates. Proteins that are not within this retention/elution pH range can be determined by expanding the pH range of the gradient. This can be done by increasing the pH of the application buffer through addition of the volatile buffer components with higher pK_a s. Expansion to higher pH ranges will also require changing the column from a weak anion-exchange column to a strong anion exchange column, so that the anion exchange ligand will be positively charged at the higher buffer pHs for retention of negatively charged proteins. There are very few proteins with pIs below 2.6 [37], but if needed, the pH of the elution buffer can be decreased further by addition of a volatile acid having a lower pK_a .

Conclusions

gCF-MS has great potential for protein characterization and proteomics work. Although there have been occasional reports of the use of volatile salts in ion-exchange HPLC interfaced to MS, there have been even fewer reports, if any, of a directly interfaced ion-exchange HPLC-MS technique for proteins. The present work

demonstrates a high-resolution ion-exchange technique directly interfaced to a mass spectrometer that can be generally applied to protein separations. It is also unique in its report of a linear pH gradient elution scheme being directly interfaced to mass spectrometry. The current work will allow for the development of ion-exchange HPLC-MS techniques to complement reversed-phase HPLC-MS techniques, particularly in the separation and determination of proteins and other charged molecules.

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