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Role of mass spectrometry in the purification of peptides and proteins

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Abstract

Experiments were carried out to evaluate the fractionation of proteins and peptides according to mass. Model mixtures were separated by either reversed-phase or ion-exchange chromatography with mass spectrometry-compatible mobile phase additives. Fraction collection was triggered by the mass/charge ratio of each one of the components of the mixture. Chromatography was additionally monitored with a UV–Vis detector in order to compare the new technique with generally accepted in separations. The results indicated that adequate purification is achieved by this new technique. Fraction collection triggered by changes in the mass/charge ratio reduces sample handling and analysis time. This study demonstrates the utility of mass-directed fractionation of peptides and proteins when mass spectrometry-compatible mobile phase additives are used.

Keywords: Mass spectrometry; Preparative chromatography; Peptides; Proteins

1. Introduction

The purification of peptides and proteins usually requires the separation of the compounds of interest from impurities that are very similar in chemical characteristics. Chromatography is the predominant mode of isolation and purification of proteins and peptides [1,2]. In particular, the fact that reversed-phase liquid chromatography offers high resolution and ion-exchange chromatography exposes the compounds to relatively mild conditions makes these two techniques the most commonly used modes of chromatography for peptides and proteins.

Traditional fraction collection consists of collecting a rather large number of fractions and then carrying out analysis of those fractions in order to identify and verify the presence of the targeted compounds. Presently, UV–Vis monitoring is the accepted manner of following the progress of a separation and it is indeed very convenient for preparative chromatography when the compounds of a given mixture can be monitored at different wavelengths. On the other hand, more often than not, targeted molecules and their impurities have to be monitored at the same wavelength making fraction collection and confirmation of compound identity tedious and time-consuming. Therefore, alternative methods for carrying out a separation need to be evaluated. Mass spectrometry (MS) has been extensively used for the analysis of peptides, proteins and their digests [3–7] as well as for the purification of combinatorial libraries [8–10]. The benefit of MS directed purification does not only rest in the fact that compounds are identified on-line by their mass but also that the number of fractions is significantly reduced to the relevant ones containing the com-

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pounds needed. Therefore, mass-directed purification of molecules is a very attractive technique for the pharmaceutical industry to develop new processes and carry them out in a more efficient manner. Phosphate and Tris buffers as well as NaCl are commonly used in preparative chromatography of proteins, however, these substances are not volatile and can quickly deposit in the MS instrument requiring frequent maintenance. While trifluoroacetic acid (TFA) is volatile, it is considered to be an ion-suppressant, and it is therefore less attractive for purifications where on-line mass spectrometers are involved.

If in fact, the process does require non-volatile components or mild ion-suppressants in the eluent, a dilution of the eluent prior to entering the MS system is an alternative to be considered in order to prolong equipment lifetime, reduce maintenance and avoid signal suppression. On the other hand, small amounts of the molecule at the beginning and end of the peak may be lost because the dilution may decrease the material concentration to a point below the detection limits of the equipment. For this reason when employing mass spectrometry for triggering the fraction collection, alternative eluent additives need to be evaluated. The work presented here examines the utility of mass-directed purification of molecules where separations of peptides and proteins are performed either by reversed-phase or ion-exchange chromatography employing MS-compatible mobile-phase modifiers.

2. Experimental

2.1. Materials

Bovine pancreas insulin chain B (oxidized form), oxytocin, vasopressin, bovine pancreas ribonuclease A, horse cytochrome *c*, bovine heart cytochrome *c*, chicken egg lysozyme, ammonium formate and formic acid were purchased from Sigma (St. Louis, MO, USA).

A 50×4.6-mm I.D. and a 50×7.8-mm Symmetry 300 C_{18}, 50×4.6 mm I.D. and a 50×7.8-mm Symmetry C_{18} as well as a 50×5-mm I.D. Protein-Pak SCX (8 μm) column (all from Waters, Milford, MA, USA) were employed in this study.

2.2. Equipment

The experiments were carried out using a Waters Autopurification system consisting of a Waters 2767 sample manager, a Waters 2525 pump, a Waters photodiode array detection (DAD) system PDA 996 and a Waters Micromass ZQ mass spectrometer. The data were acquired and the system was controlled employing Masslynx 3.5 software.

2.3. Procedures

2.3.1. Linear gradient chromatography

2.3.1.1. Reversed-phase liquid chromatography. Linear gradient experiments were carried out for model mixture 1 (bovine pancreas insulin chain B (oxidized form), oxytocin and vasopressin) using a Symmetry C_{18} column from A–B (90:10) to (60:40) for 10 min with mobile phase A composed of deionized water–1% formic acid (90:10) and B composed of acetonitrile–1% formic acid (90:10). The sample concentration was 1 mg/ml (0.333 mg/ml of each compound). The runs were performed at a flow rate and injection volume for the analytical and preparative columns of 1.8 ml/min and 1000 μl, 5.3 ml/min and 2875 μl, respectively. The runs were monitored at 215 nm. Fraction collection was carried out at a mass/charge ratio of 543, 1007.5 and 1148 for vasopressin, oxytocin and insulin bovine chain B, respectively.

Linear gradient experiments were performed for model mixture 2 (horse cytochrome *c* and ribonuclease A) using a Symmetry 300 C_{18} column from A–B (80:20) to (60:40) with mobile phase A and B prepared in the same manner as reported above. The gradient was carried out for 10 min. The feed concentration was 1 mg/ml with 0.5 mg/ml of each protein. The runs were performed at a flow rate and injection volume for the analytical and preparative columns of 1.8 ml/min and 30 μl, 5.3 ml/min and 90 μl, respectively. The runs were monitored at 215 nm. Fraction collection was carried out at a mass/charge ratio of 687.8 and 1053.8 for horse cytochrome *c* and ribonuclease A, respectively.

2.3.1.2. Ion-exchange chromatography. Linear gradient experiments were carried out for model mixture

3 (bovine cytochrome *c* and lysozyme) using an SCX Protein-Pak column with initial conditions set at 525 m*M* ammonium formate, pH 4.5, using a fixed linear gradient of 4 m*M*/min. The sample concentration was 2.5 and 5 mg/ml of bovine cytochrome *c* and lysozyme, the injection volume was varied from 10 to 200 μl. The runs were carried out at a flow rate of 1 ml/min. The effluent was monitored at 280 nm, fraction collection was carried out at mass/charge ratio of 825.2 and 1101.9 for cytochrome *c* and lysozyme, respectively. The mass spectrometer conditions were: positive electrospray ionization (ESI+), capillary 3.5 kV, cone 25 V, extractor 3 V, R_F (lens) 0.3 V; temperature: source 150 °C and desolvation 350 °C; gas flow: desolvation 500 l/h and cone 50 l/h.

2.3.2. Isocratic ion-exchange chromatography

Isocratic experiments were carried out for the model mixture 3 (bovine cytochrome *c* and lysozyme) employing ammonium formate buffer, pH 4.5, at various salt concentrations at a flow rate of 0.5 ml/min and 10 μl injections. The column effluent was monitored at 280 nm.

3. Results and discussion

The objective of this work was to examine mass-directed purification of molecules where separations of peptides and proteins were carried out using several stationary phases and employing MS-compatible mobile-phase modifiers.

3.1. Reversed-phase liquid chromatography

Two model mixtures were employed in the work involving mass-directed purification of peptides and proteins that were purified employing reversed-phase stationary phases. Model mixture 1 consisted of: bovine pancreas insulin chain B (oxidized form), oxytocin and vasopressin. Model mixture 2 consisted of the following proteins: horse cytochrome *c* and ribonuclease A.

3.1.1. Model mixture 1

Bovine pancreas insulin chain B (oxidized form), oxytocin and vasopressin are relatively small molecules and therefore chromatography can be carried out using stationary phases with relatively small pore sizes. Smaller-pore-size packing materials provide a larger surface area and are thus expected to provide a larger preparative loadability. Therefore, Symmetry C_{18} columns with a pore size of 10 nm were selected for this work. The analytical chromatogram and mass spectrometer scan carried out in ESI+ mode are shown in Fig. 1. As seen in the figure, these compounds are well separated and therefore a large amount of material could be purified. However, the solubility of some of the compounds in the initial mobile phase conditions is limited and therefore the separation load was not maximized. Fig. 2 shows the scale-up results to a 50×7.8-mm I.D. column, now with the mass spectrometer scans (performed in the ESI+ mode) for each of the components at their maximum mass/charge ratios. In particular, the collection of the fractions containing bovine pancreas insulin chain B (oxidized form), oxytocin and vasopressin was triggered at the mass/charge ratios of 1148, 1007.5 and 543, respectively. These values correspond to the 3, 1 and 2 charge states for bovine pancreas insulin chain B (oxidized form), oxytocin and vasopressin, respectively. It should be noted that by adjusting the threshold in the MS method, it is indeed possible to tune in the amount of material collected. The mass-directed fraction collection shown here illustrates the ease of collection as well as the on-line identification of each one of the components.

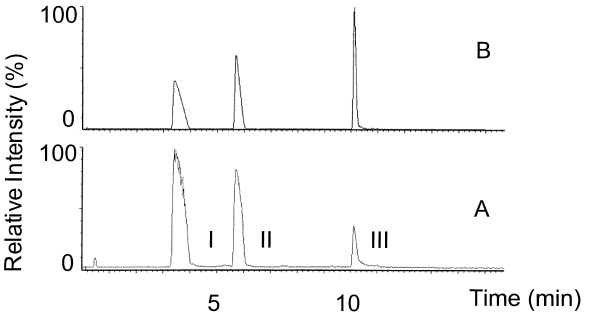

Fig. 1. Analytical results of Model mixture 1 (insulin chain B oxidized form, oxytocin and vasopressin) employing a Symmetry C_{18} 50×4.6 mm I.D. column. The compounds are: (I) vasopressin mass/charge 543; (II) oxytocin mass/charge 1007.5 and (III) insulin chain B (oxidized form) mass/charge 1148. (A) Mass spectrometry scan and (B) UV–Vis tracing at 215 nm.

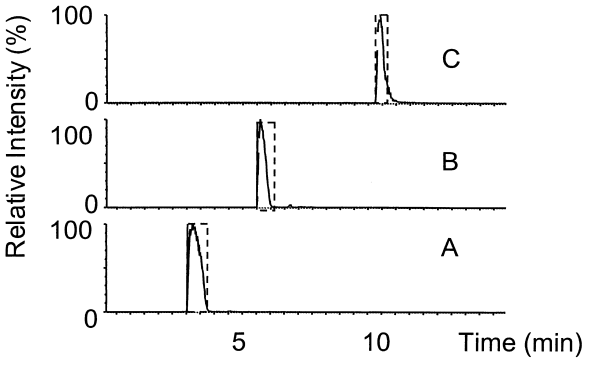

Fig. 2. Mass-directed preparative results of Model mixture 1 employing Symmetry C_{18} 50×7.8 mm I.D. column at the mass/charge ratios corresponding to each one of the compounds of the mixture. Flow-rate: 5.3 ml/min. (A) Mass spectrometry scan for vasopressin at m/z 543; (B) mass spectrometry scan for oxytocin at m/z 1007.5 and (C) mass spectrometry scan for insulin bovine chain B at m/z 1148.

3.1.2. Model mixture 2

This mixture consisted of the following proteins: horse cytochrome *c* and ribonuclease A. In the case of proteins, stationary phases with larger pores sizes are required, therefore, the Symmetry 300 C_{18} stationary phase was selected. The UV monitored chromatogram for both the analytical and the preparative run are shown in Fig. 3, with horse cytochrome *c* eluting after ribonuclease A. As seen in the figure, the performance of both the analytical and preparative columns is in good agreement with the retention times being quite similar. Also, it is noted that the method was developed to a point that good baseline resolution was achieved. The mass-directed purification results carried out in the ESI+ mode are shown in Fig. 4. As seen in the figure, there is still baseline resolution between the two proteins. While this may appear to be trivial, it is still a rather relevant point to make as due to the difference in sensitivity between the UV–Vis detector and the mass spectrometer it is possible to determine the potential contamination of one protein with respect to other compounds in the sample. In this case, it is clearly seen that the fractions are pure.

3.2. Ion-exchange chromatography

For this work a challenging protein separation consisting of compounds that have comparable chromatographic behavior was employed. Initially, several proteins were examined on the same chromatographic column employing ammonium formate buffer, pH 4.5, as the MS-compatible buffer. As a result of these experiments a model protein mixture was identified consisting of horse cytochrome *c* and chicken egg lysozyme. The isocratic runs were carried out at various salt concentrations of ammonium formate and the resulting log *k*–salt concentration plot is shown in Fig. 5. As seen in the figure, the retention of these proteins is quite similar

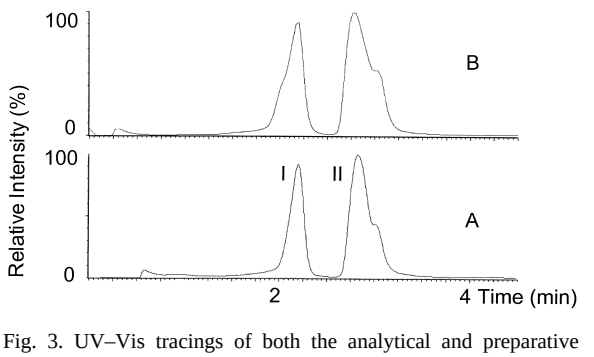

Fig. 3. UV–Vis tracings of both the analytical and preparative scale chromatograms corresponding to Model mixture 2 monitored at 215 nm. The compounds are: (I) ribonuclease A and (II) horse cytochrome *c*. (A) Symmetry 300 C_{18} 50×4.6 mm I.D. analytical column and a flow rate of 1.8 ml/min. (B) Symmetry 300 C_{18} 50×7.8 mm I.D. semi-preparative column and a flow rate of 5.3 ml/min.

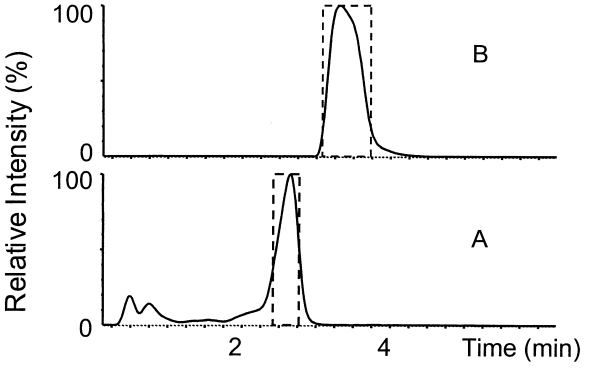

Fig. 4. Mass-directed preparative results of Model mixture 2 employing Symmetry 300 C_{18} 50×7.8 mm I.D. column at the mass/charge ratios corresponding to each one of the compounds of the mixture. Flow-rate: 5.3 ml/min. (A) Ribonuclease A mass/charge ratio 1053.3, (B) horse cytochrome *c* mass/charge ratio 687.8.

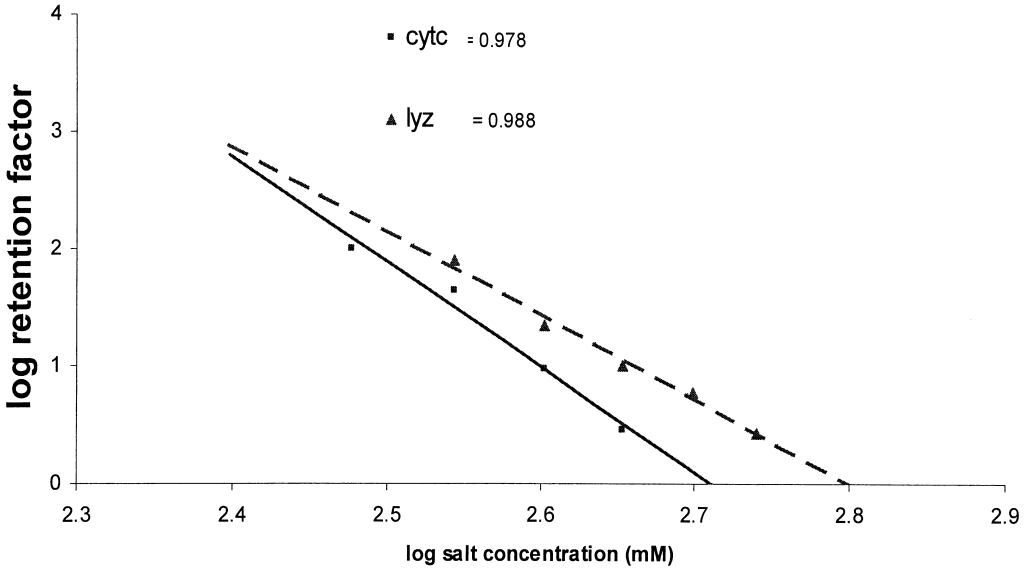

Fig. 5. Log retention–salt concentration plot for horse cytochrome *c* and chicken egg lysozyme. Experiments were carried out employing a 50×5-mm I.D. SCX Protein-Pak column. Flow-rate: 0.5 ml/min.

under linear conditions. In addition, the results shown in the figure indicate that the proteins have a diverging log *k*–salt plot. In particular, as the salt concentration in the mobile phase increases, the capacity factor difference between the two proteins increases. These types of problem mixtures separate successfully under linear gradient chromatography [11]. The linear parameters of the proteins were determined according to the steric mass action (SMA) model [12] and the characteristic charge and equilibrium constant resulted for cytochrome *c* in 8.19 and 0.649 and lysozyme in 7.18 and 2.307, respectively. While both SMA parameters for cytochrome *c* using a Waters SCX Protein-Pak column are higher than those reported earlier [11], it should be noted that data previously published have been carried out at a higher pH than the ones presented here. In fact, the pH difference between the two investigations is larger than one pH unit and it is expected that as the pH decreases there is a significant increase in the retention of cytochrome *c* (isoelectric point p*I* 9.4) and lysozyme (p*I* 11.0) [1]. Consequently both the characteristic charge and the equilibrium constant are expected to increase.

Fig. 6 shows the scans of each protein under the MS conditions in the procedure section. As seen in the figure, each scan describes a protein envelope, with a maximum mass/charge ratio of 825.3 and 1101.9 for cytochrome *c* and lysozyme, respectively. These mass/charge values correspond to 15 charges for cytochrome *c* and 13 charges for lysozyme. Furthermore, each of these mass/charge ratio values is encountered only in one of the scans, meaning that it will be possible to carry out a successful MS-triggered fraction collection with on-line identification of these molecules.

Fig. 7c shows a run monitored by UV at 280 nm and Figs. 7a and b illustrate the run monitored by a mass spectrometer as well as the collections that took place due to the triggering at mass/charge ratio of 825 and 1102. The feed load was 2.5 mg/ml of cytochrome *c* and 5 mg/ml of lysozyme in an injection volume of 200 μl. The split prior to the DAD system and mass spectrometer is such that only one tenth of the flow is directed towards the latter instrument. As seen in Fig. 7, the retention times according to both instruments are the same and indicate an overload condition. The differences in peak height between the diagrams is due to the non-linearity of each instrument and it is dependent on the chemistry of each compound. The amount of material recovered was 95.7%. The recovery was

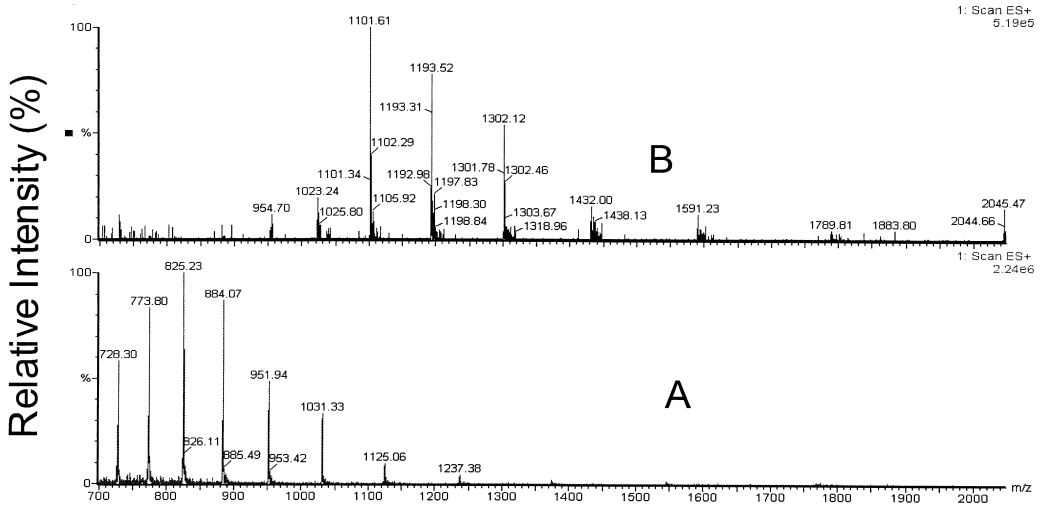

Fig. 6. Mass spectrometry scans of the proteins in Model mixture 3 indicating the maximums that were then selected for the mass-directed purification of the compounds. (A) Horse cytochrome *c*; (B) chicken egg lysozyme.

determined by re-analysis of the fractions collected and comparing those chromatographic results against standard curves generated with each one of the components in the mixture. In addition, by adjusting the threshold for fraction collection and adding timed events it is possible to adjust the amount of the purified material recovered and, therefore, tune productivity to a given purity and yield.

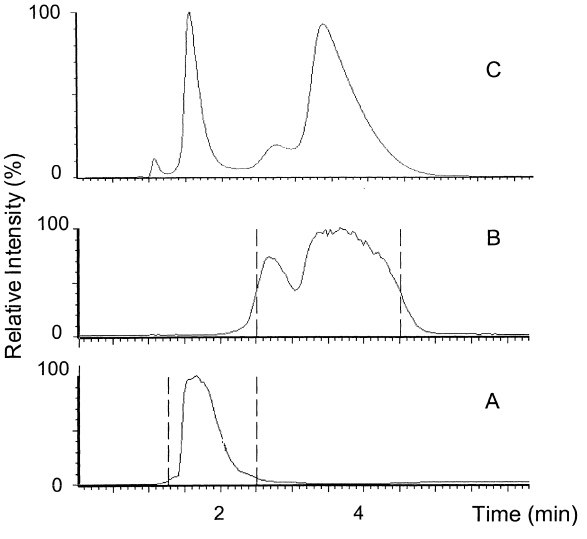

Fig. 7. Tracing results depicting the resolution between the proteins in Model mixture 3 employing SCX Protein-Pak 50×5 mm I.D. column; flow-rate: 1 ml/min. (A) Mass-directed purification of chicken egg lysozyme. (B) Mass-directed purification of horse cytochrome *c* and (C) UV–Vis trace at 280 nm.

At the end of the investigation, that included the runs with formic acid and ammonium formate, the cone of the mass spectrometer appeared to be clean indicating that maintenance of the instrument is kept to a minimum and that indeed the mobile-phase modifiers employed in this study are mass spectrometry-compatible.

The methodology presented here allows for targeted fraction collection, in situ verification of the fractions as well as automation. In addition, as long as the mass/charge ratios of the compounds in a given mixture differ more than twice the span (i.e. ±0.5 u) for the fraction collector trigger, then mass-directed fractionation of peptides and proteins is a robust tool for preparative chromatography. The experiments shown here have been carried out with a span of ±0.5 u, therefore, as long as the mass/charge ratio of compounds in a mixture differ by 1 u or more, then mass directed purification is expected to be highly effective. Consequently, due to the advantages of mass spectrometry, a wide variety of peptides and proteins can be successfully purified by mass triggered purification, including mutant and variant forms.

4. Conclusion

The work presented here examined the utility of carrying out mass-directed purification of peptides and proteins using MS-compatible mobile-phase additives. This work employed model mixtures that were purified either by reversed-phase or ion-exchange chromatography. The results indicated that successful purifications were achieved by triggering fraction collection with a mass spectrometer using MS-compatible mobile-phase modifiers. Using this technique, a decrease in the number of fractions collected is possible compared to classical fraction collection techniques. In addition, in situ verification of the fractions as well as automation is easily achieved. Further, by employing on-line identification of the eluting peaks, it is indeed possible to eliminate the requirement of analysis after the chromatographic run is completed.

Acknowledgements

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