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Journal of Chromatography B, 782 (2002) 307–316

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Surface-enhanced laser desorption–ionization retentate chromatography™ mass spectrometry (SELDI–RC–MS): a new method for rapid development of process chromatography conditions

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Abstract

Protein biochip arrays carrying functional groups typical of those employed for chromatographic sorbents have been developed. When components of a protein mixture are deposited upon an array's functionalized surface, an interaction occurs between the array's surface and solubilized proteins, resulting in adsorption of certain species. The application of gradient wash conditions to the surface of these arrays produces a step-wise elution of retained compounds akin to that accomplished while utilizing columns for liquid chromatography (LC) separations. In retentate chromatography™–mass spectrometry (RC–MS), the ''retentate'' components that remain following a wash are desorbed and ionized when a nitrogen laser is fired at discrete spots on the array after treatment with a laser energy-absorbing matrix solution. Ionized components are analyzed using a time-of-flight mass spectrometer (TOF MS). The present study demonstrates that protein biochips can be used to identify conditions of pH and ionic strength that support selective retention–elution of target proteins and impurity components from ion-exchange surfaces. Such conditions give corresponding behavior when using process-compatible chromatographic sorbents under elution chromatography conditions. The RC–MS principle was applied to the separation of an Fab antibody fragment expressed in *Escherichia coli* as well as to the separation of recombinant endostatin as expressed in supernatant of *Pichia pastoris* cultures. Determined optimal array binding and elution conditions in terms of ionic strength and pH were directly applied to regular chromatographic columns in step-wise elution mode. Analysis of collected LC fractions showed favorable correlation to results predicted by the RC–MS method.

Keywords: Surface-enhanced laser desorption; Protein purification; Protein biochip arrays

1. Introduction

The most popular method for preparative separation of proteins is liquid chromatography (LC). In LC, solid sorbents, which have complementary physicochemical properties to proteins of interest, are employed for selective adsorption. Anionic and cationic proteins adsorb, respectively, on cationic and anionic sorbents while hydrophobic proteins interact with hydrophobic surfaces. Optimal conditions of protein adsorption and desorption, how-

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00564-0

ever, have been demonstrated to be greatly dependent upon the nature of the target protein and its solvation environment of pH and ionic strength. In this situation, chromatographic conditions need to be refined every time maximum enrichment and purification of a target protein from a complex mixture is desired. Under such circumstances, ideal chromatographic conditions not only depend upon the properties of the target protein, but must also consider those properties of background impurities.

To design an LC protein separation process is not a trivial operation, but rather a relatively long and systematic task built essentially on a trial and error approach. Even when using ion-exchange resins, the choice of the type of ionic group is not always easy; strong and weak cation and anion-exchange groups are available. Guidelines of predetermined choice are totally empirical and very frequently do not correspond to the right choice for the above-mentioned reasons. Although a protein's net charge at a given pH is determined by the distribution of exposed titratable groups, most generally it is protein isoelectric point that is frequently considered for initial choice of sorbent and solvent conditions. A common approach is to prepare columns with resins that are chosen according to the ionic character of the target protein and then run separations under multiple conditions. In this manner, it is somewhat possible to identify the appropriate parameters allowing capture of the target protein while leaving the maximum number of impurities in the flow-through. Each chromatographic fraction or collected peak is then analyzed by classical electrophoretic method or by analytical HPLC to determine the purity of the target protein.

In an effort to constrain time, automatic systems can be programmed to cross over all possible conditions of separations; however, this approach is still encumbered by the need to analyze each collected fraction. Due to the large number of separation trials, large amounts of biologicals are needed even when separation columns are miniaturized. This is of significant importance, particularly when the availability of biological sample is low as during the early stages of expression for novel recombinant species. Consequently, the need for a high quality and cost effective means to work up preparative scale LC protocols has incited may groups to investigate more rational and facile processes to develop preparative chromatographic strategies.

To discriminate among various separation alternatives, heuristics have been suggested [1–3]. However, heuristics rarely consider composition of biological fluids, since such fluids are very complex and variable, and as such not subject to discretionary changes. Another approach is based upon protein separation mapping, which utilizes a large number of real chromatographic separations crossing over not only different separation mechanisms, but also discrete changes in ionic strength, buffer pH, and buffer composition within a single chromatographic column. Separation mapping is best accomplished when employing automated chromatographs that make use of intelligent software capable of elucidating ideal separation parameters from this complex matrix of conditions [4,5]. Nevertheless, the selection of an ideal sorbent and associated separation condition is ultimately verified only after accurate electrophoretic or HPLC analysis of collected fractions from each LC run.

More recently other developments have been made in attempting to calculate the best conditions of separation by using simulation software algorithms based upon the properties of both proteins and resins; including differential behavior of impurities. Such ''dry lab'' programs employ complex algorithms, which integrate thermodynamic equilibria and diffusion models [6–9]. Although all of these simulation approaches are of didactic interest, they suffer from a high level of complexity, and have never really been practically implemented with chromatographic separation devices. Furthermore, empirical verification of such algorithmic approaches also requires subsequent analysis of collected fractions.

Other researchers have applied MALDI–TOF analysis to fractions collected using non-porous HPLC separations of complex cellular lysates [10–13]. Similarly, multidimensional lysate analysis combining preparative isoelectric focusing with non-porous HPLC separations and on-line electrospray ionization TOF analysis has also been achieved [14,15]. Additionally, serial ion-exchange–reverse phase chromatography has been coupled with on-line electrospray mass spectrometry [16]. While showing promise as proteomic analytical approaches, each of these chromatographic–mass spectrometry tech-

niques do not provide a facile means to rapidly predict chromatographic behavior of nascent biologicals. The non-porous HPLC approaches rely upon denaturants and disulfide reductants to solubilize proteins, and as such resultant protein elution profiles do not correspond with those of their bioactive analogs. Furthermore, the combined ion-exchange–reverse phase HPLC methodologies have demonstrated efficacy in the purification of peptides and not intact proteins from complex biological milieus. To date, routine development of preparative chromatographic separation methods still relies upon time intensive, empirical experimentation and is heavily dependent upon the help of experienced separation specialists [17].

With the advent of mass spectrometry-based, protein biochip technology, complex biological systems have routinely been analyzed, under non-denaturing conditions, and their protein contents purified and studied for the purpose of biomarker discovery, toxicological investigation, as well as basic research [18–20]. MS–protein biochip technology is based upon the principles of surface enhanced laser desorption–ionization [21,22], and is commercially embodied in the ProteinChip® systems offered by Ciphergen Biosystems (Fremont, CA, USA). ProteinChip systems have utilized the approach of retentate chromatography–MS (RC–MS) for the purpose of performing differential protein display experiments between various sources of biological samples [23]. RC–MS has proven quite effective in cancer research, elucidating numerous protein biomarkers useful in identifying and monitoring the progression of these neoplasms [24–28]. This paper demonstrates the application of RC–MS strategies towards the development of process chromatography protocols. RC–MS will be shown to be an effective, facile, and rapid method that consumes minimal sample while clearly predicting optimal separation conditions for large scale LC purification of proteins from complex biological matrices.

2. Experimental

2.1. Chemicals and biologicals

Strong anion-exchange (SAX20), weak cation-exchange (CWX20), hydrophobic (H4), immobilized metal affinity capture (IMAC), and normal-phase (NP20) ProteinChip® arrays used in this study were provided by Ciphergen Biosystems (Fremont, CA, USA). Processed arrays were read using a PBS II, laser desorption ionization, time-of-flight mass spectrometer (Ciphergen Biosystems). *Pichia pastoris* supernatant containing expressed recombinant endostatin was provided by the National Institute of Health (Bethesda, MD, USA). CM zirconia beads (carboxymethylated sorbents for cation-exchange) were procured from Ciphergen–BioSepra (Cergy-Pontoise, France). Pre-casted polyacrylamide plates for electrophoresis came from BioRad Laboratories (Ivry sur Seine, France). All chemicals used all along the study were from Aldrich (Brussels, Belgium) and were of analytical grade.

2.2. Simulation of protein separation on arrays

Crude extracts containing expressed target protein from cell culture (Fab fragment and endostatin), were directly deposited upon ProteinChip® array surfaces. Four types of arrays were selected a priori: WCX2, SAX2, H4, and IMAC3, respectively, carrying on their surfaces carboxylic acids, quaternary amines, hydrophobic chains, and chelating chemical groups. Each array contained eight distinct spots over which the adsorption of protein could be performed. For WCX2 surfaces, the pH range investigated was between 4.5 and 6.0. Initially, all spots were equilibrated with 200 μl of a low ionic strength buffer (either a 50-m*M* acetate buffer or a 20 m*M* citrate so that to obtain a ionic strength of 5 mS/cm) by using a Ciphergen Biosystems 96-well bioprocessor. Under these conditions, selective protein surface adsorption would be dependent upon the final charge-state of both surface and solvated proteins, with the ultimate objective to preferably adsorb target proteins. After an incubation period of 30 min under vigorous shaking, each spot was then washed three times with 200 μl of the appropriate buffer of pH and ionic strength to eliminate weakly or non-adsorbed proteins.

All surfaces were then dried and prepared for SELDI–TOF MS analysis by applying two times 0.8 μl of matrix solution composed of a saturated solution of sinapinic acid in 50% acetonitrile con-

taining 0.5% trifluoroacetic acid. All arrays were then analyzed using a Ciphergen PBS II ProteinChip reader. The instrument was used in a positive ion mode, with an ion acceleration potential of 20 kV and a detector gain voltage of 2 kV. The mass range investigated was from 3 to 200 kDa while optimizing time lag focusing conditions at 48 and 20 kDa for the Fab antibody fragment and endostatin, respectively. This corresponded to respective lag times of 1055 and 1564 ns. Laser intensity was set between 200 and 280 units according to the sample tested. The instrument was calibrated with bovine serum albumin.

Once the optimal pH for target protein adsorption was determined, a second set of experiments was performed using identical arrays, but this time varying ionic strength, while maintaining constant buffer pH. In this manner, an ideal ionic strength for target protein adsorption with attendant diminution of adsorbed proteins could be determined. Furthermore, the minimal ionic strength required to elute adsorbed target protein would be concomitantly established. The concentration range explored was between zero and 1000 m*M* sodium chloride in the initial acetate buffer. All samples were loaded as previously described. Each chip surface was then washed three times with 200 μl of buffer of appropriate ionic strength and dried. Arrays were then prepared for SELDI–TOF MS analysis by applying twice 0.8 μl of a saturated solution of sinapinic acid in 50% acetonitrile containing 0.5% trifluoroacetic acid and analyzed as previously described.

After this complete set of experiments, best conditions of pH and ionic strength were identified for target protein adsorption and elution from a resin packed LC column carrying the same functional groups (weak cation-exchange: carboxymethyl). For SAX2 surfaces, the investigation was operated in a similar way, however the pH range explored was the one generally used for anion-exchange chromatography, namely between 7.5 and 9 using a 50 m*M* Tris–HCl buffer. The ionic strength was maintained at 5 mS/cm.

Hydrophobic H4 array surfaces were used according to the rules of hydrophobic interaction chromatography. Two sodium chloride concentrations (1 and 1.5 *M*) and four different pH values (4.5, 6.0, 7.5 and 9.0) were used to promote adsorption of proteins. After equilibration of each spot with 200 μl of corresponding buffer, 50 μl of crude sample previously adjusted to equilibration pH and ionic strength conditions was incubated for 30 min under vigorous shaking. Each spot was then washed three times with 200 μl of buffer at the same pH while varying ionic strength between zero and 1 *M* sodium chloride. All surfaces were then rapidly washed in deionized water in order to eliminate salts, and then dried and prepared for SELDI–TOF MS analysis by applying matrix solution as described above.

IMAC3 arrays were investigated using two different metal ions: copper and nickel. Spots surfaces were first loaded with 50 μl of a 100-m*M* solution of either copper sulfate or nickel sulfate. Excess metal ions were removed using a quick wash with 200 μl of deionized water. Spots were then equilibrated using 200 μl of 20 m*M* sodium phosphate buffer, pH 7.0, containing either 500 or 1000 m*M* sodium chloride. Fifty μl of sample previously adjusted at the same ionic strength was then incubated for 30 min under vigorous agitation. Spots were then rapidly washed with deionized water, dried and prepared for SELDI–TOF MS analysis as previously described.

2.3. Liquid chromatography

Column LC separation of target proteins from *E. coli* extract and *Pichia pastoris* culture supernatant was performed on CM zirconia beads. This choice was dictated by the results obtained from array surface investigations. Columns of 10×0.3 cm I.D. were first equilibrated with an adsorption buffer of ionic strength and pH determined by preliminary experiments using arrays systems (see above). Feedstocks were directly loaded after filtration through a 0.45-μm membrane. Elution was performed using sodium chloride concentration steps according to information obtained from the preliminary RC–MS experiments. Finally, the sorbent was regenerated by a wash with five column volumes of 1 *M* sodium hydroxide. Chromatography separations were accomplished at a linear flow-rate of 300 cm/h.

For the Fab fragment separation from *E. coli* extract, conditions of adsorption were met using a 50-m*M* acetate, 5 m*M* citrate buffer, pH 4.6 and elution was performed by increasing the ionic

strength to 150 mS/cm using sodium chloride in the same buffer. Feedstock volume loaded was 23 ml. For recombinant endostatin from *Pichia pastoris* culture supernatant, adsorption was performed in a 50-m*M* acetate buffer, pH 5, and elution was performed by increasing the ionic strength up to 800 m*M* sodium chloride. Feedstock volume loaded was 80 ml.

Fractions were then collected and analyzed either by SELDI–TOF MS using a NP20, normal-phase surface ProteinChip array, or regular SDS–polyacrylamide gel electrophoresis.

2.4. SDS–polyacrylamide gel electrophoresis

Electrophoresis of chromatography fractions was performed on a Mini-PROTEAN 3™ system (BioRad Laboratories, Ivry sur Seine, France) in classical conditions using 15-well pre-casted polyacrylamide plates of 12 or 18% concentration. Samples were prepared by a two-fold dilution in Laemelli sample buffer. Twelve μl of sample were loaded per lane, and electrophoretic migration was performed using a tension of 200 V for 45 min. Staining was achieved using Coomasssie blue solution in ethanol and acetic acid for 1–1.5 h under gentle agitation. Destaining was performed using 40% ethanol and 10% acetic acid in water.

3. Results and discussion

Ion-exchange adsorption–desorption mechanisms of proteins to a porous planar surface or to a porous bead are strictly the same. The basic principle of separating proteins from crude mixtures using protein biochip ion-exchange surfaces is schematically illustrated in Fig. 1. As per conventional column chromatography, the sample is loaded on the surface of the array in appropriate conditions of pH and ionic strength to capture the protein of interest to the solid support. A wash then follows to eliminate impurities while retaining the target protein on the array. Desorption of unwanted proteins is typically accomplished by increasing the ionic strength of the buffer or varying buffer pH. Contrary to chromatographic separations where mobile phase elutropic strength is designed to elute all proteins for subsequent downstream or off-line analysis, here retained proteins are ultimately studied. During the final stage of sample preparation, a matrix solution is added that functions to desorb adsorbed proteins from the array and entrain them in growing crystals. The crystals are irradiated by a focused pulse of laser light, which subsequently causes a phase transition, creating gaseous ions that are analyzed in the TOF MS. In this fashion, a mass spectrum can be generated, often indicating whether the protein of interest is adsorbed while additionally informing the presence of im-

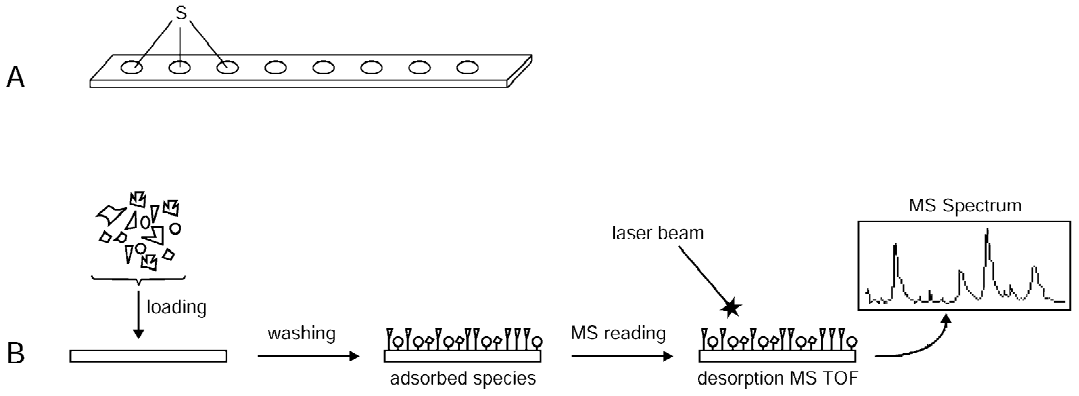

Fig. 1. Schematic representation of the technical approach to use ProteinChip® array surfaces for selective adsorption of proteins and SELDI–TOF MS analysis of captured species. (A) Array with eight spots (S) for sample loading; (B) process of loading, washing and desorption prior to SELDI–TOF MS analysis.

purities. The lower the number of impurities, the higher the selectivity of the array surfaces for the target protein in the conditions of exploitation.

3.1. Fab antibody fragment separation

The first experiment was performed with a crude extract of *E. coli* in which a recombinant Fab antibody fragment (47 kDa) was expressed at a concentration of 700 μg/ml. When using the WCX2 surface (weak cation-exchanger) the Fab fragment was effectively adsorbed at a pH range between 4.5 and 4.8. Above this last value, Fab was not present among surface adsorbed species. A major 45-kDa impurity was however retained (see Fig. 2a). At pH 5 no proteins at all were present indicating that none of the proteins from the sample were retained. Eventually optimal pH for Fab fragment retention was determined to be between 4.5 and 4.7. For all subsequent array experiments, buffer pH value was thus fixed at 4.6, while experiments proceeded examining the effect of ionic strength by varying the concentration of sodium chloride from 0 to 150 m*M* in the acetate buffer.

SELDI–TOF MS data from WCX2 arrays indicated that the Fab fragment was still present at 75 m*M* sodium chloride, but it disappeared when the concentration of sodium chloride was equal or above 150 m*M* (see Fig. 2b). These results clearly indicated that the Fab fragment could be properly adsorbed on a carboxyl-containing surface at a pH of about 4.6–4.7 and at low ionic strength. Desorption would occur when the concentration of sodium chloride would be increased to at least 150 m*M*. Since the ionic strength of the crude extract was 10 mS/cm, equivalent to a sodium chloride concentration of 50 m*M* in 50 m*M* acetate–5 m*M* citrate buffer, no changes would have to be done to get the Fab adsorbed.

Based upon the ProteinChip array results, a column LC separation was performed using a sorbent of similar composition to that of the array's surface. The sorbent, CM zirconia, was packed in a chromatography column of 10×0.3 cm I.D. Adsorption and washing buffer was 50 m*M* acetate–5 m*M* citrate buffer, containing 25 m*M* sodium chloride, pH 4.6 equivalent to an ionic strength of about 8 mS/cm and elution was accomplished at the same pH, but increasing the sodium concentration to 150 m*M*. Fig. 3 shows the chromatographic separation profile with clear indication of an adsorbed and eluted protein fraction. An analysis of the eluted fractions by mass spectrometry showed that it effectively contained Fab fragment with impurities of lower molecular mass (see Fig. 4). A major impurity present had a mass close to 37 kDa. Polyacrylamide gel electro-

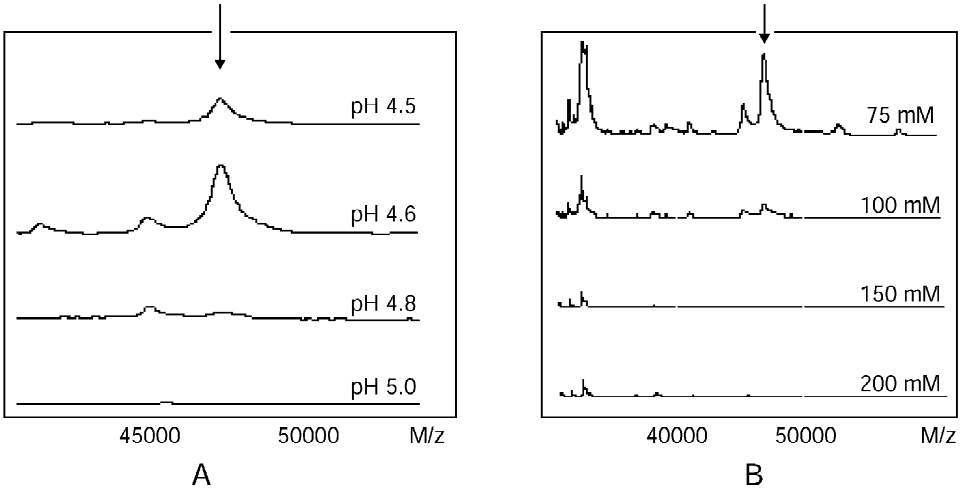

Fig. 2. SELDI–TOF MS data obtained with WCX2 array surface loaded with a crude, clarified extract of *E. coli* containing Fab antibody fragment. (A) Retained proteins at different pH values. The Fab fragment is represented by the arrow. (B) Retained proteins at different sodium chloride concentrations in a 50 m*M* acetate, 5 m*M* citrate buffer, pH 4.6. Fab fragment is represented by the arrow.

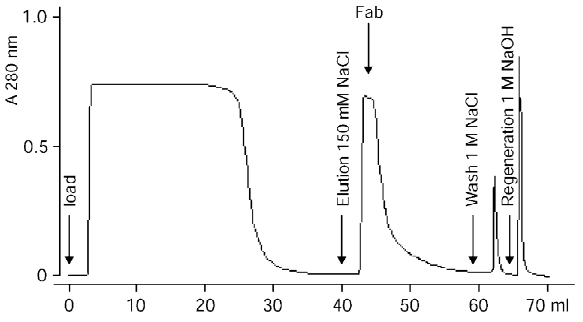

Fig. 3. Chromatography separation of crude *E. coli* extract on CM zirconia sorbent. Twenty-three ml of crude material was loaded onto the column previously equilibrated with a 50 m*M* acetate, 5 m*M* citrate buffer, pH 4.6. Elution of the Fab fragment was obtained by increasing sodium chloride concentration up to 150 m*M* in the equilibration buffer. The column was finally washed with a 1-*M* sodium chloride solution prior to regeneration with 1 *M* sodium hydroxide. Column: 10×0.3 cm I.D.; flow-rate: 300 cm/h.

phoresis analysis further confirmed the presence of the Fab fragment (results not shown). As a whole, the Fab fragment was adequately captured from crude feedstock and was effectively concentrated by 10-fold. Although protein impurities where also captured, the purpose of this experiment was to efficiently adsorb and concentrate the target expressed protein. Further purification of the Fab fragment would require orthogonal chromatographic separations.

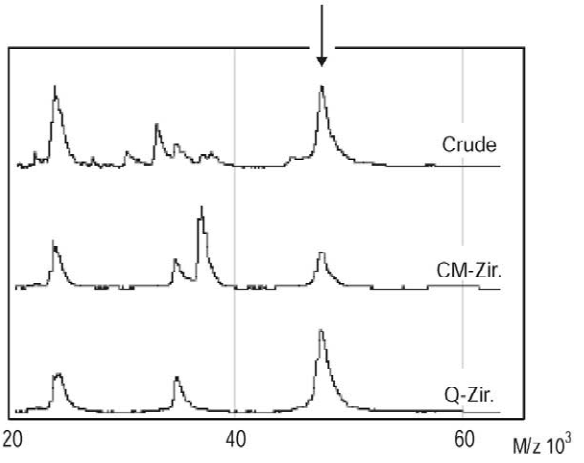

Fig. 4. Analytical results of separated Fab fractions by SELDI–TOF MS using an NP20, normal-phase ProteinChip array. From the top to the bottom: crude extract containing Fab fragment (arrow); eluate from CM zirconia chromatography column; flow-through from Q Ceramic HyperD F chromatography column.

An analysis of the crude extract at different pH and ionic strengths similar to the one described for WCX2 arrays was performed using SAX2 array surfaces. Regardless of ionic strength, the Fab fragment was not retained. As such, a SAX2 surface would provide a useful, orthogonal separation scheme to the cation-exchange resin, further purifying the Fab fraction by adsorbing impurities in lieu of target protein. This strong anion-exchange column purification step was performed using a Q Ceramic HyperD® F sorbent equilibrated with a 50 m*M* Tris–HCl buffer at pH 7.0 (column dimensions: 10×0.66 cm I.D.). Three hundred μl of eluted fraction from CM zirconia column was diluted ten times in Tris–HCl buffer at pH 7.0 in order to decrease the ionic strength to a value compatible with the column initial conditions (lower than 15 mS/cm). Under these conditions, the Fab fragment was effectively found in the flow-through. Analysis of collected fractions from the Q resin separation showed in fact that the purity of Fab fragment was significantly enhanced, however, minor impurities were still present (Fig. 4). This experiment unambiguously demonstrated the effectiveness of using ProteinChip arrays to predict conditions for liquid chromatography.

3.2. Recombinant endostatin purification

To confirm that the RC–MS approach could be extended to other proteins, a second set of experiments was performed using a culture supernatant of *Pichia pastoris* containing recombinant endostatin, a 20.1-kDa protein inhibiting endothelial cell proliferation. The total protein concentration of the feedstock was 0.6 mg/ml, while containing an endostatin concentration of 0.05 mg/ml. The crude extract was tested on four types of array surfaces as previously described: weak cation-exchange, strong anion-exchange, hydrophobic, and chelating surfaces with copper. After loading, array surfaces were washed with appropriate buffers to mimic adsorption chromatography, prior to MS analysis. As shown in Fig. 5, it appeared that WCX2 arrays adsorbed endostatin along with minor impurities, whereas SAX2, H4 and IMAC3 arrays did not show any significant interaction with the target recombinant protein. The SAX

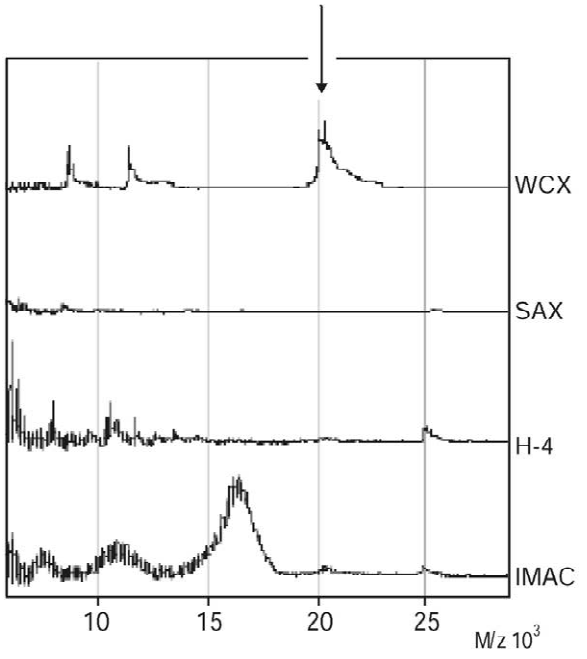

Fig. 5. SELDI–TOF MS data from different array surfaces loaded with a *Pichia pastoris* cell culture supernatant containing endostatin. WCX2: weak cationic exchange in 50 m*M* acetate–5 m*M* citrate buffer, pH 4.5; SAX2: strong anionic exchange in 50 m*M* Tris–HCl buffer, pH 8; H4: hydrophobic interaction surface in 50 m*M* Tris–HCl buffer, 1000 m*M* sodium chloride, pH 7.5; IMAC3: chelated surface with Cu^{2+} in 20 m*M* phosphate buffer, 500 m*M* sodium chloride, pH 7.0. Endostatin is indicated by the arrow.

array surface did not adsorb proteins at all while H4 and IMAC3 array surfaces showed different adsorption behavior. Both H4 and IMAC3 surfaces unambiguously interacted with other protein species excluding endostatin. H4 arrays adsorbed mainly a protein of a molecular mass close to 25 kDa while IMAC3 surfaces bound a protein of lower molecular mass (17 kDa).

In order to define conditions to purify endostatin using cation-exchange chromatography, array adsorption–desorption conditions were optimized using a narrow pH range: 5.0, 5.5, 6.0, and 6.5 (Fig. 6A). Afterwards, different ionic strengths were tested to identify a sodium chloride concentration capable of annihilating endostatin surface interaction (Fig. 6B). It was found that pH 5.0 promoted optimal endostatin surface interaction. No modification of original ionic strength was necessary. As expected, an increase in sodium chloride concentration clearly promoted endostatin desorption from WCX2 surfaces starting at about 200 m*M* of NaCl. MS analysis indicated that full desorption of endostatin was effected at NaCl concentrations ≥400 m*M*.

From these defined conditions, a column of CM zirconia was prepared and used in a preparative manner. After equilibration, 80 ml of cell culture supernatant was adjusted to pH 5.0 and was loaded onto the column. Since mass spectra indicated some desorption of recombinant endostatin at 200 m*M* sodium chloride concentration, the column was also washed with the same buffer (see Fig. 7). Under these washing conditions, the chromatographic profile did not show a large increase of absorbance, therefore the sodium chloride concentration was increased to 800 m*M* to desorb retained proteins. We take the small, observed column versus array difference in endostatin elution behavior to be attributed to expected increases in protein interactions with three-dimensional beads when compared to planar surfaces. Analysis of collected fractions evidenced in fact that all endostatin was totally adsorbed when the column was loaded and washed; elution of endostatin occurred essentially after the second increase of sodium chloride concentration. Analysis of eluted fractions (lane 3 of insert in Fig. 7) indicated that overall endostatin purity was, in all cases, significantly increased compared to the initial feedstock composition (lane 1). Impurities of lower molecular mass than endostatin were present in trace amounts. According to electrophoresis results, the elution purity of recombinant endostatin improved from less than 10 to at least 90% in a single step.

Considering that Cu^{2+} IMAC arrays exclusively adsorbed a 17-kDa protein impurity, which was still present in trace amount after CM zirconia chromatography (lane 3 of Fig. 7), it is suggested that a polishing column of chelating beads complexed with copper could be used to further endostatin fraction purity. Conversely, as suggested by preliminary results obtained using H4 array surfaces (Fig. 5), impurities with lower electrophoretic mobility (larger molecular mass) would be removed in a second polishing separation step by using hydrophobic interaction chromatography.

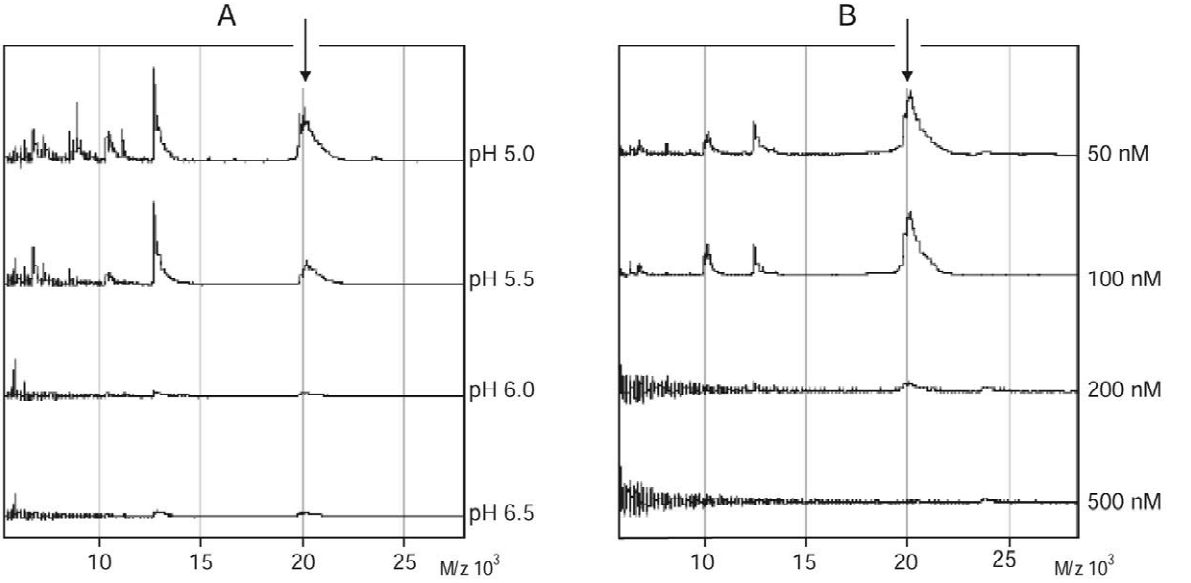

Fig. 6. SELDI–TOF MS data obtained with a WCX2 array loaded with a *Pichia pastoris* cell culture supernatant containing endostatin. (A) Retained proteins at different pH values (50 m*M* acetate, 5 m*M* citrate buffer, pH 5.0). Endostatin is indicated by the arrow. (B) Retained proteins at different sodium chloride concentrations in a 50 m*M* acetate–5 m*M* citrate buffer, pH 5.

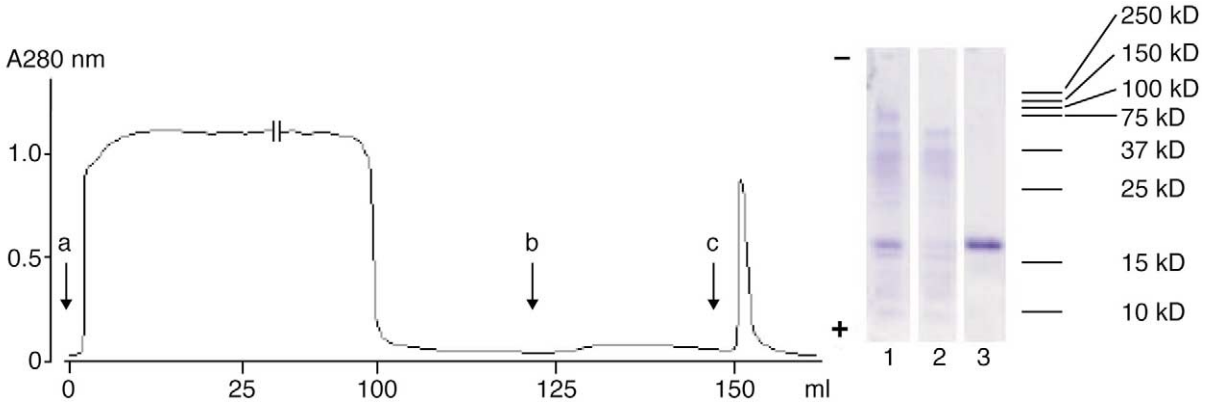

Fig. 7. Chromatography separation of crude *Pichia pastoris* culture supernatant on CM zirconia sorbent. 80 ml of crude material was loaded onto the column (a) previously equilibrated with a 50 m*M* acetate, 5 m*M* citrate buffer, 50 m*M* sodium chloride, pH 5. Elution of endostatin was obtained by increasing sodium chloride concentration in two steps: the first was done using 200 m*M* sodium chloride in the equilibration buffer (b); the second with 800 m*M* sodium chloride in the same buffer (c). Column: 10×0.3 cm I.D.; flow-rate: 300 cm/h. Insert represents electrophoresis analysis of different fractions. Lane 1: crude supernatant; lane 2: non-adsorbed fraction (flow-through); lane 3: elution fraction at 800 m*M* sodium chloride.

4. Conclusion

This work has demonstrated the ability of the RC–MS method to rapidly predict effective preparative separation conditions in short order while consuming minimal sample The results of this study indicate that RC–MS should be applicable to define preliminary separation conditions to purify many other biological liquids and tissue extracts. To begin with, the process merely requires a priori knowledge of target protein molecular mass. Alternatively, target protein molecular mass may be easily determined using the SELDI–TOF MS approach. Serial, multicolumn process separation schemes could also be designed. Although composite data from initial array operations provide enough information about the behavior of a target protein and impurities to design an entire uni-dimensional LC separation scheme, it may be also useful to start a second array analysis of the eluted fraction from the first column, to further gain insight to the nature of impurities and provide potential polishing strategies. Moreover, impurity tracking from separated fractions becomes possible with a very good level of sensitivity. Indeed, detection of species is possible in the femtomole range. Investigations in all these domains are in progress to further extend the usefulness of this approach.

Acknowledgements

The authors wish to thank Professor Joseph Shiloach, Chief of Biotechnology Unit, NIDDK, Bethesda, MD, USA, for providing crude extracts of endostatin along with data about this protein's behavior.

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