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Mapping the chromatographic behavior of a cell proteome utilizing orthogonal routines: the influence of feedstock pH

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Abstract

Surface charge, molecular weight, and folding state are known to influence protein chromatographic behavior onto ion-exchangers. Experimentally, information related to such factors can be gathered *via* two-dimensional electrophoretic (2-DE) methods.

The separation behavior depicted by the insect cultured-cells proteome, which is an important host for recombinant protein production, was explored in this study. Experimental evidence showed a correlation between apparent isoelectric point distributions and the mobile phase conductivity. It was observed that the information contained in the isoelectric point (*pI*) value(s) obtained with a 2-DE routine showed a good correlation with the IEX chromatographic behavior, for a number of commercial adsorbents. This correlation was observed irrespective of the pH of the feedstock within the range 6 to 8. An initial prediction of protein ion-exchange chromatographic behavior could be possible utilizing an experimental approach based on the mentioned orthogonal methods. This technique is providing information that more closely resembles the separation behaviour observed with a complex biotechnological feedstock.

Keywords: Insect cells, proteome, chromatography, ion-exchange, bioprocessing

Introduction

The modern life science industry is facing a real challenge to deliver new products to a highly demanding and regulated market. As a consequence, bioprocess technology options have became limited considering both total available manufacturing capacity and process efficiency and cost. The main obstacle to simplified and cost-effective bioproduct processing are the inefficiencies associated with the recovery and purification of these macromolecules. Chromatography, usually employed sequentially in various operational modes, is a wide spread method which represents the major costs is downstreaming of biotechnological relevant proteins and products (Novais et al. 2001).

Some recent progress has been made to better understand bioproduct chromatographic behaviour and to improve the modelling of this type of unit operations (Salgado et al. 2006; Salgado et al. 2006). Certainly, there are many possible routes when selecting a sequence of high-resolution chromatographic steps, especially when dealing with a complex biological protein mixture i.e. a crude or partially purified real feedstock. Expert systems have been proposed in an attempt to link protein physicochemical properties and the ability of a defined unit operation to separate the product from discrete number of contaminants. Most of these approaches to understand bioproduct behaviour alongside the downstream pathway have focused on the targeted species and often were limited to the utilisation of pure proteins as a model (Asenjo et al. 2004).

In a previous communication (Cabrera and Fernandez-Lahore, 1997), our group has described a routine to define the contaminant proteome of real recombinant expression hosts in order to provide an appropriate description of individual contaminant behaviour during purification. The development of databases describing such purification behaviour, as a function of the nature of the expression system, and in relation with the chromatographic mode utilised might open the way for easy in silico downstream processing development. A change in the current paradigm i.e. changing from focusing on the protein-product to defining the purification profile of the main protein-contaminants would make this novel approach attractive (Cabrera et al. 2007). Analytical proteomics tools, originally developed to study the complexity of biological systems in the post-genomic era (Elrick et al. 2006), are now available to help in understanding complex separation process situations (Cabrera et al. 2008). The underlying mechanisms governing the chromatographic separation of proteins and other bioproducts is not completely understood, particularly when complex protein mixtures are involved.

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Ion-exchange chromatography (IEC) is nowadays a widely utilized chromatographic method within the pharmaceutical and biotech industry due to its good resolution capability, high binding capacity, and versatility. In this chromatographic mode, macromolecule retention is manly determined by Coulomb-type interactions. However, with macromolecules such as proteins, interaction mechanisms with charged surfaces are often complex and the fundamental understanding of such processes is limited (Yao et al. 2005; Malmquist et al. 2006). In addition, non-electrostatic interactions, such as hydrophobic interactions and hydrogen bonding, or other factors like the nature of the buffer ions are also known to influence protein separation with ion-exchangers. Moreover, protein-protein association may lead to non-expected protein chromatographic behavior in a chemical environment supporting native protein conformation.

This paper further advanced previous studies aimed at the evaluation of proteomic tools in order to understand global separation performance in anion-exchangers. Particularly, the influence of sample pH was evaluated employing a complex protein mixture obtained from *D. melanogaster* S2 cells in culture. This expression system is gaining importance for the production of bio-pharmaceutics in the light of recent European legislation which favors intellectual property protection for insect-cell derived bio-products.

Materials and methods

Materials

The insect cell line, the cultivation media, and other cultivation additives were from Invitrogen Europe (Karlsruhe, Germany). Acrylamide, Dithiothreitol (DTT) and (3-[(3-Cholamidoprpyl) dimethylammonio]-1-prpanesulfonate) (CHAPS) were purchased from Carl-Roth GmbH (Karlsruhe, Germany). The molecular weight markers were purchased from Pierce Biotechnology Inc. (Rockford, IL). Chromatographic materials and pre-casted gels were purchased from GE Healthcare Europe GmbH (Munich, Germany). All other buffer-salts and process chemicals were analytical grade and were obtained from AppliChem (Darmstadt, Germany).

Methods

Cell cultivation procedure: *Drosophila melanogaster* S2 cells (D.Mel-2 ATCC #CRL 1963) were grown under aerobic conditions in liquid media by incubating in 1 L baffled flasks at 150 rpm, in 300 ml serum free medium with 5% GlutaMAX™, 10,000 U/ml Penicillin G (sodium), and 10 mg/ml Streptomycin sulphate at 27°C ± 0.5°C. Cells were harvested in the late exponential growth phase (1-2 x 107 cell/ml) by centrifugation at 200 g for 15 min. The resulting cell pellet was stored at –20°C.

Preparation of the crude cell extract: One gram (wet weight) of the cell pellet was suspended in 8 ml of chromatographic mobile phase. One hundred µl of protease inhibitor cocktail (Sigma P 8849, St. Louis, MO) was added per gram of cells. Cells were disrupted by sonication with pulses having duration of 10 s and which were delivered at 30s intervals. Cell disruption was performed at 4°C. Cell debris was separated by centrifugation at 3220 g for 20 min. The soluble fraction remaining in the supernatant was subsequently filtered with a 0.45 µm membrane (Minisart Sartorius, Göttingen, Germany) and utilized as sample for further chromatographic fractionation.

Chromatographic fractionation: Chromatographic experiments were performed in an AKTA FPLC system equipped with a Frac-900 fraction collector and UNICORN 4.10 Software for data collection and analysis (GE Healthcare Europe GmbH, Munich, Germany). The HiTrap™ IEX Selection Kit (1ml matrix bed volume) was employed as adsorbent phase. The kit included pre-packed columns containing Q- and DEAE- Sepharose™ Fast Flow, and ANX Sepharose™ 4 Fast Flow high sub. Chromatography was performed at a flow rate of 117 cm/h and at 20°C. The mobile phase consisted in 20 mM sodium phosphate buffer (pH 5 to 8; 4 mS/cm). Samples (1.0 ml; 40 mg protein) were loaded onto the columns. Unbound biological material was washed out with the same buffer solution and retained protein fractions were eluted stepwise utilizing 0.1, 0.3, 0.6 and 1 M sodium chloride in running buffer as eluent. Collected fractions were desalted by size exclusion chromatography in an open column packed with Sephadex™ G-25 Medium and subsequently concentrated at 40°C under vacuum (Vacufuge Concentrator 5301 Eppendorf AG, Hamburg, Germany). Chromatographic fractionation experiments were performed in triplicate and fraction pooled before analysis.

Two-dimensional polyacrylamide gel electrophoresis: Chromatographic samples were solubilized in a lysis buffer (7 M urea, 2 M tiourea, 4% w/v CHAPS, 1 % w/v DTT, and 2% v/v Pharmalyte™ 3-10). First dimension isoelectrofocusing (IEF) was performed using 7-cm Immobiline™ DryStrips pH 3–10 NL which were placed in a flatbed Multiphor II unit (GE Healthcare Europe GmbH, Munich, Germany) at 20°C. The IPG strips were hydrated for 12 h in Rehydration buffer (6 M urea, 2 M thiourea, 1% CHAPS, 0.4 % DTT and 0.5 % v/v Pharmalyte™ 3-10). Fifty ml of sample was applied in the strip by cup loading. IEF was performed for 10,000–20,000 Vh. Before running the second SDS-PAGE dimension, the Immobiline strips were first equilibrated for 15 min in buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% w/v SDS, 10 mg/ml DTT) and then for 15 min in the same buffer with 25 mg/ml iodoacetamide instead of DTT. After equilibration, the strips were placed on top of a12.5 % SDS vertical slab and embedded in 0.5 % hot low-melting point agarose in SDS electrophoresis running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). SDS-PAGE was performed in a Hoefer™ SE260 gel electrophoresis unit in 0.75 mm thick 10cm x 10.5cm gels at 20 mA per gel. The gels were stained with colloidal Coomassie blue (Neuhoff et al. 1988). Stained 2-D gels were scanned using a 48-bit (400 dpi) Epson color scanner (Epson perfection 4990 Photo) and images analyzed with ImageMaster 2D platinum 6.0 (GE Healthcare Europe GmbH, Munich, Germany). Electrophoretic evaluation of chromatographic fractions was performed in triplicate.

Protein content of the samples: The protein concentration in the samples was determined by the BCA protein assay kit (Pierce, Rockford, IL) as per the manufacturer instructions. This procedure is based on the well known bicinchoninic acid assay (Smith assay), which is a colorimetric method for determining the total level of protein in a solution.

Results and discussion

Chromatographic experiments

Scouting experiments were performed to evaluate the chromatographic behavior of the insect cell soluble proteome obtained as described in the materials and methods section. In doing so, a variety of ion-exchange materials were employed. All the materials under study represent beaded hydrogel-type supports based on agarose. Weak and strong anion-exchangers were

represented among the materials tested. The characteristics of the mentioned ion-exchange supports are given by the provider. On the other hand, other chromatographic conditions were kept the same so as to assure standard operational conditions and parameters. Elution of bound protein fractions were performed stepwise thus resembling industrial practice were gradient elution is not favored. The experimental approach presented here opens the opportunity for a direct comparison between adsorbents in terms of selectivity towards protein naturally occurring in a complex feedstock. Information of this kind is seldom available in the open literature since most studies on material selectivity or protein chromatographic behavior are usually restricted to one or a few purified model proteins (Lagerlund et al. 1998).

-angers tended to behave alike at alkaline pH values. On the other hand, DEAE-Sepharose showed better resolution at acidic pH e.g. 5 peaks can be noticed at pH 6.

Proteomic routines

The chromatographic fractions obtained by fractionation of the total soluble Drosophila cell proteome were analyzed on the basis of 2D-PAGE, as well as, the crude feedstock loaded into this system. Fig. 2 shows, as an example, the 2D-PAGE analysis of the Q-Sepharose fractions. These studies, when applied to the various adsorbents and feedstock pH values, gave information on the overall number of potential contaminants present in each of these fractions and on their

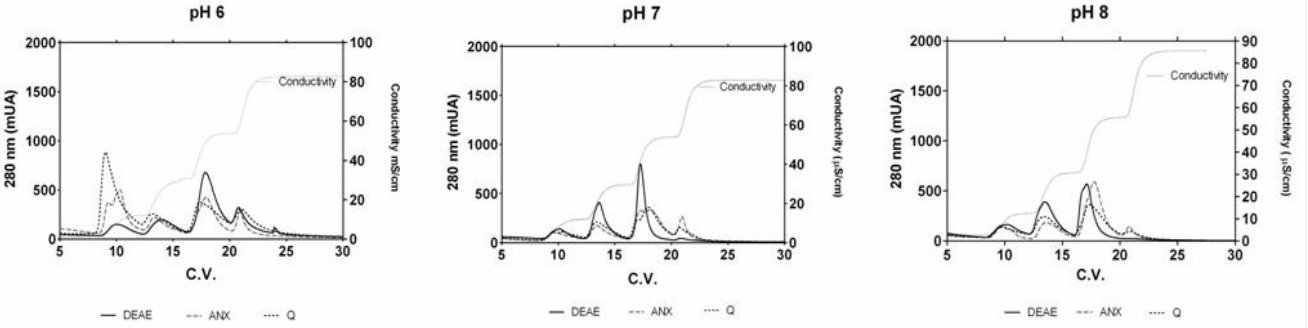

Figure 1: Chromatographic profiles obtained on various commercial adsorbents and with several initial feedstock pH values.

Anion exchanger materials were comparatively studied in relation with the Drosophila cell soluble proteome. Dynamic adsorption experiments were performed in phosphate buffer at pH 7.5 at a superficial velocity of 117 cm/h. Fig. 1 depicts the separation attained utilizing weak (DEAE- and ANX-) or strong (Q-) anion-exchange chemical moieties onto agarose beads. Total protein load in such systems was limited to approximately the expected average dynamic binding capacity for these materials. It was observed that ~ 70 % of the protein load in all cases was lost in the column flow-through as non retained components of the mixture. On the other hand, the bound material was fractionated by elution via stepwise increase in the mobile phase ionic strength by adding increasing concentrations of sodium chloride i.e. measured conductivity was 11, 29, 54, and 86 mS/cm in the four collected fractions, respectively. As judged by UV absorbance @ 280 nm, four main eluting peaks were distinguished for ANX- and Q- adsorbents but only tree in the case of the DEAE- support. This is not anticipated on the basis of the functional ligands involved since DEAE- and ANX- (tertiary ammonium group) but not Q- (quaternary ammonium group) are considered to be weak anion-exchangers. However, there is a clear match between the separation profiles obtained for ANX- and Q- materials as opposed to DEAE-Sepharose FF.

Changes introduced in the initial pH of the mobile phase would alter chromatographic separation not only due to changes in protein charge but as a function of the titration profiles of the different materials that could be utilized to exert a certain purification task. As shown in Fig. 1, chromatographic experiments were performed at three levels of pH: acidic (pH 6), neutral (pH 7), and basic (pH 8). It was observed that the fraction of protein material, as judged by @ 280 nm, which is very weakly retained (i.e. because elutes at very low salt concentration) was increased at acidic pH values. Differences between materials were exacerbated at such pH. Increasing pH values to 7 and further to 8 has resulted in a much smaller weakly-retained protein fraction and in chromatographic profiles more closely resembling each others. Therefore, anion-exch

distribution in a proteome space defined by coordinates of apparent isoelectric point values and protein molecular weights, under chemically denaturing conditions. Each sample, which represents a set of potential contaminants within a certain operational window, was subjected to concentration keeping an identical concentration factor. This would directly reflect the total mass of the contaminating material in each recovered fraction. Similarly, protein load onto the 2D gels was related to the total protein concentration in each of the collected chromatographic fractions. This allowed for direct comparison between 2D gels after staining with Colloidal blue. The selection of this particular staining method reflected the intention of revealing major protein spots since priority should be given during any downstream process for the removal of major

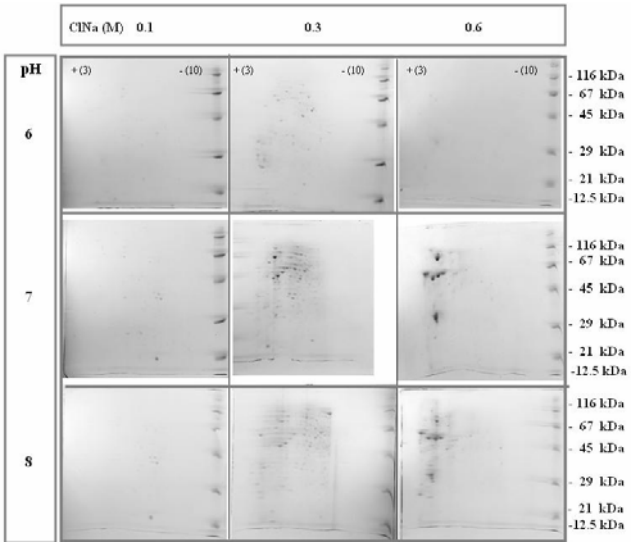

Figure 2: Analysis of the chromatographic fractions obtained after separation with Q FF- Sepharose at different pH. Each fraction was obtained by pooling three different chromatographic runs. An orthogonal proteomic routine was utilized to fractionate the proteins existing in each of the mentioned samples.

contaminants at first. Ion exchange is a moderate resolution method, which usually finds place as an early-to-intermediate isolation step and thus, optimization of this method on the basis of major contaminants accompanying a certain product, is duly justified.

A first insight on overall chromatographic behavior can be gathered after analyzing the number of spots revealed by 2D electrophoresis. Fig. 3 depict the spot number as a function of material type, feedstock pH, and concentration of sodium chloride required for elution. After colloidal blue staining, the total number of spots observed in the crude feedstock was 824. Fractions obtained after elution of weakly bound material i.e. the material eluting from the column a low salt concentration (0.1 M sodium chloride in running buffer) presented a limited number of spots. In Table 1 it can be observed that, in most cases, the number of spots is maximum in the fraction eluted with 0.33 M of sodium chloride. The DEAE-material showed a more even distribution in spot number among the fractions analyzed. On the contrary, ANX- and Q- showed a more unequal distribution: a) a larger number of spots was observed at acidic pH values and low salt concentration for ANX-Sepharose, and b) a larger number of spots was observed at neutral-alkaline pH values and high salt concentration.

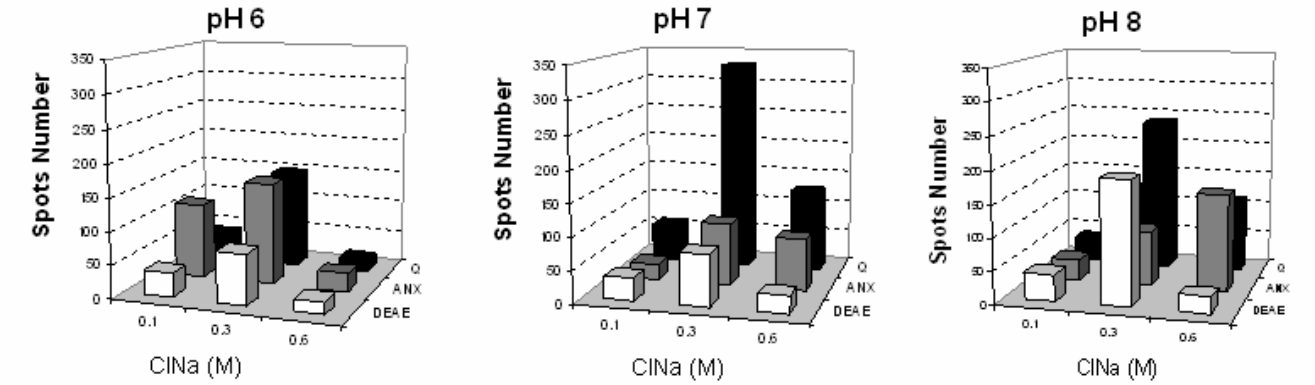

Figure 3: Spot number in each chromatographic fraction, as reveled by 2D SDS-PAGE analysis, as a function of adsorbent type, feedstock pH, and mobile phase conductivity. 2D gel electrophoresis was performed in triplicate, for each of the samples under analysis. Stained 2D gels were scanned using a 48-bit (400 dpi) Epson color scanner (Epson perfection 4990 Photo) and images analyzed with Image Master 2D platinum 6.0 (GE Healthcare Europe).

Table 1: Spot number in each chromatographic fraction.

Adsorbent Material	ClNa (M)	pH (-)		
		6	7	8
DEAE	0.1	37	35	41
	0.33	77	80	192
	0.66	19	27	27
ANX	0.1	113	25	33
	0.33	155	96	86
	0.66	28	80	150
Q	0.1	39	60	38
	0.33	147	328	238
	0.66	19	128	112

Apparent pI distributions

The quantitative and qualitative information obtained after the 2D electrophoretic evaluation of the chromatographic fractions in terms of spot number and their mass-relevance can be complemented by considering the distribution of isoelectric point values -among the observed spots and in relation to the ionic strength levels required for elution. A second level of data evaluation can be done on the basis of apparent isoelectric point distributions. These could be linked to desorption behaviour in a way that not necessarily reflects total (or major) spot number(s). Figure 4 shows the relationship between pI value distributions and salt concentration relationship between pI value distributions and salt concentration in the eluting buffer. Overall, a tendency was observed for the pI values to be displaced to the range of acidic pH values. This was a general trend observed from very rich data obtained after analyzing a large number of chromatographic fractions: samples (3 initial pH values / 3 pH values for the mobile phase) were run onto 3 types of anion-exchangers, and fractions collected at 3-5 conductivity intervals. These observations are in agreement with the known retention principle of proteins onto ion-exchangers which is manly determined by Coulomb-type interactions with a charged surface: for a fixed adsorbent type it is expected to have strong-negatively charged proteins binding with more affinity to the positively charged anion-exchangers. Consequently, it is expected that more acidic proteins i.e. those with the lower pI values would elute at

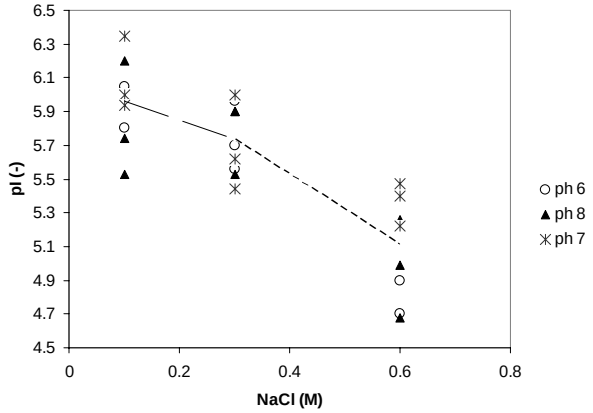

Figure 4: Isoelectric point value distribution as a function of salt required for elution. 3 anion-exchangers and three sample pH values are considered. 2D gels were run in triplicate and scanned images statistically analyzed with Image Master 2D platinum 6.0 (GE Healthcare Europe). Each point represents the mean isoelectric point for a certain spot population, i.e. fractions obtained as a function of buffer conductivity required for elution and pH of the mobile phase. The trend line was drawn by averaging such mean isoelectric point values, at each buffer conductivity value.

higher salt concentrations. However, non-electrostatic interactions can also affect protein separation during ion-exchange chromatography. Solid phase effects, as well as, nonspecific interactions with the immobilization chemical structures or the ligand itself can not be ruled out.

Ion exchange chromatography at near-neutral pH values, in relatively low salt polar buffers is non-denaturing and therefore, proteins in a real sample might have the ability to form complexes (Butt et al. 2001). Protein-protein complexes usually will have a different pI than the one of the individual proteins. Association of two or more proteins can also mask charged patches at the surface of these proteins thus, impacting on the nature of the interaction with the solid phase.

Interaction of proteins with chromatographic supports is an often complex physicochemical event(s) and little is known about the fundamental mechanism behind protein binding to charge surfaces. It is still uncertain which model best describes the ionic strength dependence of this interaction since retention changes caused by changing the salt concentration in the eluent depend mainly on the protein charge or the charge distribution on its surface (Hallgren et al. 2000) as well as the geometry (Malmquist et al. 2006). The nature of the chromatographic support also influences protein interaction and separation.

Conclusion

Chromatographic runs performed with a cell proteome utilizing 3 types of anion-exchangers and mobile phases having pH values within the range 6 to 8 were analysed by orthogonal proteomic techniques.

A clear tendency between isoelectric point distributions and the amount of salt required to elute sample sub-proteomes was clearly observed.

The information provided in this work can help in understanding the chromatographic behavior of natural (complex) protein mixtures, usually found in a real process feedstock. . Our studies could be particularly helpful for the design of separation process of recombinant proteins produced in the intracellular space in cultured insect cells. However, the observed trends in separation behavior might be partially extended to other cell proteomes. Studies are under way in our laboratory to more closely understand the chromatographic behavior of proteins derived from microbial and mammalian cell systems.

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