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A study on the nature of interactions of mixed-mode ligands HEA and PPA HyperCel using phenylglyoxal modified lysozyme

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ABSTRACT

Mixed mode chromatography, or multimodal chromatography, involves the exploitation of combinations of several interactions in a controlled manner, to facilitate the rapid capture of proteins. Mixed-mode ligands like HEA and PPA HyperCel™ facilitate different kinds of interactions (hydrophobic, ionic, etc.) under different conditions. In order to better characterize the nature of this multi-modal interaction, we sought to study a protein, lysozyme, which is normally not retained by these mixed mode resins under normal binding conditions. Lysozyme was modified specifically at Arginine residues by the action of phenylglyoxal, and was extensively studied in this work to better characterize the mixed-mode interactions of HEA HyperCel™ and PPA HyperCel™ chromatographic supports. We show here that the adsorption behaviour of lysozyme on HEA and PPA HyperCel™ mixed mode sorbents varies depending on the degree of charge modification at the surface of the protein. Experiments using conventional cation exchange and hydrophobic interaction chromatography confirm that both charge and hydrophobicity modification occurs at the surface of the protein after lysozyme reaction with phenylglyoxal. The results emanating from this work using HEA and PPA HyperCel sorbents strongly suggest that mixed mode chromatography can efficiently separate closely related proteins of only minor surface charge and/or hydrophobicity differences.

1. Introduction

Chromatographic ligands were initially assumed to be interacting with proteins through a single mode of interaction. However, chromatographic interactions are often multi-modal between the interacting protein and the ligand, ranging from ionic, hydrophobic, to specific affinity. In addition, the support matrix could also form non-specific "secondary" interactions with the proteins, affecting their adsorption. Mixed mode chromatography, or multimodal chromatography, involves the exploitation of combinations of these interactions in a controlled manner, to facilitate the rapid capture of proteins. The ligands used in mixed-mode chromatography are chosen such that they allow different kinds of interactions under different conditions.

Mixed mode ligands have long been in use, long before the term was coined. Hydroxyapatite chromatography is the oldest known form of mixed-mode system [1], involving a combination of ionic and metal-affinity interactions [2,3]. Histidine-affinity is another well-studied mixed-mode method [4], whose versatility arises from the unique nature of the amino acid L-Histidine [5]. Other pseudo-affinity chromatography like dye-affinity [6–9] and peptide-affinity [10,11] also involve multi-modal interactions. The first bases for mixed-mode chromatography originate from the development of thiophilic chromatography [12], exemplified by its multi-modal interaction owing to its pyridine ring structure containing a sulphur moiety [13–15]. Following thiophilic chromatography, a number of mixed-mode ligands were invented, consisting of mercaptoheterocycles, heteroatomic cycles and aromatic groups. The Mercapto-ethyl pyridin group, found in MEP HyperCel™ resin, was the first report on Hydrophobic Charge Interaction Chromatography (HCIC) [16–19].

Classical affinity media like Protein A, have high binding strengths and affinity owing to multimodal interactions with their target proteins, ranging from hydrogen bonding, to hydrophobic

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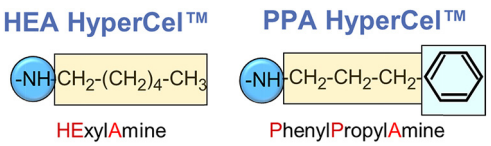

Fig. 1. Structure of HEA and PPA HyperCel™ mixed mode ligands.

and ionic interactions, much like in the case of mixed mode ligands [20]. However, the major difference between classical affinity and mixed-mode ligands is the ability of the latter to interact with a wide range of proteins, and the ability to regulate these interactions by the buffer conditions provided. The major advantage of these mixed mode resins over traditional hydrophobic interaction resins is their ability to function at lower salt concentrations [21–28], and their capacity to bind proteins at near physiological conditions, which is always preferred for the capture of proteins, especially when the confirmation is essential for its function.

A wide range of target proteins: ranging from serum albumin, recombinant proteins [29–32], to antibodies [33–42] have been successfully purified by involving mixed-mode ligands. However, owing the complexity of mixed mode ligands, the conditions of binding and elution need to be optimized for each protein studied.

Here we demonstrate the application of HEA HyperCel™ and PPA HyperCel™ (Fig. 1) in purifying native and modified lysozyme. Binding under hydrophobic conditions, followed by elution using charge repulsion is a characteristic method for separating proteins using these mixed mode sorbents. The different interactions that mixed mode resins pose have been evaluated by using protein standards. By varying the pH and ionic strength of the buffers used, and by addition of organic compounds, two major modes of interactions were highlighted: hydrophobic and electrostatic interactions.

In order to better characterize this multi-modal interaction, we sought to study a protein (lysozyme) that is normally not retained by these mixed mode resin under normal conditions. Lysozyme was modified specifically at Arginine residues by the use of phenylglyoxal [43–48], and has been extensively studied in this paper in characterizing mixed-mode interactions of HEA HyperCel™ and PPA HyperCel™ chromatographic supports.

2. Experimental

2.1. Materials

2.1.1. Resins, chemicals and columns

HEA HyperCel, PPA HyperCel resins were kind gifts by Pall Life Sciences (Cergy, France). HiPrep™ 26/10 Desalting, Hitrap Desalting (5 ml) and Resource S (1 ml) pre-packed columns were purchased from GE Healthcare (Saclay, France). Tricorn 5/50 and Tricorn 10/50 empty columns were purchased from GE Healthcare (Saclay, France). Hexyl and Phenyl Toyopearl resins were from purchased from Tosoh Biosciences (Stuttgart, Germany). μ-Precolumn™ (300 μm ID × 5 mm), C4 PepMam™ (75 μm ID × 5 cm) and C4 analytical columns were purchased from Dionex (Voisins Le Bretonneux, France). Phenylglyoxal, salts and buffers were from Sigma–Aldrich (Saint Quentin Fallavier, France).

2.1.2. Instrumentation

AKTA Purifier 100 and 10 workstations from GE Healthcare (Saclay, France) were used for chromatographic experiments monitored with Unicorn software. Protein presence was monitored at 280 nm. Samples for quantification were analyzed using NanoDrop 1000 (Thermo Fisher Scientific, Villebon sur Yvette, France). Blood Tube rotator SB1 was from Bibby Stuart Scientific (Nemours, France), Orbital shaker was from Infors HT (Massy, France). Mass spectrometry analyses were carried out on LTQ Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany).

2.2. Methods

2.2.1. Packing of columns

Resins HEA HyperCel, PPA HyperCel were packed into Tricorn 10/50 (1 cm × 5 cm) columns with a 5 cm bed height corresponding to 4 ml of chromatographic gel, according to the manufacturer's recommendations. They packing efficiency was determined, and both columns presented similar numbers of theoretical plates (data not shown). Hexyl and Phenyl Toyopearl were packed into Tricorn 5/50 (0.5 cm × 5 cm) according to the manufacturer's recommendations.

2.2.2. Chemical modification of lysozyme by phenylgloxal

Chemical modification of lysozyme was performed at 4 °C, 25 °C and 37 °C under agitation at different time (6 h, 9 h, 18 h, 24 h, 30, 48 h, 54 h, 72 h and 96 h). Several concentrations of lysozyme and phenylgloxal were evaluated to choose the most appropriate ratio (data not shown). For a volume of 120 ml, 134 mg of phenylglyoxal and 120 mg of lysozyme (corresponding to 0.7 μmole of lysozyme and 73.4 μmole of phenylgloxal) were used resulting in a phenylglyoxal:arginine ratio of 9.527. The chemical modification was stopped by subjecting the mixture to size exclusion chromatography by injection of 5 ml of reaction mixture on a HiPrep™ 26/10 Desalting column.

2.2.3. Mixed mode chromatography of chemical modified lysozyme

Retention of the modified lysozyme on mixed mode columns was studied on HEA HyperCel and PPA HyperCel resins packed in Tricorn 10/50 columns. Columns were equilibrated in 50 mM Tris–HCl, pH 8. Two ml of the desalted modified fractions from the above reaction mixture were passed over columns in each experiment, after which columns were washed with equilibration buffer for at least 10 column volumes to recover non-retained proteins. After the absorbance at 280 nm reached baseline, elution was achieved by the application of pH gradient over 20 column volumes with 50 mM sodium phosphate citrate buffer, pH 3. The pH drop was a convex gradient showcasing a pH drop from 8 to 4 in the first 5 CV, followed by the pH drop from 4 to 3 in the next 13 CV. Fractions of 1 ml each were collected through the gradient, and each fraction was analyzed by mass spectrometry.

2.2.4. Mass spectrometry analysis

Online nanoLC–MS/MS analyses were performed using an Ultimate 3000 system (Dionex, Amsterdam, The Netherlands) coupled to a nanospray LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Fractions from the mixed mode chromatography experiments were first acidified with formic acid (1% final) and 10 μl of each sample were loaded on a PepMap C4 precolumn (ID 300 μm × 5 mm; LC Packings, Dionex, USA) at a flow rate of 30 μl/min. After 3 min of desalting, samples were separated online using a C4PepMap™ column (ID 75 μm × 5 cm; LC packings, Dionex, USA) with a 5–75% linear gradient of solvent B in 22 min (solvent A was 0.1 formic acid in 5% ACN and solvent B was 0.1% formic acid in 80% ACN), at a flow rate of 250 nl/min. The mass spectrometer operated in positive ion mode at a 1.96-kV needle voltage and a 14-V capillary voltage. Data were acquired in a MS scan survey over the range m/z 300–2000 and deconvoluted using Bioworks 3.3.1 (Thermo Finnigan). The average theoretical mass of chicken lysozyme is 14305.14 Da and one molecule of phenylglyoxal contributes to an additional 116 Da to the theoretical mass of lysozyme. Based on this analysis, the predominating population in

each fraction and number of added phenylglyoxal moieties were determined.

2.2.5. Evaluation of the effect of phenylglyoxal on cation exchange and hydrophobic interaction chromatography

2.2.5.1. Cation exchange chromatography. Resource S cation exchange column (1 ml) (GE Healthcare) was used for cation exchange chromatography experiments. The column was equilibrated with 50 mM sodium acetate buffer, pH 6 and desalted samples from the mixed-mode experiments were injected. The bound proteins were eluted by a linear gradient of 0–1 M NaCl over 20 column volumes. A constant flow rate of 4 ml/min was used.

2.2.5.2. Hydrophobic interaction chromatography. Conventional hydrophobic interaction chromatography was performed with Hexyl and Phenyl Toyopearl resins (Tosoh Biosciences), which were close in mimicking HEA and PPA HyperCel supports. The resins were packed in Tricorn 5/50 columns of 1 ml following the manufacturer's instructions.

During the chromatographic run, the column was equilibrated with 20 mM sodium phosphate buffer pH 7.0, containing 1.5 M ammonium sulphate. The samples were brought to the same conductance by mixing in equal volume of equilibration buffer containing 3 M ammonium sulfate. Elution was achieved using 20 mM sodium phosphate buffer, pH 7.0 in a linear gradient over 20 column volumes. A constant flow rate of 1 ml/min was used.

3. Results and discussion

3.1. Chemical modification of lysozyme by phenylglyoxal

Lysozyme was chosen as a candidate for this study, owing to its positive nature contributed by eleven arginine residues [49]. In fact, the guanidinium moiety of arginine is protonated over a wide range of pH. This behaviour was exploited, by selectively eliminating these positive charges in a controlled manner, to observe the selectivity of mixed mode resins. Specific elimination of positive charges by arginine was achieved by reacting with phenylglyoxal (PG) [43,44]. This protein modification technique has been mainly described to inactivate enzymes such as hydroxylase, hydroxybenzoate [45] or tryptophanase [46], suggesting that the modification of arginine residues in binding sites of the enzyme disrupts function. Therefore, many experiments highlighting the change in the reactivity of enzymes and loss of attachment of coenzymes in the presence of phenylglyoxal have demonstrated the involvement of arginines [47,50–56]. Although we found some modifications of hyper-reactive lysines and terminal amines in rare instances (when very high concentrations of phenylglyoxal were used over prolonged reaction times), this modification was largely specific to the guanidium group in arginine residues.

3.2. Interaction of phenylglyoxal-modified lysozyme with HEA and PPA HyperCel

We prepared different populations of modified lysozyme under different conditions (by altering reaction time, temperature) to vary the number of modified arginine residues. The modified samples were then subjected to mixed mode chromatography and their behaviours were followed in terms of adsorption and retention times. The different fractions from each chromatographic run were analyzed by mass spectrometry to determine the exact number of phenylglyoxal moieties on lysozyme, which in turn correlates to the number of modified arginines.

As anticipated, unmodified lysozyme rapidly exited without any interactions on HEA or PPA HyperCel resins at pH 8 in the absence of salt. However, one population of phenylglyoxal-modified lysozyme

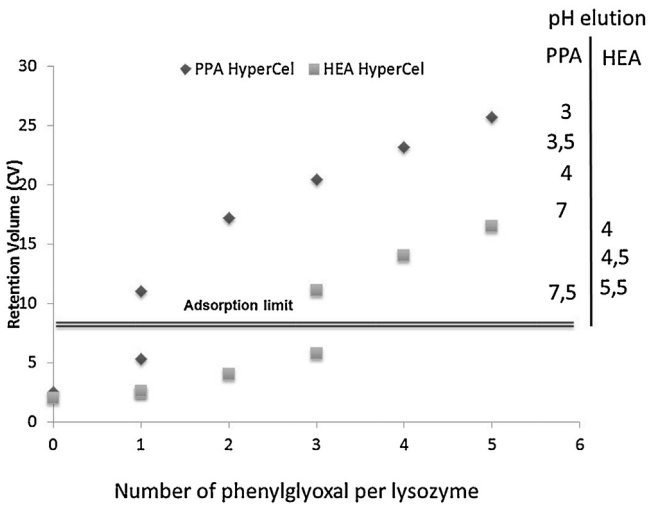

Fig. 2. Influence of chemical modification of lysozyme upon interaction with HEA and PPA HyperCel™ mixed-mode chromatographic supports. 2 ml of modified lysozyme (desalted after reaction with phenylglyoxal) was injected over 4 ml of HEA or PPA HyperCel™ packed in a Tricorn 10/50 column equilibrated with 50 mM Tris–HCl, pH 8.0 at a flow rate of 1 ml/min. Elution was achieved by a gradient with 50 mM phosphate-citrate buffer, pH 3.0 over 20 column volumes. The fractions obtained were analyzed by mass spectrometry.

exhibited a delayed retention time of more than 5 CV. Analysis by mass spectrometry revealed the presence of a one molecule of phenylglyoxal in this fraction of lysozyme (Fig. 2). This led to the conclusion that the modification of even a single charge led to changes in retention of the protein on the mixed mode resins, highlighting their condition-dependent selectivity.

The remaining of modified lysozyme was bound to the mixed mode resins and required a reduction in pH in order to be eluted. Mass spectrometric analysis of this fraction revealed the presence of larger number of modified arginine residues. Hence it was evident that modification of arginines allowed the adsorption of lysozyme on the HEA and PPA HyperCel resins at pH 8 under low ionic strength. By varying the reaction conditions, we were able to vary the number of molecules of phenylglyoxal fixed per protein molecule. In our experiments, we observed a number of peaks delayed and/or eluted at different pH conditions. We were able to determine and compare retention times under different conditions as the mixed-mode chromatographic experiments had been carried out using reproducible methods. From the mass spectrometry analysis, we were able to correlate retention time to the number of phenylgloxal fixed. We show in Fig. 2 the increase in protein retention as a function of the amount of phenylglyoxal attached to lysozyme. After a certain number of phenylglyoxal moieties were bound to lysozyme, viz., number of modified arginine residues, retention became adsorption and it was necessary to use a pH gradient to desorb the modified proteins (adsorption limit highlighted in Fig. 2). Elution was achieved by application of a linear gradient (over 20 column volumes) with 50 mM sodium phosphate citrate buffer, pH 3 – resulting in a convex gradient with pH decreasing from 8 to 4 in the first 5 CV, and from 4 to 3 in the next 13 CV. Fig. 2 also correlates the drop in pH in terms of column volumes.

The retention also differed between the two mixed mode resins. The strength of retention with increasing number of Phenylglyoxal moieties increased faster for PPA HyperCel™ in comparison to HEA HyperCel™. Likewise, the number of charges that needed to be eliminated per protein molecule to ensure adsorption on PPA HyperCel™ was lower than for HEA HyperCel™. This is in agreement with previous reports showing that interaction on PPA HyperCel is stronger than on HEA HyperCel™. The surface charge on the protein in combination with the hydrophobic effect is

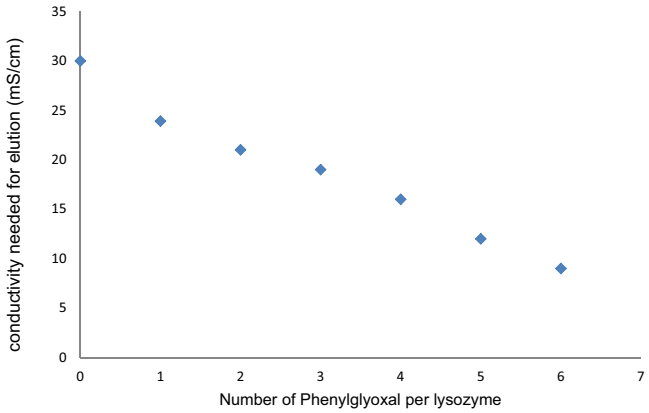

Fig. 3. Influence of phenylglyoxal on the surface charges of lysozyme and its adsorption on cation exchange resin. 2 ml samples from various fractions of mixed-mode experiments were injected on a 1 ml Resource S cation exchange column equilibrated with 50 mM sodium acetate buffer, pH 6.0 at a flow rate of 4 ml/min. Elution was achieved by using a linear gradient with the equilibration buffer containing 1 M NaCl.

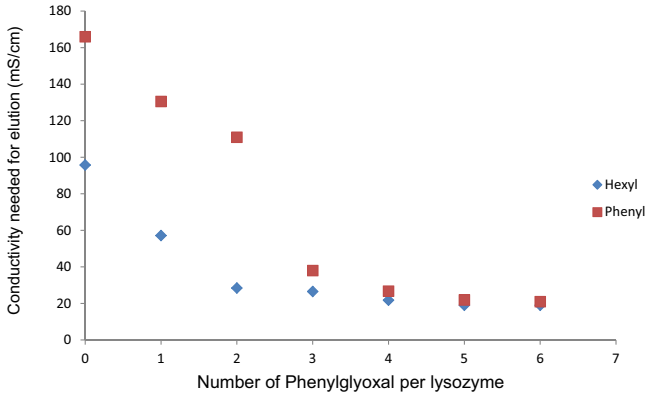

Fig. 4. Influence of phenyl glyoxal on the lysozyme hydrophobicity and its adsorption on hydrophobic interaction chromatography resins. 4 ml samples from various fractions of mixed-mode experiments (2 ml sample diluted with equal volume of 20 mM sodium phosphate buffer, pH 7.0 containing 3 M ammonium sulphate) were injected on 1 ml Hexyl or Phenyl Toyopearl resins packed in Tricorn 5/50 columns equilibrated with 20 mM sodium phosphate buffer, pH 7.0 containing 1.5 M ammonium sulphate at a flow rate of 1 ml/min. Elution was achieved by using a linear gradient of the equilibration buffer without salt.

extremely important in the mechanism of separation, and defines selectivity of the resins. The retention observed with lysozyme at basic pH conditions seems to be well supported by the presence of local positive charges near the binding site disturbing interaction, and modification of these charges in turn promote adsorption.

3.3. Evaluation of the interactions of phenylglyoxal-modified lysozyme on cation exchange and hydrophobic interaction chromatography

In order to understand the differences in retention/adsorption exhibited by different populations of phenylglyoxal-modified lysozyme on mixed-mode resins, we sought to see if this changed binding was due to the reduction in charge and/or the increase in hydrophobicity. Each eluted fraction from the HEA and PPA HyperCel™ experiments (based on retention times) were subjected individually to conventional cation exchange chromatography and to conventional hydrophobic interaction chromatography using supports that mimic the ligand of HEA and PPA HyperCel™ resins.

3.3.1. Phenylglyoxal-modified lysozyme and cation exchange chromatography

In order to demonstrate the change in the charge distribution on the surface of lysozyme, we subjected eluted fractions from HEA and PPA HyperCel™ experiments to conventional cation exchange chromatography. Each eluted fraction from the mixed mode experiment (containing different number of modified arginines) was individually subjected to cation exchange chromatography, after which the bound proteins were eluted by a linear salt gradient.

We showed that with greater number of phenylglyoxal groups, lesser salt was required to elute the modified protein from the cation exchange column (Fig. 3). The reduction in salt needed to elute the modified lysozyme confirms the diminution of electrostatic interaction between the support and proteins. The reduction in surface charges upon treatment with phenylglyoxal was evident from these experiments.

3.3.2. Phenylglyoxal-modified lysozyme and hydrophobic interaction chromatography

The addition of phenylglyoxal moieties to proteins introduces aromatic rings, which in turn could contribute to hydrophobic interactions (π–π). In order to verify if the altered retention in mixed-mode resins was also contributed by changes in hydrophobicity of lysozyme, we subjected each of the eluted fractions to conventional hydrophobic interaction chromatography (HIC). The HIC ligands chosen for this study were Hexyl and Phenyl resins, which mimic the mixed mode ligands HEA and PPA HyperCel™ respectively.

Under normal conditions, lysozyme, which is a relatively hydrophilic molecule, requires substantial salt concentrations (1.5 M ammonium sulfate) to adsorb on to hydrophobic interaction resins. The elution of the native protein was obtained by setting up decreasing linear gradient of salts. We observed that with increasing number of phenylglyoxals, the ionic strength required for the elution of the protein decreases (Fig. 4). This irrefutably established that the surface hydrophobicity of the lysozyme increased upon addition of phenylglyoxals, and this increase in hydrophobicity contributed significantly to binding to hexyl and phenyl HIC supports.

With these additional studies, we were able to establish that the interactions of phenylglyoxal-modified lysozyme on mixed mode HEA and PPA HyperCel™ chromatographic resins have been favoured by two factors: the reduction in repulsive positive charges on the surface by arginine residues; and by the increase in hydrophobicity of the protein surface.

4. Conclusion

In this study on the evaluation of mixed mode interactions using PPA and HEA HyperCel™ chromatographic media, phenylglyoxal was used to modify lysozyme – both by reducing the number of positive charge exposed at the surface of the protein (elimination of arginine's charge) and increasing local surface hydrophobicity where the charge was removed.

The balance between the different interactions, particularly between the hydrophobic and ionic effects (attraction and/or repulsion), is the keystone of separation in mixed mode chromatography. The distribution of these interactions is directly dependent on the physico-chemical environment posed under given chromatographic conditions [57,58]. We show here that the adsorption behaviour of lysozyme on HEA and PPA HyperCel™ mixed mode sorbents varies, depending on the degree of charge modification at the surface of the protein. While native lysozyme is not retained on HEA and PPA HyperCel™ resins at pH 8 without salt, attributed to the large number of arginine residues in its surface, the removal of even a single positive charge at the surface of the protein

significantly increased retention on the mixed mode resins. Experiments using conventional cation exchange and hydrophobic interaction chromatography confirm that both charge and hydrophobicity modification occured at the surface of the phenylglyoxal-modified lysozyme, and that this modification contributed to the changed binding to mixed-mode resins. These results obtained using HEA and PPA HyperCel™ sorbents strongly highlight the potential of mixed mode chromatography in separating closely related proteins with just minor surface charge and/or hydrophobicity differences.

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