PALL Life Sciences

Characterizing the Separation Properties of Two New Mixed-Mode Sorbents

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INTRODUCTION

Mixed mode chromatography offers new selectivities compared to conventional techniques such as ion exchange and hydrophobic interaction. New mixed-mode chromatography sorbents combine in a single tool the ability to exploit both the ionic and hydrophobic characteristics of a protein, overcoming the salt issue.

HEA and PPA HyperCel™ sorbents carry mixed-mode synthetic ligands, immobilized on a robust and scalable cross-linked cellulose matrix (HyperCel) that confers high porosity, chemical stability and low non-specific binding. The ligands include aliphatic (HEA – hexylamine) and aromatic (PPA – phenylpropylamine) amines. The aromatic group on PPA HyperCel sorbent confers an enhanced hydrophobicity compared to HEA HyperCel sorbent.

A panel of proteins of different isoelectric points (pI) and hydrophobicity (GRAVY[1] index) was used at different pH and ionic strength to characterize the interaction mechanisms with HEA and PPA HyperCel sorbents. The mixed-mode sorbents were also used to purify industrially relevant proteins. Their unique selectivity, illustrated by the separation of protein or peptide isoforms, was demonstrated.

CHARACTERIZATION OF INTERACTIONS WITH MODEL PROTEINS

Four different proteins with different pI and hydrophobicity were used to characterize the interactions between the mixed-mode sorbents and proteins:

Acidic proteins: bovine serum albumin (BSA, pI = 4.7, GRAVY[1] = -0.433) and ovalbumin (pI = 4.6, GRAVY = -0.008). Basic proteins: α-chymotrypsinogen (α-chymo, pI = 8.5, GRAVY = +0.051) and lysozyme (pI = 9.1, GRAVY = -0.314).

*(1) **GRAVY** = **GR**and **AV**erage of h**Y**drophobicity - Gasteiger, E., et al, Protein Identification and Analysis Tools on the ExPASy Server; John M. Walker Ed.: The Proteomics Protocols Handbook, Humana Press (2005) 571-607.*

Method used

- Salt and pH influence: α-chymo in different pH and salt conditions (Figure 1).
- Protein separation: capture of a four-protein mixture in PBS, pH 7.4. Elution by pH step and gradient (Figure 2).

Results

- **Influence of salt and pH on binding of α-chymo on HEA and PPA HyperCel sorbents** (Figure 1):
  - Limited or no salt (presence or not) influence on binding
  - Limited binding at acidic pHs
- **Separation of protein mixture** (Figure 2):
  - Only acidic (BSA and ovalbumin on both sorbents) or hydrophobic basic (α-chymo on PPA HyperCel sorbent) protein bound in PBS, pH 7.4.
  - Basic proteins (α-chymo on PPA HyperCel sorbent) are eluted first, followed by acidic ones (BSA and ovalbumin).

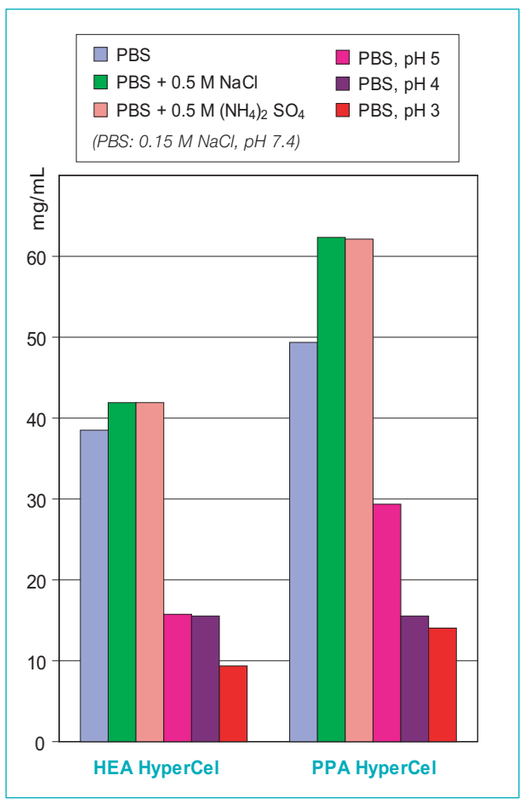

Figure 1. Influence of salt and pH on binding of α-chymo on HEA and PPA HyperCel sorbents.

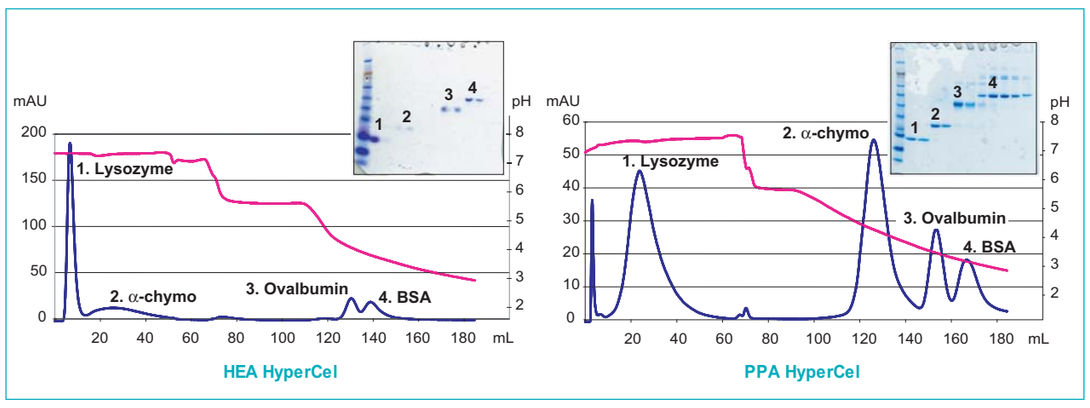

Figure 2. Separation of a protein mixture on HEA and PPA HyperCel sorbents. Load: PBS, pH 7.4, step elution at pH 5.4, then pH gradient from 5.4 to 2.6.

- **Binding relies more on hydrophobic than on electrostatic interactions.**
- **Different interaction compared to standard IEX or HIC sorbents.**
- **Electrostatic repulsion between positive charges on protein and sorbents for elution** (Figure 3).

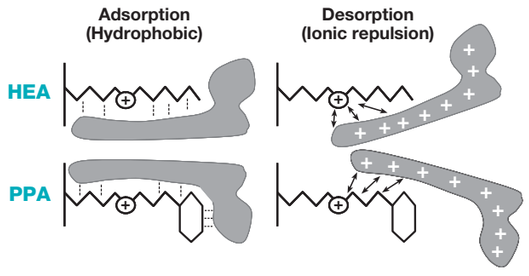

Figure 3. Adsorption and desorption mechanism on HEA and PPA HyperCel mixed-mode sorbents.

SEPARATION OF RECOMBINANT CHEMOKINE ISOFORMS

PPA HyperCel sorbent was used for the purification of a chemokine (cytokine) from a PEAK cell culture supernatant with 10 % fetal calf serum *(courtesy of NovImmune)*.

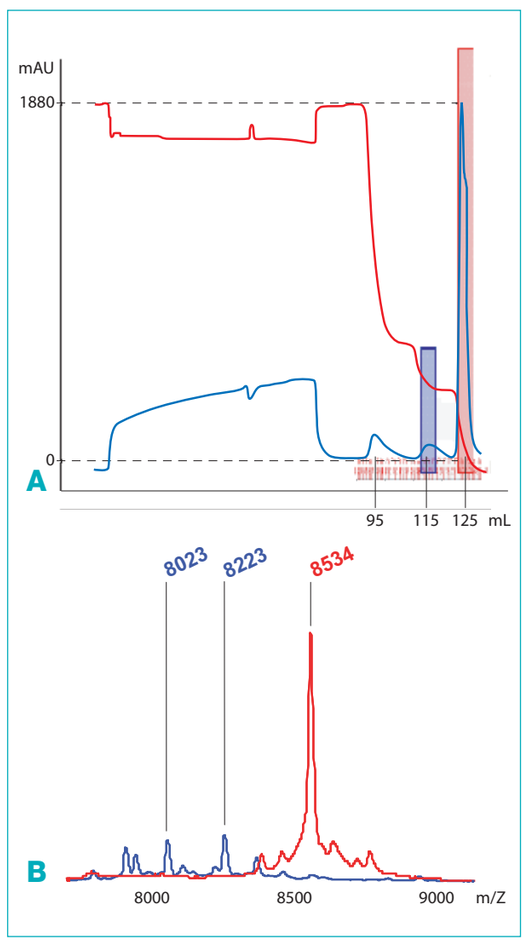

Figure 4. **A** = Separation of chemokine isoforms (MW 8.0 – 8.2 kDa and 8.5 kDa) by a pH gradient on PPA HyperCel sorbent. **B** = SELDI analysis of the fractions eluted at **115 mL (blue)** and **125 mL (red)**.

Method used

- Load in PBS, pH7.4 on PPA HyperCel sorbent. Elution at pH 5.0, 4.0 and 2.6.
- Analysis by ELISA (test developed at NovImmune) and SELDI-MS.

Results

- Fractions eluted at 95, 115 and 125 mL (Figure **4A**) were analysed on ELISA (not shown) and SELDI-MS (Figure **4B**).
- ELISA: Fractions 115 mL and 125 mL positive (higher quantity for 125 mL).
- SELDI-MS analysis: Chemokine in fractions 115 mL and 125 mL but presence of molecules of different molecular weight: 8.0 – 8.2 kDa in fraction 115; 8.5 kDa in fraction 125.

- **PPA HyperCel sorbent can separate chemokine isoforms of minor difference.**
- **Mixed-mode sorbents can differentiate closely related molecules, which may not be done by ion exchange (IEX) or hydrophobic interaction chromatography (HIC).**

PURIFICATION OF RECOMBINANT F(ab')$_2$ FRAGMENT

HEA HyperCel sorbent was used as a capture chromatography step to purify a recombinant F(ab')$_2$ fragment obtained through baculovirus expression in insect cells (SF9).

Method used

- Load on HEA HyperCel sorbent at pH 6, pH 4 and pH 2.
- Analysis by SDS-PAGE, ELISA and BCA assays.

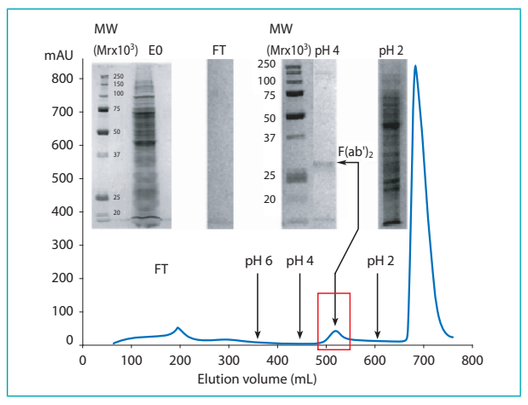

Figure 5. Purification of F(ab')$_2$ fragment using HEA HyperCel sorbent. The fractions identified on the chromatogram during load and elution were analysed using ELISA (not shown) and SDS-PAGE to identify and quantify the F(ab')$_2$.

Results

- No protein in the flowthrough or elution at pH 6.
- F(ab')$_2$ fragment eluted at pH 4 while HCPs eluted at pH 2.
- F(ab')$_2$ fragment recovered at 82 % and with a 39-fold purification factor (ELISA analysis, data not shown).

HEA HyperCel sorbent is very efficient to selectively isolate industrially relevant proteins such as recombinant F(ab) $_2$ fragments expressed in insect cells, while maintaining a good yield.

CONCLUSION

- **HEA and PPA HyperCel mixed-mode sorbents provide different selectivities compared to existing chromatography techniques and offer new options to purify industrially relevant proteins.**
- **Proteins and peptides with only minor differences can be discriminated (e.g., isoforms).**

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