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Simplifying Progress

Modulating and Understanding Retention of Proteins on Chromatographic Support by Changing Cation-Exchanging Ligand

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1. Introduction

Ion-exchange chromatography is a key chromatographic technique for the separation and purification of large biomolecules; however, its performance strongly depends on the nature of the ligand and the underlying support matrix. Our research investigates the effects of sulfonate (CIM® SO3) and multimodal cation-exchange sulphate (CIM SO4) ligands on the chromatographic separation of two basic proteins: equine cytochrome c (Cyt) and chicken egg-white lysozyme (Lys).

When performing protein separation under an ascending salt gradient, the elution of both Cyt and Lys were dramatically shifted to higher NaCl on sulfate ligand. The effect was more pronounced for Lys despite their comparable molecular sizes and isoelectric points (pI). Since Lys is reported to be slightly more hydrophobic than Cyt, we hypothesize that CIM SO4, due to reported higher hydrophobicity of SO4 groups, interacts with Lys stronger through additionally expressed "hydrophobic" interactions [1, 2].

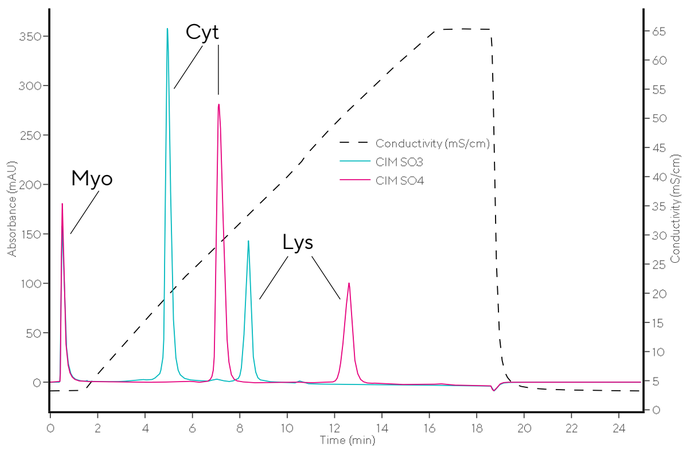


Figure 1: Separation of myoglobin (Myo), Cyt and Lys on CIM SO3 and on CIM SO4 columns in NaCl gradient.
Buffer A: 20mM phosphate, pH 7.5
Buffer B: 20mM phosphate, 0.7 M NaCl, pH=7.5
Detection: UV at 280 nm and conductivity
Method: Linear ascending NaCl gradient from 100% buffer A to 100% buffer B over 30 CV

2. Experimental Approach

The goal of this study was to describe the chromatographic differences by performing and interpreting thermodynamic analyses for Lys and Cyt adsorption at different binding conditions. Two complementary methods were used: (i) chromatographic analysis using the Van't Hoff equation, and (ii) isothermal titration calorimetry (ITC).

(i) Van't Hoff thermodynamic analysis of retention data

Protein	Cytochrome	Lysozyme
Column	CIM SO3 and CIM SO4	
Mode	isocratic	
Temp.	25, 30, 35, 40 °C	
Buffer	20 mM phosphate, 0.15 M NaCl, pH 7.5	20 mM phosphate, 0.29 M NaCl, pH 7.5
Sample	0.60 mg/mL myoglobin, 0.75 mg/mL Cyt in the Buffer	0.70 mg/mL Lys in the Buffer

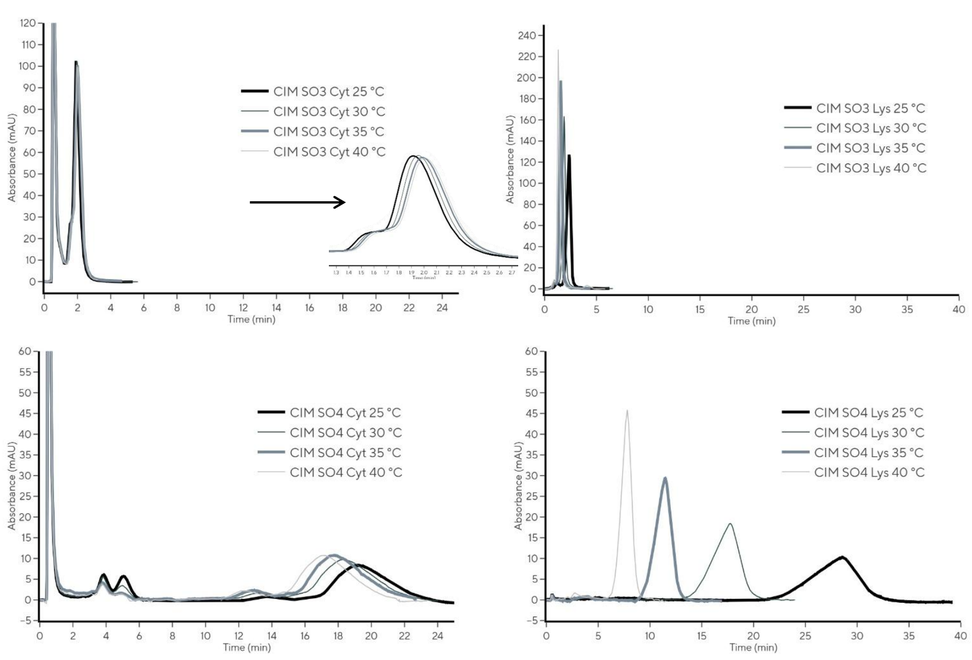


Figure 2: Chromatograms obtained at 25, 30, 35 and 40 °C injecting Myo (nonbinding tracer) with Cyt or Lys onto a CIM SO3 and CIM SO4 under isocratic chromatographic conditions.

The Van't Hoff equation enables the calculation of thermodynamic binding parameters directly from the retention time of the protein:

$$\ln k' = a + \frac{b}{T} + \frac{c}{T^2} + \ln \varphi$$

$$\Delta H^\circ = -R\left(b + \frac{2c}{T}\right)$$

$$\Delta S^\circ = R\left(a - \frac{c}{T^2}\right)$$

$$\Delta C_p^\circ = \frac{2Rc}{T^2}$$

Where k' is the retention factor, T the absolute temperature and φ the phase ratio, a, b and c represent empirical coefficients, which were used to calculate the termodynamic parameters [3].

(ii) Isothermal titration calorimetry (ITC)

ITC experiments were performed using a 1 mL sample cell containing a slurry of the ground monolithic stationary phase, while the 250 µL syringe was filled with protein solution (5 mg/mL Lys in 20 mM phosphate, 0.10 M NaCl, pH 7.5). Titrations were carried out at 25 °C. For each measurement, 25 injections of 10 µL were performed under continuous stirring.

The power (heat rate) required to compensate for the heat released or absorbed upon each injection of protein into the ligand solution was recorded and integrated (raw signal shown in Figure 3). The resulting heats (Figure 4) were analyzed using Wiseman fitting to determine the thermodynamic binding parameters.

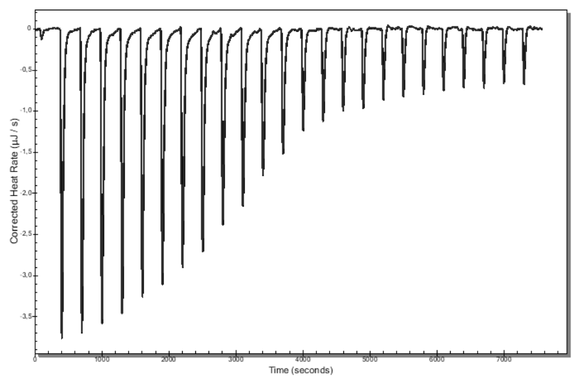


Figure 3: Raw ITC signal obtained for titration of Lys sample solution into 5 % CIM SO3 slurry at 25 °C

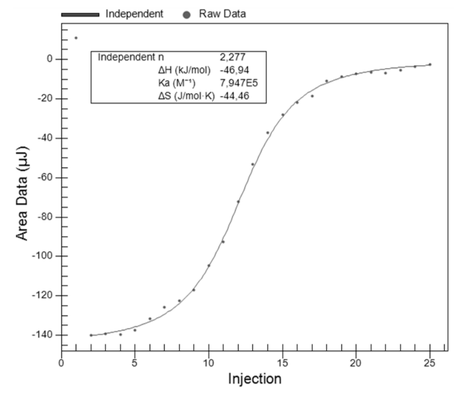
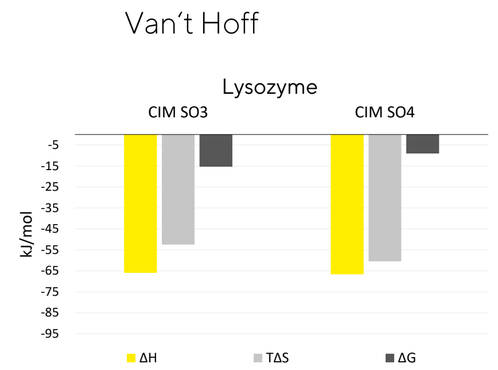
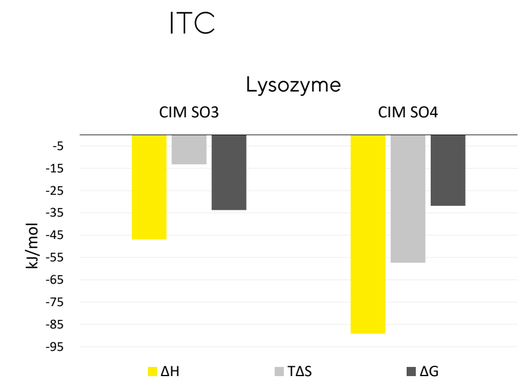


Figure 4: Tiration curve for CIM SO3 slurry

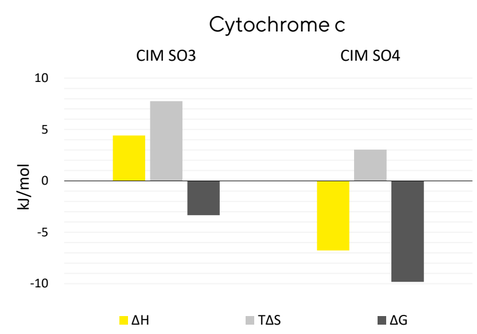
3. Results



Buffer: 20 mM phosphate, 0.29 M NaCl, pH 7.5, T = 25 °C



Buffer: 20 mM phosphate, 0.1 M NaCl, pH 7.5, T = 25 °C



Buffer: 20 mM phosphate, 0.15 M NaCl, pH 7.5, T = 25 °C

Lys	Cyt
CIM SO3 and SO4: ▪ enthalpy-driven process ▪ Strongly exothermic process, dominated by electrostatic interactions ▪ negative ΔS values → hydrogen bonds formation and a decrease in conformational freedom upon adsorption.	CIM SO3: ▪ entropy-favorable process ▪ endothermic process + high positive ΔS → disruption of hydrogen bonds and release of bound water molecules CIM SO4: ▪ exothermic process + positive ΔS → ionic interaction + dehydration

Van't Hoff vs ITC analysis of Lys adsorption

- Both Van't Hoff and ITC analyses confirmed enthalpy-driven lysozyme binding to both cation-exchange monoliths, indicating the domination of attractive electrostatic and hydrogen-bonding forces .
- The Van't Hoff analysis revealed only minor differences between the two ligands, with slightly stronger enthalpic contribution observed for the multimodal CIM SO4 ligand, while ITC clearly discriminate between the two exchangers. Possible reason 1: Due to its indirect nature and the assumption of ideal retention behavior, the Van't Hoff approach provides a less sensitive differentiation between binding modes. Possible reason 2: The two methods were performed at different salt concentrations (ITC at 0.1 M NaCl, Van't Hoff at 0.29 M), affecting the mechanism of adsorption.
- ITC data reveal that the stronger binding on the multimodal SO_4^- ligand originates from intense ionic interactions and specific hydrogen bonding between the arginine residues of lysozyme and the negatively charged sulfate groups. The difference in total heat release between SO_3^- and SO_4^- ligands reflects the additional stabilizing interactions provided by the multimodal ligand.

4. Conclusion

- Under identical isocratic conditions, Lys showed a 12-times higher retention time using CIM SO4 compared to CIM SO3.
- Van't Hoff thermodynamic analysis revealed no major differences between the exchangers for Lys, whereas Cyt exhibited a distinct adsorption mechanism depending on the ligand type.
- ITC clearly differentiated the ion exchangers: CIM SO3 demonstrated a more entropy-driven adsorption, while CIM SO4 showed a higher negative TΔS, indicating a more enthalpy-dominated interaction. Based on these unexpected observations, we propose that protein adsorption is influenced not only by the ligand chemistry, but that the backbone of the matrix (polymethacrylate polymer) itself plays an equally important role in determining the binding mechanism.

5. References

[1] J. C. Simoes-Cardoso et al. Langmuir 2020, 36, 3336–3345. https://doi.org/10.1021/acs.langmuir.0c00197
[2] Begić, M. Et al. Biotechnol. J. 2021, 16, e2100100. https://doi.org/10.1002/biot.202100100
[3] F.S. Marquesa et al. Colloids and Surfaces B: Biointerfaces 2014, 122, 801–807. https://doi.org/10.1016/j.colsurfb.2014.08.024