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# Surface charge distribution: a key parameter for understanding protein behavior in chromatographic processes

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## abstract

Multi-component adsorption of proteins still requires a better understanding of local phenomena to improve the development of predictive models. In this work, all-atom Molecular Dynamics (MD) simulations were used to investigate the influence of protein charge distribution on the adsorption capacity. The simultaneous adsorption of  $\alpha$ -chymotrypsin and lysozyme on a cation exchanger, SP Sepharose FF, was studied through MD simulations and compared to macroscopic isotherm experiments. It appears that the charge distribution is a relevant information to better understand specific phenomena, such as a multilayer adsorption caused by the particular electrostatic profile of  $\alpha$ -chymotrypsin. Therefore, MD simulations seem to be an interesting way to visualize and highlight these behaviors.

of the main drawback of these methods is that it does not provide information on the distribution of charges around the protein surface [12]. Indeed, an

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## 1. Introduction

Ion-exchange chromatography is one of the most used process for protein purification [1–3]. However, even though this process is effective for separating simple media, their optimization may remain difficult when complex media with multiple proteins need to be purified [4,5]. While most industrial processes are still based on empirical models, phenomena such as interactions and competition between proteins still require a better understanding at molecular level.

The adsorption equilibrium of macromolecules on a porous media is usually described by empirical or semi-empirical models such as Langmuir isotherm [6,7], the distributed pore model [8] or the Steric Mass Action (SMA) law [9,10] with varying degrees of efficiency [4,11,12]. Those models can be useful to predict the salt effect [12] but, meanwhile, can show unreliable results at high protein concentrations for instance [4]. Nowadays, SMA law seems to be one of the most widely used in the specific case of ion-exchange [13] as it considers the displacement of counterions in solution, and is applied to macromolecules such as proteins and accounts for the multipoint nature of the interaction [14] through the value of a characteristic charge. However, it appears that one

heterogeneous distribution may affect interactions with the environment, in particular with surrounding proteins, and then change adsorption behaviors [15]. Therefore, most models used in ion-exchange chromatography may fail to describe multicomponent adsorption based on single-component observations [4,12]. A coupling of different models or methods appears to be an interesting strategy, such as Poisson-Boltzmann calculations [16] coupled with stoichiometric models to better predict electrostatic effects [17]. Finally, a better understanding of mechanisms involved in multicomponent adsorption, and especially interaction mechanisms between two proteins, could help in the development of predictive models [18] and more generally in the understanding of intermolecular interactions in various processes such as complex coacervation [19].

This preliminary work aims to highlight the potential of Molecular Dynamics (MD) simulations as a theoretical method to improve knowledge on simultaneous adsorption of two proteins. In particular, the effect of protein charge distribution on their adsorption is analyzed. For this reason, simultaneous retention of  $\alpha$ -chymotrypsin and lysozyme on a chromatographic surface, SP Sepharose FF, has been studied. First, an experimental study was conducted, involving adsorption isotherm measurements. In parallel, MD simulations were performed and then compared to observations drawn from experiments, showing the interest of considering the charge distribution instead of the net charge to better understand ion-exchange behavior.

## 2. Materials and Methods

### 2.1. Charge distribution on protein surface

Protonation states of titrable residues at different pH were determined using

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PROPKA from PDB2PQR web server [20]. The APBS (Adaptive Poisson-Boltzmann Solver) tool [21,22] was used to determine the charge distribution on protein surfaces and all surface representation figures were obtained using PyMol 1.7 [23].

## 2.2. Protein adsorption

**2.2.1. Multicomponent isotherm experiments**  $\alpha$ -chymotrypsin (C-4129, purity  $\geq 85\%$ ) and lysozyme (L-6876,  $\geq 90\%$ ) were purchased from Sigma (St. Louis, MO, USA) and used without prior purification. The salts used for buffer solutions (citric acid, trisodium citrate dihydrate and sodium chloride) were also obtained from Sigma and were of analytical grade. The ion-exchange resin, SP Sepharose FF, was obtained from GE Healthcare (Uppsala, Sweden).

Multicomponent isotherms were measured using static batch experiments at  $T = 20^\circ\text{C}$ ,  $\text{pH} = 5$  (50 mM citrate buffer) and without sodium chloride in solution [24]. The resin was first equilibrated with the buffer solution. A known amount of drained resin, ranging from 0.01 to 0.85 g, was then transferred into several flasks containing 5 mL of protein solution. The initial concentration of each protein  $C_{0,i}$  was set to about 2 g/L. The sealed flasks were shaken during at least 8 hours in order to reach the equilibrium state. The supernatant was then collected and analyzed. Protein concentrations  $C_i$  were determined using HPLCSEC (size-exclusion chromatography) according to the protocol described in [24]. Finally, the amount of adsorbed proteins  $q_i$  was calculated by mass balance, following the equation:

$$q_i = (C_{0,i} - C_i) \frac{V}{V_R} \quad (1)$$

where  $V$  is the protein solution volume,  $V_R$  the resin volume and  $i$  the protein.

### 2.2.2. Molecular dynamics simulations

The system setup for *in silico* experiments and data analysis methods are the same as described in the previous work [24]. In addition to  $\alpha$ -chymotrypsin (PDB-ID: 1YPH, resolution 1.34 Å), lysozyme was added in the simulation box at the beginning of each simulation. The atomic coordinates of lysozyme were obtained from the X-ray structure (PDB-ID: 1AKI) at a resolution of 1.5 Å. This protein is composed of one chain, containing 129 residues.

Three initial configurations were studied (Figure SI.1 in Supplementary Information) in order to observe the influence of the starting orientation on the adsorption. First, two proteins were introduced in the simulation box with two different initial orientations (Figures SI.1.A and B). Both proteins are rotated at  $180^\circ$  on the y-axis from Configuration 1 (Figure SI.1.A) to obtain Configuration 2 (Figure SI.1.B). Then, four proteins, i.e. two lysozymes and two  $\alpha$ -chymotrypsins, were introduced in the simulation box: one protein of each was already adsorbed on the surface at the beginning of the simulation and two other proteins were added in the simulation box (Figure SI.1.C). Thus, the effect of adsorbed proteins on the adsorption capacity can be highlighted. MD time length was set to 200 ns for all simulations.

## 3. Results and Discussion

### 3.1. Charge distribution

Figure 1 represents the charge distribution on protein surfaces from APBS calculations. At pH 5 (Fig. 1.A and 1.B), both proteins are globally positively charged, with a net charge equal to +7 for  $\alpha$ -chymotrypsin and +10 for lysozyme. Fig. 1.C and 1.D show the electrostatic potential at protein surfaces when the pH is close to the isoelectric point  $pI$ , i.e. pH 9 for  $\alpha$ -chymotrypsin ( $pI \approx 8.3$  [25]) and pH 11 for lysozyme ( $pI \approx 11.3$  [26]). Thus, the net charges are estimated at +0 for both proteins.

This representation allows to identify and visualize areas on the protein surface that may be involved in the ion-exchange mechanism and how the charges are distributed on the surface. At pH 5,  $\alpha$ -chymotrypsin shows a strongly positively charged region, already identified in previous work [24] to be the predominant adsorption patches. Despite some local neutral or negatively charged areas, the net protein charge is positive. As the working pH gets closer to  $pI$ , two distinct and opposite sides with positive and negative charges appear, resulting in a net neutral charge. However, it does not exclude the possibility that the protein may still adsorb to a cationic surface when considering only electrostatic effects. At pH 5, the charge repartition on lysozyme surface seems to be more evenly distributed with positive charges located on the whole surface. At pH close to  $pI$ , neutral, positive and negative charges appear homogeneously without forming two distinct zones. Nevertheless, variations such as protein

conformational changes may slightly influence the charge distribution on protein surface.

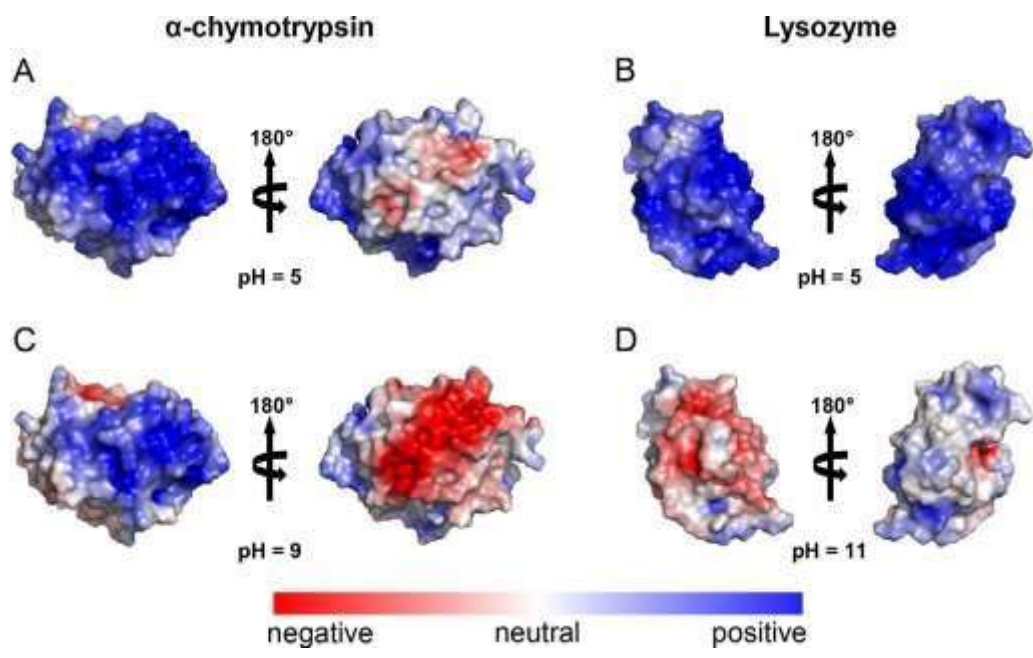
### 3.2. Adsorption behavior in multicomponent system

#### 3.2.1. Equilibrium isotherms

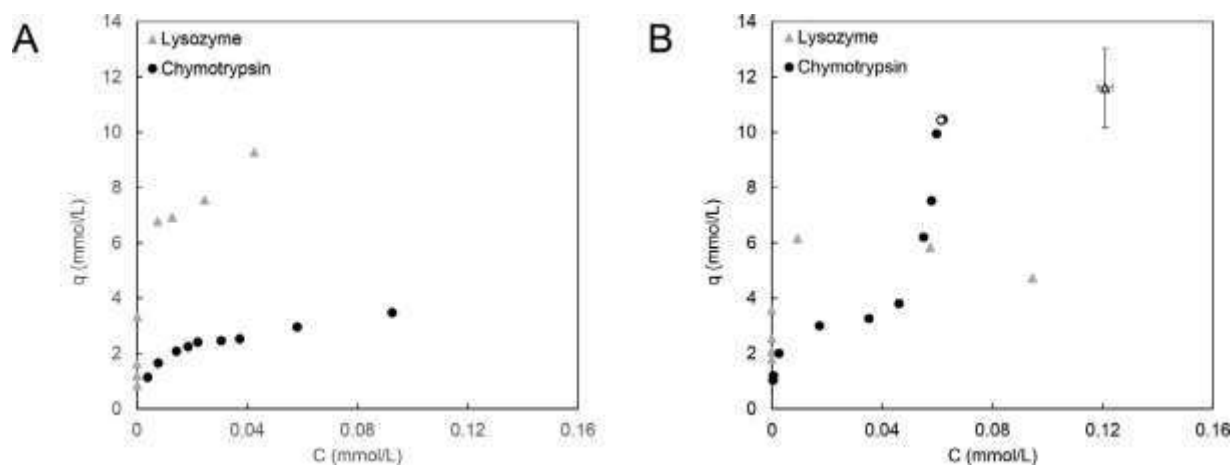
The adsorption isotherms, measured at pH 5 in a 100 mmol/L sodium solution, are presented on Figure 2. Both single-component (Figure 2.A) and multicomponent (Figure 2.B) isotherms are shown, in order to highlight the influence of protein competition on the adsorption efficiency.

At high concentrations in lysozyme single-component isotherm (Figure 2.A), data dispersion increases, which is mainly due to the uncertainties generated by the batch experiments. It appears that the concentration variations during the experiment are equivalent to these uncertainties. In addition, to obtain this data, a very small amount of resin have to be introduced in the tubes, thus increasing the uncertainty of this measurement. This isotherm seems to show a Langmuir-type behavior, which is consistent with results from Dismar et al [27] that studied lysozyme in similar conditions (SP Sepharose FF, pH 5). Lysozyme appears to have a stronger affinity with the resin than  $\alpha$ -chymotrypsin regarding the curve shape. This result is consistent with observations made in the previous paragraph 3.1. Indeed, the global charge of lysozyme is strongly positive and well distributed on the surface, which favors electrostatic interactions with the negatively charged resin. As showed in the previous work [24],  $\alpha$ -chymotrypsin isotherm does not show any atypical behavior in mono-component system.

In multi-component system (Figure 2.B), the  $\alpha$ -chymotrypsin isotherm has the shape of a stepwise curve: when the concentrations of both proteins in solution are high, i.e. from about 0.04 mmol/L, the shape of the isotherm changes significantly. This type of isotherms can be considered to reflect a multilayer adsorption [28]. However, the abrupt change in the slope of the  $\alpha$ -chymotrypsin isotherm is only the result of a strong disappearance of the protein in solution, because in the experiments only the concentration  $C$  is directly measured,  $q$  is calculated by mass balance. The assumption made during the calculation of  $q$  is that the quantity of protein no longer in solution is retained on the



**Fig. 1.**  $\alpha$ -chymotrypsin and lysozyme surface representations colored according to electrostatic potential (-5 keV, red ; +5 keV, blue), at pH = 5 (Figures A and B) and pH  $\approx$  pI (Figures C and D), which corresponds to pH 9 for  $\alpha$ -chymotrypsin and 11 for lysozyme.



**Fig. 2.** Adsorption isotherms of  $\alpha$ -chymotrypsin and lysozyme on SP Sepharose FF at pH 5, (50 mM sodium citrate buffer). A: Isotherms measured independently in single-component system and B isotherms measured in multi-component system (simultaneous adsorption). The empty symbols with standard deviations on Fig. B are averaged data measured in duplicate with high amount of resin, in order to confirm isotherm shape.

chromatographic support. Thus, this isotherm can reflect several phenomena such as an interaction between proteins, but also an aggregation or precipitation of  $\alpha$ -chymotrypsin, which would also explain a decrease in the amount of solubilized protein [28]. In solution, no precipitation of the protein was observed, but it is also possible that these phenomena could take place inside the resin, which in this case is difficult to identify experimentally. At this point, two observations are noted:

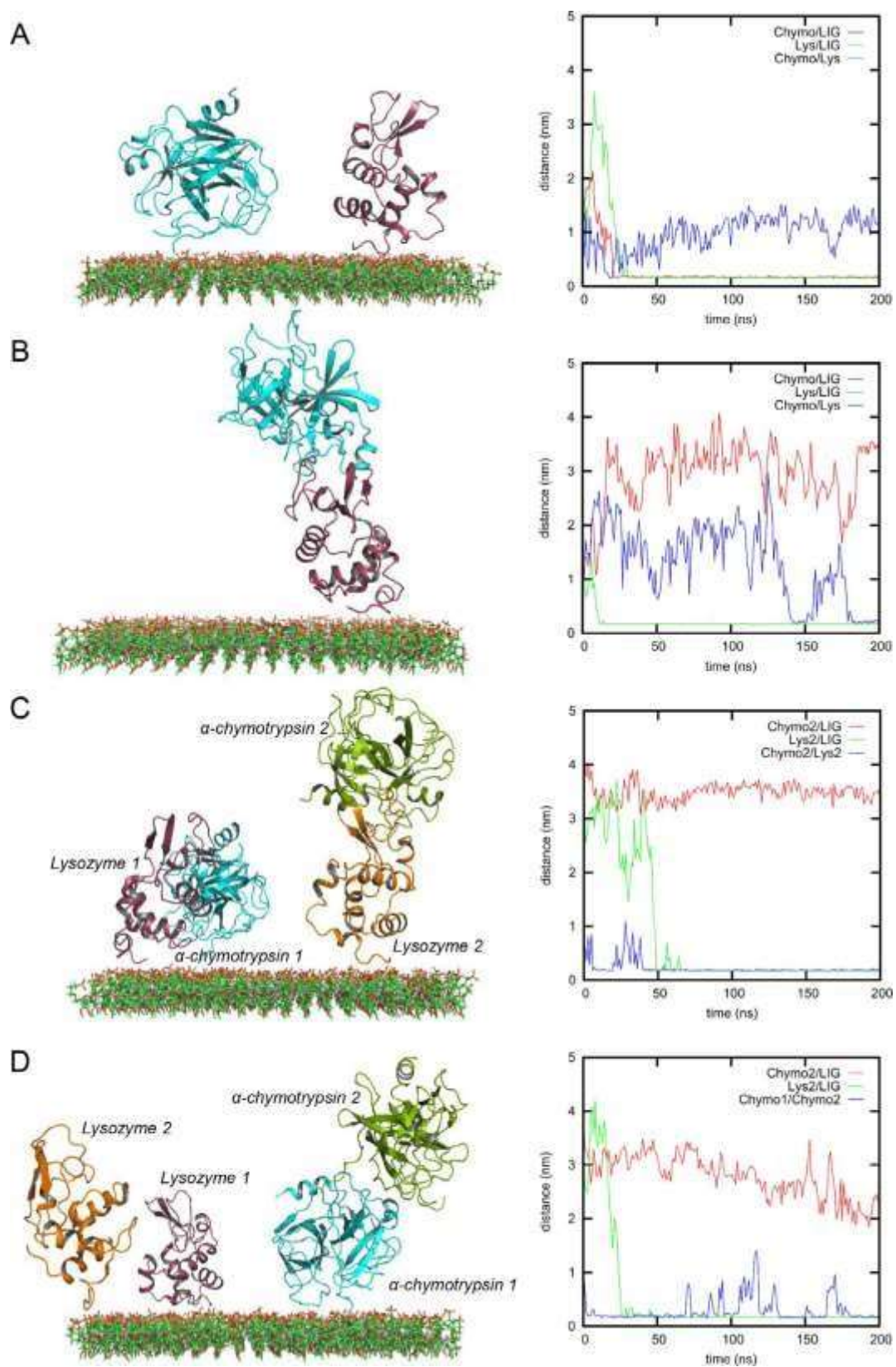
- The lysozyme seems to have a similar behavior in single and multicomponent systems showing Langmuir-type isotherms. Moreover, this protein shows a stronger affinity with the SP Sepharose FF resin than  $\alpha$ -chymotrypsin.
- The  $\alpha$ -chymotrypsin has a different behavior at high concentrations (over 0.04 mmol/L) in multi-protein system.

### 3.2.2. MD simulations

The adsorption isotherms performed on a multi-protein system highlighted two distinct situations. The first situation seems to show a weak competition

between proteins (concentrations below 0.04 mmol/L). During the simulation, this case was represented by the configurations with only two proteins in the box. Although there is still enough space on the chromatographic surface (around 65% available) for the two proteins to adsorb independently, these conditions still represent a competitive situation and may more likely represent a transition between competitive and non-competitive adsorption. In these conditions, four MD simulations were run and analyzed. The second situation highlighted by the experiments (protein concentrations higher than 0.04 mmol/L) traduces a high competitive adsorption. The configuration containing four proteins in the simulation box was then used to study a higher protein competition. In these conditions, the available surface area ratio is around 30%. Three MD simulations were run from this initial configuration.

Some representative results are presented on Figure 3, showing the last frame of each simulation as well as protein-protein and protein-ligands minimum distances over time (complete re-



**Fig. 3.** Last frames of four MD simulations and evolution of protein-protein and proteins-ligands minimum distance over time. A and B: MD simulations performed from initial configurations 1 and 2, with lysozyme in red and  $\alpha$ -chymotrypsin in cyan. C and D: MD simulations performed from initial configuration 3, with lysozyme in red or orange, and  $\alpha$ -chymotrypsin in cyan or green.

sults are available in Supplementary Information Figures SI.2 et SI.3). **Fig. 3 .A** and **B**, representing results from the *2-proteins* configuration, show that both proteins were adsorbed during the simulations. However, if the lysozyme was always adsorbed onto the chromatographic surface (green curves), the  $\alpha$ -chymotrypsin adsorbed on the surface (**Figure 3 .A**) and on the adsorbed lysozyme forming a multilayer (**Figure 3 .B**). The evolution of minimum distances over time seems to show that  $\alpha$ -chymotrypsin adsorption is hindered by the lysozyme electrostatic field. Indeed, when lysozyme is the first protein adsorbed,  $\alpha$ -chymotrypsin tends to adsorb in multilayer as suggested with the isotherms. Results corresponding to the *4-proteins* configuration are presented on **Figs. 3 .C** and **D**. In this situation of stronger competition between proteins,  $\alpha$ -chymotrypsin adsorbed on other proteins ( $\alpha$ -chymotrypsin or lysozyme) during each simulation.

In both conditions, a preferential adsorption of lysozyme on the chromatographic surface is observed and could be explained by the strong distribution of positive charges on its surface. Experimentally, lysozyme also has more affinity with the resin than the  $\alpha$ -chymotrypsin. Conversely,  $\alpha$ -chymotrypsin appears to be able to form a multilayer in both conditions: when the protein concentration is relatively low (two proteins in the simulation box), and at high concentration (four proteins in the simulation box). In this case, the adsorption of the second  $\alpha$ -chymotrypsin on another protein could be emphasized by the limited surface area available, caused by the placement of adsorbed proteins on the chromatographic surface. The heterogeneous charge distribution of  $\alpha$ -chymotrypsin, studied in paragraph 3.1, could explain its ability to interact with the positively charged surface of lysozyme and  $\alpha$ -chymotrypsin. Indeed, most of  $\alpha$ -chymotrypsin residues in interaction with lysozyme (see Table SI.1) are not located on the highly positively charged area on  $\alpha$ -chymotrypsin surface. As an example, Figure SI.4 shows electrostatic distribution of adsorbed proteins.

Finally and specially, in those situations where surface area available for adsorption is limited, MD simulations show that  $\alpha$ -chymotrypsin is able to form a multilayer, both with the lysozyme and with itself, which is possible due to the particular charge distribution on the protein surface. These results agree with the observations made at macroscopic scale.

#### 4. Conclusion

MD simulations were successfully used to explore molecular mechanisms involved during simultaneous adsorption of two proteins, showing a good agreement with macroscopic experiments. The charge distribution on protein surface appears to be a relevant information to understand phenomena that are difficult to predict with empirical or semi-empirical models such as the Steric Mass Action law. Indeed, according to both macroscopic experiments and MD simulations,  $\alpha$ -chymotrypsin adsorbs as a multilayer in competitive situation (high concentrations). Despite a positive net charge in the chosen working conditions,  $\alpha$ -chymotrypsin shows two distinct zones at its surface, one of them predominantly negative and involved in the interaction with lysozyme, or even with another  $\alpha$ -chymotrypsin.

However, it is important to notice that these simulations show the appearance of a multilayer phenomenon when the proteins are already close to the surface as a first result. More simulations will be conducted to better understand these interactions, especially at low concentration. Furthermore, in order to quantify the strength of protein-protein interactions, binding energies will be calculated and compared to protein-ligands interactions.

#### Declaration of Competing Interest

The authors declare that they have no competing interests.

#### CRediT authorship contribution statement

**Marine Tournois:** Investigation, Writing - original draft, Methodology, Software, Formal analysis, Validation. **Stéphane Mathé:** Supervision, Conceptualization. **Isabelle André:** Project administration, Supervision, Resources. **Jérémy Esque:** Methodology, Software, Writing - review & editing, Conceptualization. **María A. Fernández:** Project administration, Supervision, Resources, Conceptualization.

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#### Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi: [10.1016/j.chroma.2021.462151](https://doi.org/10.1016/j.chroma.2021.462151)

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