Influence of acid-induced conformational variability on protein separation in reversed phase high performance liquid chromatography

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Abstract

Influence of acid concentration in the mobile phase on protein separation was studied in a wide concentration range of using trifluoroacetic acid (TFA) and formic acid (FA). At low, 0.001-0.01 v/v% TFA concentration and appropriate solvent strength proteins elute before the column's dead time. This is explained by the proteins having a structured, but relatively extended conformation in the eluent; and are excluded from the pores of the stationary phase. Above ca. 0.01-0.05 v/v% TFA concentration proteins undergo further conformational change, leading to a compact, molten globule-like structure, likely stabilized by ion pairing. Proteins in this conformation enter the pores and are retained on the column. The results suggest ~~a novel, conformational exclusion~~ a pore exclusion induced ~~based~~ separation ~~mechanism,~~ related to protein conformation. This effect is influenced by the pH and type of acid used, and is likely to involve ion-pair formation. The TFA concentration needed to result in protein folding (and therefore to observe retention on the column) depends on the protein; and therefore can be utilized to ~~dramatically~~ improve chromatographic performance. Conformation change was monitored by circular dichroism spectroscopy and mass spectrometry; and it was shown that not only TFA, but FA can also induce molten globule formation.

Keywords

TFA-protein adduct, protein conformation, RP-HPLC, protein separation mechanism

Highlights

- Unusual, ion-pairing related RP-chromatographic behavior of proteins was observed
- Ion-pairing induced conformation change influences protein elution
- This finding could be utilized in separation of proteins

1. Introduction

Reversed phase chromatography of biological macromolecules is a promising separation technique in the pharmaceutical field [1,2], for isolation [3,4], in proteomics [5-7], and in various related areas [8-12]. In the separation both the stationary phase and mobile phase play an important role. Recently, Fekete et al. published a review on key parameters of the stationary and mobile phases [2]. In the chromatography of proteins and peptides the type and concentration of ion pairing agents in the mobile phase also have an important role [2,13-19]. Biological macromolecules are typically separated in acidic conditions. Below the pI of a protein or peptide, basic amino acid residues (lysine, arginine, histidine, and also the N-terminus) will become protonated, which may form ion pairs with anions present in the mobile phase. Ion pairing increases hydrophobicity of the protein, which changes the interaction with the reversed phase [20,21]. Trifluoroacetic acid (TFA) is a widely used ion pairing additive, but its effect on protein structure and the retention mechanism is not clearly understood.

Several peptide and protein specific retention mechanisms have been suggested in the literature. Guiochon [22] mentions two mechanisms, one related to hydrophobic, the other to ion-exchange interactions. Geng and Regnier [23] assumes that the three-dimensional structure of a protein is a major factor affecting protein separation. In the present manuscript we have studied these mechanistic aspects in some detail.

Acid titration of proteins helps to understand protein retention in RP-HPLC. It was found that proteins may unfold to an open conformation when titrated with strong acids [24], losing their tertiary structure. This behavior is related to repulsive electronic forces between protonated side chains. In some cases, further titration caused a subsequent conformation change, explained by ion pair formation of the acid with the protonated side chains. This decreases the influence of repulsive forces, and may result in refolding the protein. Stigter described this refolded state as a molten globule, with a high degree of hydrophobic clustering [25]. It was found that properties of the refolded state varied significantly among different proteins [24]. The effectiveness of different anions in the refolding process was also studied [26]. Protein precipitation, induced by strong acids like trichloroacetic acid, may be due to a similar mechanism [27]. Trichloroacetic acid induced protein precipitation results in a reversible association of molten globule-like, partially structured, flexible intermediate states.

Mass spectrometry (MS) has been widely applied for detection of species separated by HLPC with on-line coupling (HPLC-MS). Besides being an analytical tool, mass spectrometry also gives information on protein conformation [28-31] and on the structure of non-covalent protein complexes [32,33]. Charge distribution in electrospray ionization is a prime indicator of protein conformation. Folded proteins show low charge states (high m/z values) and narrow charge state distributions, in contrast to unfolded proteins, commonly observed in electrospray ionization under acidic conditions [34]. This is explained by the lower surface area of globular proteins, which allows them carrying less charge, than unfolded structures. Protein conformation is typically studied for pure samples, and rarely in HPLC-MS, where conformation may vary with solvent composition (e.g. organic modifier, pH, TFA concentration) [35,36].

Circular dichroism (CD) spectroscopy is also a widely utilized method to probe structural changes of proteins and peptides under different conditions. CD curves recorded in the far- (<250 nm) and near-UV (250-320 nm) region provide valuable information on the content of the secondary structure as well as on the changes of the conformational states of aromatic residues (tertiary structure) [37].

In our study to optimize proteins separation in reversed phase HPLC(-MS), we have observed unusual chromatographic features as a function of TFA concentration. These suggest conformation change, and may reveal a novel separation mechanism. Here we describe chromatographic behavior of two model proteins (transferrin and lysozyme) in detail using HPLC-UV-MS; describe conformational changes based on the observed charge state distributions; and evaluate the observed features in terms of separation mechanism.

2. Experimental

2.1. Instrumentation

Chromatographic experiments were performed on a Waters Acquity UPLC system (Waters, Milford, MA, USA) coupled to a Waters Micromass Q-ToF Premier (Waters, Milford, MA, USA) mass spectrometer. For mass spectrometric experiments electrospray ionization was used, in positive ion mode. The UPLC instrument was equipped with a binary solvent manager, autosampler, thermostated column compartment, and TUV detector. Most experiments were performed both with MS and with TUV detector; chromatographic behavior was identical in both cases. The autosampler was equipped with a 5µl loop, operating in full loop injection mode. The temperature of the Aeris WIDEPORE XB-C18 (2.1 mm x 150 mm, 3.6 µm, 200 Å) column (Phenomenex, (Torrance, CA, USA) was set to 50 °C. The TUV

detector operated with a 500 nL flow cell, set to 280 nm and 10 Hz sampling rate. Data acquisition, data handling and instrument control were performed by MassLynx V4.1. (Waters, Milford, ~~Ma~~MA, USA) software.

Circular dichroism (CD) spectra were recorded on a JASCO J-715 spectropolarimeter at 25 (± 0.2) °C. Temperature control was provided by a Peltier thermostat equipped with magnetic stirring. CD in the far-UV region (185-250 nm without TFA and 195-250 nm with TFA) was monitored using a rectangular quartz cell of 0.1 cm path length (Hellma, USA) with a protein concentration of 10 μM. Near-UV ellipticity signals were recorded between 250-330 nm using a 1.0 cm quartz cell with a protein concentration of 35 μM. Far- and near-UV CD spectra (five accumulations for each sample) were acquired at a scan speed of 50 and 100 nm/min with response time of 2 and 1 sec, respectively.

2.2. Chemicals and samples

Water was obtained from a Milli-Q Purification System (Bedford, MA, USA). Acetonitrile of HPLC gradient grade, trifluoroacetic acid (≥99%) and formic acid (≥96%) were purchased from Sigma-Fluka (Budapest, Hungary). Protein standards of transferrin (human, 79.5 kDa, pI = 6.1-5.3) and lysozyme (chicken, 14.3 kDa, pI = 11) were obtained from Sigma-Fluka (Budapest, Hungary). Stock solutions of proteins were prepared in water at 230 pmol/μl, respectively. Stock solutions were stored at -20 °C. It was controlled (in comparison with freshly prepared samples) that storing the stock solution in the course of our work did not influence the chromatographic behavior of proteins. Furthermore, mass spectra of the intact proteins did not show the presence of impurities and protein aggregates were also not observed. Working solutions of 20 pmol/μl were diluted from the stock solutions with water. The working solutions were kept at 4 °C between and under the chromatographic runs.

2.3. Methods

Eluent A was water, eluent B was acetonitrile. In gradient elution experiments, initial mobile phase composition was 30% B, final mobile phase composition was 60% B. TFA was added to both eluents in the range of 0.001-0.3 v/v%. Gradient slope was 5% B/min. Flow rate was set to 0.3 ml/min. Chromatographic runs were recorded from low TFA to high TFA concentrations. When changing the eluents, the column was flushed with 10 column volumes of the next eluent. A blank was run before injecting the proteins onto the column. The repeatability of the results were checked. In isocratic experiments, 35% B was used as eluent, TFA concentration ranged between 0.001-0.08 v/v%.

Mass spectrometry scans were carried out in the following circumstances. Scan range was set from m/z 600 to m/z 4000. Scan time was 1.5 sec., interscan delay time was 0.02 sec. Capillary voltage was set to 2.8 kV, sampling cone voltage was 35 V, source temperature was 90 °C, desolvation gas temperature was 250 °C, desolvation gas flow was 800 L/h. Note that using TFA additive is unfavorable for mass spectrometry detection. In our case we found at 0.001 v/v% TFA two-fold, at 0.1 v/v% TFA five-fold sensitivity decrease compared to formic acid.

CD data of physiological, and 35% acetonitrile containing lysozyme solutions were recorded. TFA was added to the organic containing solutions systematically, to set 0.001, 0.05 and 0.1 v/v% concentration. CD curves of lysozyme were corrected by digital subtraction of baseline spectra of 35% acetonitrile containing aqueous blanks at the same TFA concentration, measured under identical operating conditions and are expressed in terms of ellipticity (mdeg).

3. Results

3.1. Retention properties as a function of TFA concentration

RP-HPLC (with UV detection) and RP-HPLC-MS analysis of the intact proteins (transferrin and lysozyme) have been performed using conventional gradient elution using TFA containing water and acetonitrile (see Experimental for details). Protein elution as a function of TFA concentration in a wide range, from 0.001 to 0.3 v/v% have been studied. When no TFA was added to the eluent, the protein did not elute from the column even at 60% acetonitrile content. When TFA additive was varied in a wide range (from 0.001 v/v% to 0.3 v/v% concentration) and gradient was changed from 0% to 60% B, the proteins eluted from the column in the usual retention window. The retention time of the proteins showed the well-known logarithmic type increase as the function of TFA concentration in the 6.1-8.7 min time window (See supporting information). ~~This retention time varied with TFA concentration, but variation was within a relatively narrow, ca. ±20% time frame.~~ We have repeated the experiments using a narrower gradient (from 30% to 60% B), we have observed ~~a surprising phenomenon~~unusual chromatographic behavior, which is described below.

In order to improve chromatographic performance, most studies use TFA additive at relatively high concentration (0.1-0.3 v/v% range) in the case of protein separations. In this TFA concentration range the proteins are retained on the column, behavior is as expected. At low TFA concentrations retention times change significantly (Fig. 1 and 2). At very low TFA

concentration both proteins elute before the dead time (t_0=0.97 min, t_0 was determined by the solvent disturbance method [38,39]). When TFA concentration is slightly increased (but when it is still less than ca. 0.01 v/v%), two peaks appear in the chromatograms, one before, the other after the dead time. Mass spectrometric analysis confirmed that both peaks are due to transferrin and lysozyme, respectively. Note, that pure protein standards were used, and that the mass spectra of the 'split' peaks (see e.g. Fig. 3.) clearly show signals due to the same, pure protein. Note also, that signals due to protein aggregates (dimers, trimers) are absent (presence of even few % dimers or trimers would be clearly observable). UV spectra of the two peaks are also identical. These experiments exclude the possibility that one of the two peaks is due to the presence of an impurity or protein adduct. We have also compared various ways and conditions for measuring dead t_0, which showed a variability of less then ±0.02 min. This confirmed, that in the above mentioned conditions transferrin and lysozyme indeed elute significantly before the dead time.

In this range even small increase in the TFA concentration induces various changes: (a) Retention time of the 'early' peak increases, but nevertheless elutes before the dead time. (b) Relative intensity of this peak decreases with increasing TFA concentration. (c) Retention time of the 'late' peak increases significantly with TFA concentration. (d) Relative intensity of the 'late' peak increases with TFA concentration. These changes in the chromatographic behavior are shown in Figs. 1.and 2. in detail.

Note that the two proteins behave in an analogous manner, but the transition from the 'early' to the 'late' chromatographic peak comes at different TFA concentrations. Transition of lysozyme peaks was observed in the ~0.02-0.005 v/v% TFA range, while transferrin peak showed this behavior below ~0.005 v/v% TFA. The above described chromatographic tests have been repeated maintaining the same pH, but replacing TFA with formic acid (pH and concentration values are listed in Table 1). Similar peak splitting have been observed. Results showed only minor differences in the chromatographic behavior using TFA or FA used at the same pH. The respective chromatograms are shown in Supplementary data.

3.2. Mass spectrometry of the proteins

The chromatographic behavior described above has been followed by mass spectrometric detection as well. Mass spectra showed a significant change in the observed charge distributions, suggesting conformational change. Protein conformation may change both due to TFA content and to amount of organic solvent in the eluent (which changes in the course of gradient elution). To exclude the possible influence of organic solvent on protein

conformation, the influence of TFA was studied using isocratic elution. Concentration of eluent B (acetonitrile) was set to 35%. Isocratic elution had the additional advantage that (in the present case) the chromatographic peaks were better resolved.

Fig. 3A shows the chromatograms, 3B the corresponding mass spectra of lysozyme at various TFA concentrations, using isocratic elution mode. At 0.001 v/v% TFA concentration lysozyme elutes before the dead time. The spectrum shows the charge state distribution of a protein under typical ESI mass spectrometry conditions: relatively high charge states, with a wide distribution. This is known to correspond to a non-native, unfolded conformation (or mixture of conformations), induced mainly by the acidic media. Increasing TFA concentration to 0.01 v/v% practically does not change neither the retention time, nor the charge state distribution. At a TFA concentration of 0.05 v/v% there is a major change, two peaks appear in the chromatogram. Both were proved to be corresponding to lysozyme (based on the m/z values, Fig 3B). The first peak elutes at 0.76 min, somewhat later than in the case of 0.01 v/v% TFA, but still before dead time. The second peak elutes at 0.96 min, slightly after the dead time (t_0=0.95 min, in isocratic LC-UV-MS experiments). Presence of the two peaks suggests that under the conditions applied two protein conformations may be present in the solution, possibly also influenced by interaction with the stationary phase [40-43]. This suggests slow kinetics of equilibria between the two protein conformations. The mass spectra are characterized by low charge states and a narrow distribution suggesting a folded, possibly molten-globule type conformation. Increasing TFA concentration slightly to 0.08 v/v%, the first peak (i.e. the unfolded, highly charged structure) disappears from the chromatogram, the retention time is increased somewhat to 0.97 min, and the mass spectra shows low charge states (i.e. folded conformation).

At low (5+, 6+) charge states, observed at relatively high (>0.01- v/v%) TFA concentration, the mass spectra shows the presence of TFA adducts: (Fig. 3B; part of the spectra are blown up in Fig. 4). In the 6+ charge state only few TFA adducts with relatively low abundance are observed. In the 5+ state at a higher TFA concentration (Fig. 4) addition of as many as 5 TFA molecules were also observed $[M+nTFA+5H^+]^{5+}$, showed as a peak series at m/z 2861.9, m/z 2885.0, 2907.8, 2930.2, 2953.1 and 2476.7, corresponding to the addition of 0-5 TFA molecules to protonated lysozyme (Fig. 4).

The experiments performed clearly show that TFA addition has a marked influence on protein conformation. To establish whether it is an effect of pH or that of an ion pairing reagent, further experiments have been performed using formic acid instead of TFA. The effect of pH on the mass spectra was also checked in direct injection mode. The pH value of

TFA containing eluents was set with formic acid in the range of pH 2.1-3.2 (Table 1).When the pH was adjusted by formic acid, charge states showed similar, but less pronounced shift to higher m/z values, indicating lower charge states. This suggested, that not only TFA, but also FA effected the conformation. These results showed, that conformational change monitored by the shift of charge state distributions might be influenced by both the pH and the quality of the acid anion. The same experiments were repeated for transferrin as well, and those showed an analogous behavior (see Supplementary information).

3.3. Circular dichroism spectroscopic study of TFA induced structural changes of lysozyme

The aim of the CD studies was to investigate possible conformational changes under conditions, when the unusual chromatographic behavior was observed (using isocratic elution, described in section 3.2). The CD spectra of lysozyme were recorded in the far- and near-UV region to monitor the secondary and the tertiary conformational changes, respectively. At native conditions (phosphate buffer, pH 7.4) and in water solution containing 35 v/v% acetonitrile both far- and near-UV CD curves of the protein were very similar. In line with earlier reports, the near-UV CD spectrum of lysozyme is dominated by positive peak intensities above 275 nm complemented with a more intense negative region with a broad shoulder between 275 and 255 nm (Fig. 5). This CD pattern arises from the tertiary structure of the protein and reflects the additive contributions of asymmetric environment of the aromatic residues (6 Trp, 3 Tyr, 3 Phe). Addition of 0.001% TFA does not induce gross spectral changes, but the vibrational fine structure of the CD curve becomes less resolved. Increasing TFA concentration to 0.05%, however, dramatically transforms the spectrum: intensity of both negative and positive ellipticity signals are greatly reduced, the positive band completely vanishes and only a weak, noisy, negative residual curve can be measured. There was no further change in CD spectroscopic behavior upon increasing TFA concentration to 0.1%. The effect of formic acid was also investigated. Results showed similar behavior compared to TFA, when the pH of the solutions were the same (shown in Supplementary data). Under native conditions the far-UV CD spectrum of lysozyme displays a strong positive peak at 192 nm, a deep negative minimum around 208 nm and a shoulder about 222 nm (Fig. 5). The most intense, positive-negative band pair at shorter wavelengths is attributable to the π–π^* transition of the amide groups while the asymmetrically perturbed n–π^* transitions give rise to weaker ellipticity contributions at longer wavelengths. The signal intensity is greater at 208 nm than at 222 nm, which is a characteristic of α+β class of

proteins. Indeed, secondary structure estimations indicated the major contribution of α-helix and β-sheet components [44]. Addition of TFA (0.001 v/v%) slightly increases the ellipticiy value of the 208 nm band but does not alter other spectral regions. TFA added in higher concentrations (0.05 and 0.1 v/v%) results in ~2 nm blue shift of the negative minimum at 208 and intensity loss of the positive peak.

3.4. Discussion and application of the method

In this study we suggest, that, in a typical RP-HPLC experiment, TFA and FA induce protein conformation change. This is likely a similar effect to that described in the case of protein precipitation using tricholoroacetic acid, as described above. This conformational change can be monitored by changes in charge state distributions observed in ESI mass spectra. Slightly acidic mobile phases produce 'typical' ESI charge state distributions (Fig. 3), indicative of an unfolded (i.e. not native) protein conformation. This is observed also, when TFA or FA is added at a very low concentration (less than ca. 0.01 v/v%).

Less resolved vibrational fine structure of the near-UV CD curve measured at very low TFA content (0.001 v/v%) is the early sign of the perturbation of the tertiary structure of lysozyme. Charging the protein side chains in slightly acidic conditions increases the conformational flexibility of aromatic residues resulting less intense vibronic CD peaks. This is in line with mass spectrometric charge distribution, which suggests an increase in the surface area of lysozyme. In contrast to these subtle alterations, 50-fold increase of the TFA concentration (0.05 v/v%) provokes abrupt, dramatic diminution of the near-UV CD signals which refers to the nearly total disruption of the restricted asymmetric environment of aromatic residues and thus a global loss of the tertiary structure. Contrary to this, the far-UV CD curve at characteristic wavelengths exhibited only slight changes which indicates the retention of most of the native secondary elements. In accordance with the mass spectrometric analysis, such a conformational modification that is devoid of tertiary structure but contains extensive secondary components is characteristic to the molten globule state of lysozyme [45]. It is to be noted that far-UV CD spectra of lysozyme are indicative of CD contributions from aromatic side chains [46]. Thus, enhanced conformational fluctuation of the aromatic residues in the molten globule state of lysozyme may affect the far-UV CD profile as well. Therefore, the increase of the ellipticity values below 215 nm might be associated with the cancellation of the positive contributions of aromatic chromophores to the respective far-UV CD bands [45].

The typically observed protein unfolding may be due to the organic content, slightly acidic conditions, and low salt concentration in the mobile phase. Under such conditions, the unfolded protein is suggested to be excluded from the pores of the stationary phase. Due to pore exclusion and the applied eluent composition (i.e. eluent strength), the protein elutes before dead time (Fig. 1). Higher TFA concentration (in the order of 0.01-0.1 v/v%) induces structural transition to a more compact, molten globule-like form. This was also observed for FA. Analogous phenomenon was reported by Liu et al. [47]. Increase of the retention time in size exclusion chromatography of monoclonal antibodies was observed, when applying similar mobile phase conditions (increasing TFA concentration from 0 to 0.1 v/v% in 20% acetonitrile). These results assume the reduction of the hydrodynamic radius of the antibody with increasing TFA concentration. The TFA concentration needed for this protein folding depends significantly on the protein. Note, that TFA concentration may depend on the pI of the protein. In the present study we have studied lysozyme (pI=11) and transferrin (pI=6.1-5.3, depending on iron saturation [48]), so the present results may not be extrapolated to strongly acidic proteins. Results of the CD studies, mass spectrometric studies on charge state distribution and the observation of TFA adduct formation (Fig. 3B and 4) are in good agreement with this hypothesis. This refolded, molten globule like conformation is likely needed to obtain a reasonable protein separation in RP-HPLC; and that is the reason for commonly using relatively high (0.1-0.3 v/v%) TFA addition to the mobile phase in most applications described in the literature. We suggest that the relative conformational stability of the molten globule, possibly with ion-pair formation between TFA and the protein plays a key role in the efficiency of separation.

Note, that pore exclusion can be observed only if gradient elution starts at relatively high organic solvent content (30% B in the present case). When gradient starts from 0% B, proteins show ‘conventional’ retention on the column, and elute around 25-30% B concentration. This suggests, that at low organic solvent concentration the proteins are retained on the column (possibly even without entering the pores).

From a practical point of view, the observed large variation in retention of proteins at very low acid concentrations and be advantageously utilized to improve separation efficiency. Here we present an example of the conformational exclusion based separation (CEBS) of transferrin and lysozyme on the Aeris WP column. The gradient used was described in the experimental section. Under conventional conditions in a reversed phase system (0.1 v/v% TFA), the retention times of these two proteins are close. By using a much lower (0.005 v/v%) TFA concentration, the two proteins can be separated very efficiently (Fig. 5), using

the CEBS mechanism described above. This separation principle may be particularly useful for preparative separation of proteins.

4. Conclusion

In this study we describe an unusual RP-HPLC behavior of proteins. This might relate to ion-pair formation with additives like TFA or FA, which strongly influence protein conformation. At low TFA concentration proteins have an extended conformation (as shown by the charge distribution observed in the mass spectra), and are excluded from the pores of the stationary phase. This, and the applied eluent composition causes the protein to elute *before* the dead time. At higher TFA or FA concentration ion pair formation between a protonated site of the protein and the acid anion becomes likely. This makes the protein more hydrophobic, induces conformation change leading to a compact, 'molten globule' type structure. This compact structure is retained on the column and elutes after the dead time. Note that formation of the 'molten globule' like structure (which is apparently needed for a reasonably good RP-HPLC of proteins) is suggested to be influenced by both the pH and the type of the acid anion.

The TFA concentration needed to induce conformation change depends on protein structure, and was significantly different for the two proteins studied. This effect can be advantageously utilized, as a conformational exclusion based separation method. This could be helpful in protein isolation or purification – an example is shown in Fig. 6. The same effect may be utilized to separate proteins and small molecules: at low TFA content and appropriate eluent strength proteins will elute before dead time, while small molecules – independently of their polarity – can diffuse into the pores and elute after dead time. Note also, that most chromatographic separations of acidic or basic compounds depend on the pH. Keeping the pH constant, but substituting the acid additive (like FA and TFA), may influence the conformation of some compounds, which effect may be utilized in the case of difficult separations.

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Figure and table captions:

Fig. 1. UV chromatograms of lysozyme (panel A) and transferrin (panel B) at different TFA concentrations in gradient runs on the Aeris WP column.①: peak before dead time,②: solvent disturbance, ③: peak after dead time

Fig. 2. Retention properties of the proteins on the Aeris WP column (dotted line: lysozyme, dashed line: transferrin). Shaded area represents the concentration range of TFA, where two peaks (before and after dead time) are present in the chromatograms.

Fig. 3. Mass spectra of lysozyme in isocratic elution mode. A: UV-chromatograms at different TFA concentrations in the mobile phase. B: MS spectrum of the peaks shown in A.*Solvent disturbance (no proteins detected in these peaks neither by UV, nor by MS detection).

Fig. 4. Mass spectra of lysozyme-TFA adducts $[M+nTFA+5H^{+}]^{5+}$ at A: 0.08 and B: 0.01 v/v% TFA (Zoomed spectra from Fig. 3.). *Impurities.

Fig. 5. Top panel: far-UV CD spectra of 10 mM lysozyme in the absence and in the presence of increasing concentrations of TFA (trifluoroacetic acid). Bottom panel: near-UV CD spectra of 35 mM lysozyme in the absence and in the presence of increasing concentrations of TFA (water:acetonitrile 65:35 v/v%, 25 ºC).

Fig. 6. Conformational exclusion based separation of lysozyme and transferrin. See experimental details in section 2.3. ~~UV chromatograms were recorded on the Aeris WP column at A: 0.1 v/v% TFA, B: 0.005 v/v% TFA in the mobile phase.~~

Table 1. Eluent pH values set with trifuoloroacetic acid and formic acid

Supplementary Fig. 1. UV chromatograms of A: transferrin, B: lysozyme using gradient elution described in section 2.3, pH was adjusted with formic acid instead of TFA (Table 1).

Supplementary Fig. 2. Mass spectra of lysozyme at different pH values, set with formic acid and trifluoroacetic acid. *Lysozyme-TFA adducts, detailed in the paper (Fig. 4.).

Supplementary Fig. 3. Mass spectra of transferrin at different pH values, set with formic acid and trifluoroacetic acid.

Supplementary Fig. 4. MaxEnt deconvolution of the spectra from Figure 1. and 2. A: lysozyme, B: transferrin. Protein-additive adducts are represented as M+n.

Supplementary Fig. 5. Near-UV CD spectra of 35 mM lysozyme in the absence and in the presence of increasing concentrations of formic acid (water:acetonitrile 65:35 v/v%, 25 ºC).

Supplementary Fig. 6. Retention properties of the proteins on the Aeris WP column (dotted line: lysozyme, dashed line: transferrin) starting gradient elution from 0%B.