Effect of surface hydrophobicity distribution on retention of ribonucleases in hydrophobic interaction chromatography

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Abstract

The effect of surface hydrophobicity distribution of proteins on retention in hydrophobic interaction chromatography (HIC) was investigated. Average surface hydrophobicity as well as hydrophobic contact area between protein and matrix were estimated using a classical thermodynamic model. The applicability of the model to predict protein retention in HIC was investigated on ribonucleases with similar average surface hydrophobicity but different surface hydrophobicity distribution. It was shown experimentally that surface hydrophobicity distribution could have an important effect on protein retention in HIC. The parameter "hydrophobic contact area," which comes from the thermodynamic model, was able to represent well the protein retention in HIC with salt gradient elution. Location and size of the hydrophobic patches can therefore have an important effect on protein retention in HIC, and the hydrophobic contact area adequately describes this.

1. Introduction

Hydrophobic interaction chromatography (HIC) is an important technique for protein purification, which exploits the hydrophobic character of proteins promoting their separation based on hydrophobic interactions between stationary phase ligands and hydrophobic regions on the protein surface [1]. In HIC, proteins are induced to bind to a weakly hydrophobic ligand attached to a stationary phase under high salt concentration conditions. Elution is achieved either isocratically or by decreasing the ionic strength of the mobile phase in a linear way [2]. HIC is widely used in the downstream processing of proteins, as it provides separation properties complementary to other protein purification techniques [3].

The main system characteristics affecting protein retention in HIC are concentration and type of salt [1,4] and density and type of hydrophobic ligand attached to the matrix [5]. However, only little information exists about the way these factors affect behavior of proteins in HIC [6].

On the other hand, the main physicochemical property of proteins that determines chromatographic behavior in HIC is hydrophobicity, which relies on no absolute definition. There is consensus in that protein hydrophobicity is determined by the hydrophobic contribution of the amino acids the protein consists of [5,7]. It was recently proposed to estimate this property as "average surface hydrophobicity" [8] starting from the protein three-dimensional structure data and considering the hydrophobic contribution of the exposed amino acid residues as a weighed average. Based on this definition, a methodology was proposed to predict the retention time of proteins in HIC with salt gradient elution [9]. The disadvantage of this methodology is that it does not consider the effect of the distribution of the surface hydrophobicity on protein retention.

Mahn et al. [10] have reported that some proteins, like conalbumin and ribonuclease A, showed a not expected behavior in HIC when using phenyl-Sepharose and 2 M ammonium sulfate in the gradient elution buffer, having a very similar surface hydrophobicity but showing rather different retention time. This varying behavior can be attributed to differences in the surface hydrophobicity distribution. It is well known that the surface of globular proteins can have hydrophobic patches, which affect the retention in HIC [5].

The effect of single amino acid mutations on protein retention in HIC has been studied for different bird lysozymes [11]. It was concluded that only the amino acids located in the zone opposite to the active site have an effect on protein

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retention in HIC. The effect of the surface properties on protein retention has also been broached in a study about ion-exchange chromatography, where the effect of surface charge distribution of proteins was investigated [12]. Recently, the effect of surface hydrophobicity distribution of proteins related to protein–polymer association has been investigated through molecular dynamics simulations [13], using theoretical protein models. However, it has not been possible to establish a clear relation between protein retention in HIC and their surface hydrophobicity distribution.

In the present work, the effect of surface hydrophobicity distribution of proteins related to their retention in HIC was investigated. To isolate the effect of surface hydrophobicity distribution, we used proteins with very similar average surface hydrophobicity, but which distinctly differ on their surface hydrophobicity distribution. First, we investigated the chromatographic behavior in HIC of ribonuclease T1 and a variant of that enzyme that presents a symmetric amino acid mutation (Fig. 1). By "symmetric mutation," we mean to exchange the position of two exposed amino acids, without changing their exposure level. In RNase T1 variant, the residue tryptophan, which in the RNase T1 wild type occupies position 59, was moved to position 45, and the tyrosine originally located in position 45 was changed to position 59. In this way, the average surface hydrophobicity of the protein remains invariable, while the surface hydrophobicity distribution is indeed affected. To complete the present study, we investigated the chromatographic behavior in HIC of two different additional ribonucleases, showing similar average surface hydrophobicity, but different surface hydrophobicity distribution.

The protein retention time in HIC was related to the average surface hydrophobicity ($\phi_{surface}$), the hydrophobic accessible area (HAA) and the hydrophobic contact area (HCA) (contact area between the stationary phase and the protein when attached to the HIC resin). The total accessible area (TAA) and the hydrophobic accessible area were estimated starting from the protein three-dimensional structure data, considering that the most hydrophobic amino acids are alanine, valine, leucine, isoleucine, methionine, phenylalanine and proline. The hydrophobic contact area was estimated from isocratic retention data, using the simplified thermodynamic model proposed by Melander et al. [14] for hydrophobic and electrostatic retention.

2. Theory

2.1. Thermodynamic model for hydrophobic interaction

The simplified thermodynamic model proposed by Melander et al. [14] to describe the effect of salt concentration on retention in biopolymer chromatography was used. This model can be applied to stationary phases that have a highly hydrated surface with fixed charges or weakly hydrophobic binding sites or both.

In the treatment of electrostatic interactions, the Manning's counter ion condensation theory was used [15], while the hydrophobic interactions were treated using an adaptation of the Sinanoglu's solvophobic theory [16] to the salting out of proteins and their retention in HIC [1].
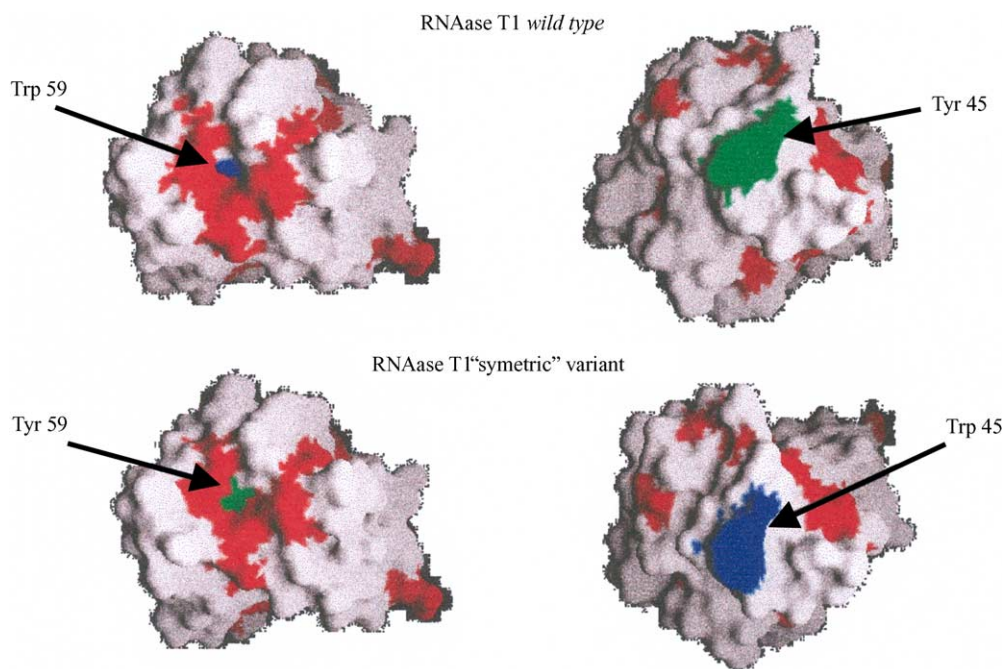

Fig. 1. Snapshot for ribonuclease T1 *wild type* and its symmetric variant trp 59 tyr/tyr 45 trp. Hydrophobic aminoacids are colored red, tyrosine residues are colored green and triptophane residues are colored blue.

2.1.1. Electrostatic interaction

The retention thermodynamics in electrostatic interaction chromatography was analyzed based on the counter ion condensation theory of Manning [15]. Melander et al. [14] derived the following expression for the free energy of binding (ΔG°_{es}) of the protein to a stationary phase in the presence of the salt counter ion:

$$\frac{-\Delta G^{\circ}_{es}}{2.3RT} = \log\left(\frac{N_{AV}b^2\delta_p}{1000e}\right) + \frac{Z_p}{Z_s}\log\left[\frac{1000e}{(N_{AV}b^2\delta_s m_s)(1-Z_s\xi)}\right] \quad (1)$$

where m_s is the molal salt concentration, N_{AV} the Avogadro's number, "e" the base of the natural logarithm, "b" the average spacing of fixed charges on the surface, δ_p the thickness of the condensation layer over the surface of the stationary phase where each fixed charge occupies an area of b^2, δ_s the layer thickness of salt counter ion, Z_p the characteristic charge of the protein, Z_s the valence of the salt counter ion, and ξ a dimensionless structural parameter that characterizes the charged surface. R is the universal constant of gases and T the absolute temperature.

2.1.2. Hydrophobic interaction

The retention due to hydrophobic interactions was assumed to occur due to contact between hydrophobic patches at the protein's surface and the hydrophobic binding sites of the stationary phase [14]. The free energy of hydrophobic interactions $(\Delta G^{\circ}_{h\phi})$ was expressed as a function of the molal surface tension increment of the salt (σ_s) as follows [14]:

$$\Delta G^{\circ}_{h\phi} = \Delta G^{\circ}_{aq} - \Delta A'\sigma_s m_s \quad (2)$$

where m_s is the salt molality, ΔG°_{aq} represents the reduction in free energy due to other effects different form hydrophobic interactions, $\Delta A'$ the difference between molecular surface area of the free protein (A_M) and the molecular surface area of the bound protein (A_s). $\Delta A'$ corresponds to the surface contact area between the bound protein and the hydrophobic site of the matrix. The relatioship shown in Eq. (2) is valid only in the absense of specific salt effects.

2.1.3. Combined electrostatic and hydrophobic interaction

In order to represent the retention factor k' as a function of the salt molality when hydrophobic and electrostatic interactions are present, Melander et al. [14] combined the previous equations to yield:

$$\log k' = \log\left(\frac{N_{AV}b^2\delta_p}{1000e}\right) + \frac{Z_p}{Z_s}\log\left[\frac{1000e}{(N_{AV}b^2\delta_s m_s)(1-Z_s\xi)}\right] + \frac{\Delta G^{\circ}_{aq}}{2.3RT} + \frac{\Delta A'\sigma_s m_s}{2.3RT} + \log\alpha \quad (3)$$

where α is the phase ratio (stationary phase/mobile phase).

Eq. (3) can be simply expressed as follows:

$$\log k' = A - B\log m_s + Cm_s \quad (4)$$

where A is a constant determined by all the system characteristics, B the electrostatic interaction parameter and C the hydrophobic interaction parameter. The parameter C, which is of interest in this work, is given by:

$$C = \frac{\Delta A'\sigma_s}{2.3RT} \quad (5)$$

This simplified thermodynamic model can be used to evaluate the three parameters involved in Eq. (4) depending on the salt concentration used. At low salt concentration, hydrophobic interactions can be neglected, thus through a linear regression between isocratic retention factors obtained with different salt molaliies the parameters A and B can be estimated. At high salt concentration, electrostatic interactions are negligible, then the parameters A and C can be obtained. The parameter C accounts for the hydrophobic surface contact area between the protein and the matrix. The HCA can easily be obtained from the slope of the limiting plot of $\log k'$ and salt molality.

In the present work, our interest is set on the hydrophobic interactions, then HIC isocratic experiments were carried out using different salt concentration, in a high range of molality.

3. Experimental

3.1. Materials

Ribonuclease T1 wild type (PDB ID 1RGC) and the variant Y45W/W59Y (PDB ID 1TRP) were obtained by expressing both enzymes in *Escherichia coli* strain DH5α. Competent cells were transformed with the corresponding plasmids. RNase variants were produced and purified after the protocol published by Grunert et al. [17]. Both plasmids were kindly donated by Professor Dr. Ulrich Hahn (University of Hamburg, Germany). Ribonuclease A (1AFU), ribonuclease S (1RBC) and Tris buffer were purchased from Sigma (St. Louis, MO, USA). Water prepared from a Milli-Q water cleaning system (Millipore, Bedford, MA, USA) and analytical-reagent grade ammonium sulfate (Merk) were used in the preparation of the buffers.

3.2. Equipment

The high-performance liquid chromatography system employed was of a fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden). The chromatographic matrix used was butyl-Sepharose fast flow (a gift of Amersham Pharmacia Biotech, Uppsala, Sweden) packed in a 1 ml column. The experiments were performed at room temperature (23.5 °C), using a flow rate equal to 0.75 ml/min.

3.3. Void volume determination

The void volume of the column was determined using an indirect method [18]. The column was filled and weighed in triplicate with a light solvent, in this case methanol ($\rho_M = 0.7842$ g/ml) and a heavy solvent, in this case ethyleneglycol ($\rho_E = 1.1074$ g/ml). The total volume of the mobile phase in the column, V_0, was calculated by:

$$V_0 = \frac{m_2 - m_1}{\rho_E - \rho_M} \tag{6}$$

where m_1 and m_2 are the masses of the column filled with the light and the heavy solvents, respectively. We decided to use this technique because it is the only hold-up volume measurement with a physical meaning, as it represents the volume within the column accessible to the two solvents and an analyte molecule of a size comparable to that of the solvent molecule [18].

3.4. Experimental conditions

Isocratic HIC runs were performed at room temperature, using 20 mM Tris buffer pH 8.0 plus different ammonium sulfate concentrations, in the range of 0.5–2.5 m. Samples were injected through a 50 μl loop. The samples were prepared starting from 3 mg/ml protein stock solutions. Concentrated ammonium sulfate buffer was added, depending on the final salt concentration needed. Retention volume was recorded. All the experiments were made in triplicate.

Gradient elution HIC experiments were carried out using a decreasing salt gradient, with a steepness of 7.5% B/min (a 10 column volume gradient). The initial eluent was 20 mM Tris, pH 8.0, plus a maximum salt concentration of 2 M ammonium sulfate. The final eluent was 20 mM Tris, pH 8.0 (buffer A). Samples, which contained 0.5 mg/ml protein, were injected through a 200 μl loop. Retention volume was recorded. All the runs were made in triplicate.

All buffers were filtered through 0.22 μm Millipore filters after preparation and degassed with helium for 10 min.

3.5. Determination of protein average surface hydrophobicity

The average surface hydrophobicity was estimated after the methodology proposed elsewhere [8,19]. The proteins were characterized based on their amino acid sequences. These sequences and the three-dimensional structures were obtained from the Protein Data Bank (PDB, http://www.rcsb.org/pdb) [20]. Using the PDB file as input for the program "Graphical Representation and Analysis of Surface Properties" (GRASP) [21], the three-dimensional structure of the proteins was visualized allowing to depict the surface distribution of amino acids. The protein's solvent accessible area and that of each kind of amino acid were calculated by GRASP. Finally, the hydrophobicity of each protein ($\phi_{surface}$) was obtained using the surface acces-

sible area of each amino acid in the protein, the normalized Miyazawa–Jernigan [22] amino acid hydrophobicity scale and Eq. (7):

$$\phi_{surface} = \frac{\sum s^*_{aai}\phi_{aai}}{s_p} \tag{7}$$

where $\phi_{surface}$ is the surface hydrophobicity, "i" indicates the different standard amino acids, s_{aai} the solvent accessible area occupied by amino acid "i," ϕ_{aai} the hydrophobicity value assigned to amino acid "i," and s_p the total solvent accessible area of the protein [8,10,19]. "s_p" has also been called "total accessible area," TAA.

3.6. Determination of the hydrophobic accessible area

The hydrophobic accessible area of the proteins was obtained starting form the three-dimensional structure data, given in the corresponding PDB file. The HAA was calculated as the solvent accessible area of the hydrophobic residues on the protein surface, using the program GRASP. This program considers that the hydrophobic residues are alanine, valine, leucine, isoleucine, methionine, phenylalanine and proline.

3.7. Determination of the hydrophobic contact area

The hydrophobic contact area of each protein was determined starting from their isocratic retention factors obtained using different ammonium sulfate molalities. The HCA was obtained from Eq. (5). The value of the parameter C was obtained with linear regression with the respective data; the value of σ_s, which corresponds to the molal surface tension increment of ammonium sulfate, was obtained from literature, and was equal to 2.6×10^3 dyn g/cm mol [23].

4. Results and discussion

4.1. Surface hydrophobicity and hydrophobic contact area of different ribonucleases

Surface hydrophobicity of proteins was evaluated as a weighed average using Eq. (7) and the normalized amino acid hydrophobicity scale developed by Miyazawa and Jernigan [22]. The estimated $\phi_{surface}$ values of the proteins used are shown in Table 1. The hydrophobicity scale used gives $\phi_{surface}$ values ranging from 0.18 to 0.32, when thirteen different globular monomeric proteins were used [8,10]. In the case of the ribonucleases, this value fluctuated between 0.222 and 0.269, which corresponds to 33% of the complete surface hydrophobicity range.

The ribonucleases showed relatively similar surface hydrophobicity. RNase T1 wild type and the RNase T1 variant showed almost equal surface hydrophobicity, differing only in 1.5%. This is because both proteins differ only in the surface hydrophobicity distribution. They have the

Table 1
Surface properties and molecular mass of the different ribonucleases

Protein	PDB Id[a]	M_r[b]	$\phi_{surface}$[c] (–)	TAA[d] (Å^2)	HAA[e] (Å^2)	HCA[f] (Å^2)
RNase A	1AFU	13 574	0.230	6955.2	1136.6	64.5
RNase S	1RBC	13 196	0.222	6666.1	1040.3	257.3
RNase T1 wild type	1RGC	11 072	0.265	5487.7	783.5	112.4
RNase T1 variant	1TRP	11 072	0.269	5476.4	775.5	386.8

[a] PDB ID is the file code given by The Protein Data Bank [20].
[b] M_r is the molecular mass of the protein.
[c] $\phi_{surface}$ is the average surface hydrophobicity of proteins estimated by Eq. (7).
[d] TAA is the total accessible area.
[e] HAA is the hydrophobic accessible area.
[f] HCA is the hydrophobic contact area.

same three-dimensional structure but the position of the residues 45 and 59 are exchanged symmetrically [24]. Surface hydrophobicity of RNase A and RNase S differ only in 3.5%, since these proteins have also very similar average surface hydrophobicity. RNase S is a complex that consists of two proteolytic fragments of RNase A: the "peptide S" (residues from 1 to 20) and the "protein S" (residues 21–124). These fragments are non-covalently connected by a sulfate molecule [25], thus RNase S is dynamically more flexible and unstable than RNase A, despite their identical structures and similar enzymatic activities [26].

The total accessible area (equal to "s_p" in Eq. (7)) and the hydrophobic accessible area of each ribonuclease are shown in Table 1. RNase T1 wild type and RNase T1 variant showed very similar TAA and HAA. The same was observed for RNase A and RNase S. The parameters HAA and TAA are directly related to the molecular weight of the proteins; similar molecular weight implies similar TAA and HAA.

The hydrophobic contact area was estimated from isocratic retention data, using the thermodynamic model for hydrophobic and electrostatic interaction [14] (Eq. (4)). Fig. 2 shows the plots of $\log k'$ versus salt molality for RNase T1 wild type (Fig. 2a) and RNase T1 variant (Fig. 2b), and Fig. 3 shows the plots corresponding to RNase A (Fig. 3a) and RNase S (Fig. 3b). For all the proteins studied, the correlation coefficients (r^2) were higher than 0.92 (average = 0.97). Different slope values were obtained for the different proteins, despite the similar surface hydrophobicity, what makes us expect a different chromatographic behavior in HIC for each protein. Starting from these slope values, the HCA was calculated (Table 1) using Eq. (5). The biggest HCA corresponded to the RNase T1 variant, and the smallest one was that of RNase A. There was no linear correlation between the HCA either with the molecular weight of the proteins or with the parameter TAA. This situation is opposite to that reported by Katti et al. [23], who claimed that the "hydrophobic parameter" (which corresponds to the parameter 'C' in Eqs. (4) and (5)) has a linear dependence on the surface accessible area of the proteins. These different results can be attributed to the different methodology those authors used to estimate protein surface accessible area. They used the algorithm developed by Lee and Richards [27] implemented on their own routine. They considered a probe

radius equal to 4 Å, leading to higher solvent accessible area estimations. The algorithm of Lee and Richards [27] has been adapted in the program GRASP [21], allowing the use of a probe radius equal to 1.4 Å (which was used in our calculations of accessible areas). In addition, Katti et al. [23] used only three proteins to develop the correlations between solvent accessible area and the hydrophobic parameter. In the present work, we used four proteins.

We found no linear correlation between the HCA and the HAA; thus we can suppose that the location of the hydrophobic patch would have an important effect on protein retention in HIC. This is the case of RNase A. This
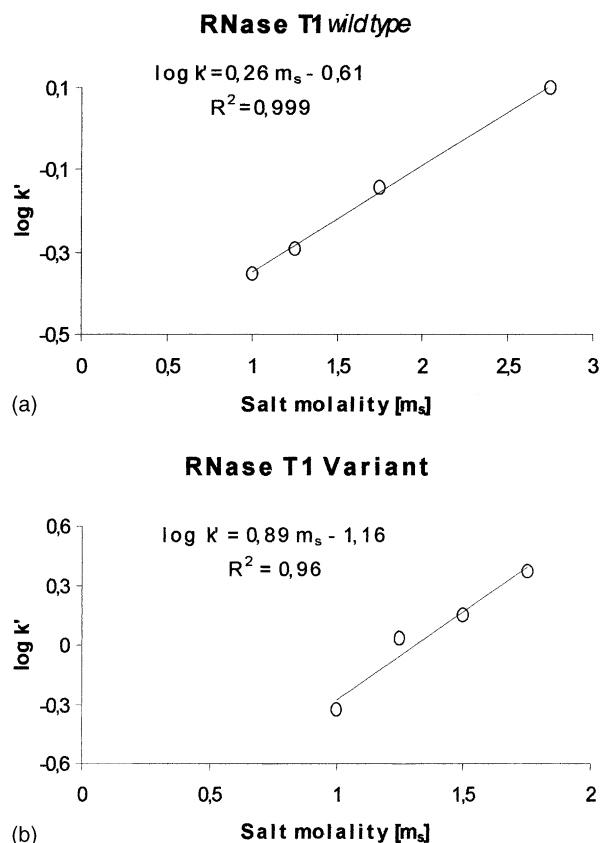

(a)

(b)

Fig. 2. Linear regression between $\log k'$ in isocratic HIC experiments and the different salt concentration (molal) for RNase T1 wild type (Fig. 2a) and RNase T1 variant (Fig. 2b). The points are the experimental results and the continuous line is the tendence line.
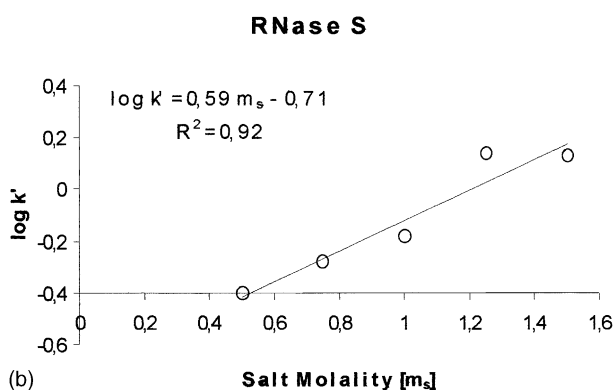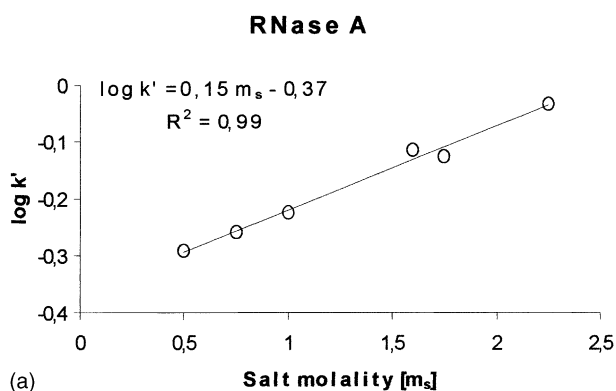
Fig. 3. Linear regression between $\log k'$ in isocratic HIC experiments and the different salt concentration (molal) for RNase A (a) and RNase S (b). The points are the experimental results and the continuous line is the tendence line.

protein has a big hydrophobic accessible area but a small hydrophobic contact area, possibly because the largest hydrophobic patch is located in the hydrophobic pocket (concave zone) that corresponds to the active site. In this way, the largest hydrophobic patch would be less accessible to the hydrophobic ligand of the matrix possibly causing a low retention time.

4.2. Retention time prediction starting from average surface hydrophobicity

The dimensionless retention time (DRT) of the different RNases was predicted starting from surface hydrophobicity (ϕ_{surface}) using a quadratic empirical correlation previously
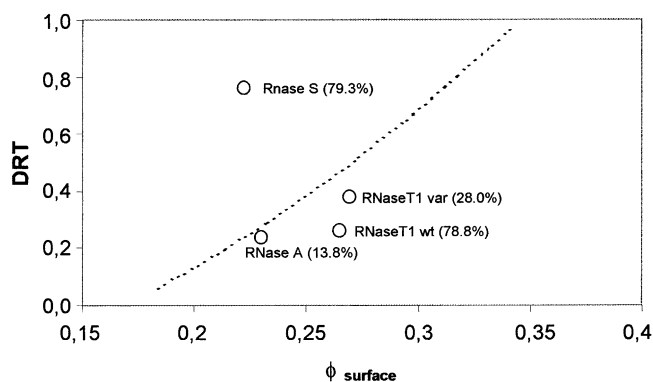

Fig. 4. Experimental dimensionless retention time (DRT) of the RNases used in the present study (individual points), compared to the quadratic model based on average surface hydrophobicity to estimate protein's DRT (dotted line). The deviation from the model is given in brackets. The quadratic model presented here is that published by Lienqueo et al. [8] for gradient elution in butyl-Sepharose and 2 M ammonium sulfate in buffer B: $\text{DRT} = 10.02\phi_{\text{surface}} + 0.54\phi_{\text{surface}} - 0.38$, where ϕ_{surface} is the average surface hydrophobicity of proteins and DRT the dimensionless retention time calculated by Eq. (8).

reported by Lienqueo et al. [8]. The parameter DRT was calculated using Eq. (8), where t_R is the time corresponding to the peak maximum in the chromatogram, t_0 the time corresponding to the start of the elution gradient, and t_f the time corresponding to the end of the salt gradient.

$$\text{DRT} = \frac{t_R - t_0}{t_f - t_0} \qquad (8)$$

The predicted DRT values were compared with the experimental DRTs and the deviation between these values was calculated. Theoretically, if the surface hydrophobicity distribution has no effect on protein retention, the deviation should be smaller than 15%, based on the results published before [8]. Table 2 shows the DRT values for each protein and the deviation from the predicted ones.

Fig. 4 shows the empirical model for DRT as a function of ϕ_{surface} and the experimental DRT values obtained for the different RNases used in this study. Clear differences between predicted and experimental DRT can be observed, the biggest being RNase S. This protein showed a different behavior in HIC possibly due to its high dynamic flexibility. Another possible explanation to this situation is that in solution three different species can be found at equilibrium:

Table 2
Predicted and experimental dimensionless retention time (DRT) of different ribonucleases

Protein	ϕ_{surface}[a] (–)	DRT predicted[b]	DRT experimental[c]	Deviation (%)[d]
RNase A	0.230	0.272	0.239	13.8
RNase S	0.222	0.233	0.760	69.3
RNase T1 wild type	0.265	0.465	0.260	78.8
RNase T1 variant	0.269	0.489	0.382	28.0

[a] Surface hydrophobicity estimated using Eq. (7) and the normalized Miyazawa–Jernigan [22] amino acid hydrophobicity scale.
[b] Predicted DRT starting from surface hydrophobicty using the quadratid model published by Lienqueo and collaborators [8].
[c] Experimental DRT obtained in butyl-Sepharose and 2 M ammonium sulfate in buffer B.
[d] Deviation $= \frac{|\text{DRT}_{\text{experimental}} - \text{DRT}_{\text{prediced}}|}{\text{DRT}_{\text{experimental}}} \times 100$.

RNase S as a complex, peptide S and protein S [28]. The empirical model used is not applicable to all the RNases used in this study, only for Rnase A the deviation factor was smaller than 15% (equal to 13.8%). RNase A is a very stable protein, due to the high number of cistines present in its tertiary structure [29]. RNase A has four cistines in its tertiary structure, and two peptidic bonds proline–tyrosyne and proline–asparagine. In this way, it results more difficult to change the protein conformation to increase the contact area between the protein and the hydrophobic matrix in HIC. In addition, it can be expected that very stable proteins have a structure in solution similar to that of the crystallized protein. If this is the case, then the empirical model would represent in a correct way the surface hydrophobicity of very stable proteins, because the estimation of this property would be based on a crystal structure similar to that found in solution.

On the other hand, the largest hydrophobic patch in RNase A is located in a hydrophobic pocket; thus it can be expected that the probability of collision with the hydrophobic ligands of the resin would be reduced. In the convex zone opposite to the hydrophobic pocket, the hydrophobic patch distribution is relatively homogeneous [29]; therefore it can be expected that the average surface hydrophobicity can represent in a correct way this homogeneous distribution.

The previous observations lead us to propose that the empirical model would be adequate to very stable proteins, as in the case of RNase A.

Proteins with almost identical average surface hydrophobicity showed different dimensionless retention time in HIC. RNase T1 wild type and the variant only differ 1.5% in their surface hydrophobicity, but they differ 47% with respect to their DRT. A similar situation was observed for RNase A and RNase S. These proteins have very similar surface hydrophobicity (with a difference of 3.5%) but very different DRT, with a deviation of 69%. It can be supposed that the surface hydrophobicity distribution of proteins can be an important factor affecting their retention in HIC.

4.3. Relation between protein retention in HIC and hydrophobic areas

The dimensionless retention time of proteins in HIC was related to the hydrophobic accessible area and the hydrophobic contact area of the ribonucleases used in the present study. Fig. 5 shows the correlation level between DRT and HAA (Fig. 5a) and HCA (Fig. 5b). Fig. 6 shows the relation between DRT and HCA/HAA ratio (Fig. 6a) and HCA/TAA ratio (Fig. 6b). It was observed that no linear correlation exists between DRT and the hydrophobic accessible area of the proteins. The correlation coefficient (r^2) considering the four RNases was equal to 0.05, and neglecting RNase S it was equal to 0.39.

The correlation between DRT and the hydrophobic contact area gave r^2 equal to 1, which means that this parameter (HCA) can represent very well the retention of these proteins in HIC using gradient elution. Besides, this correlation
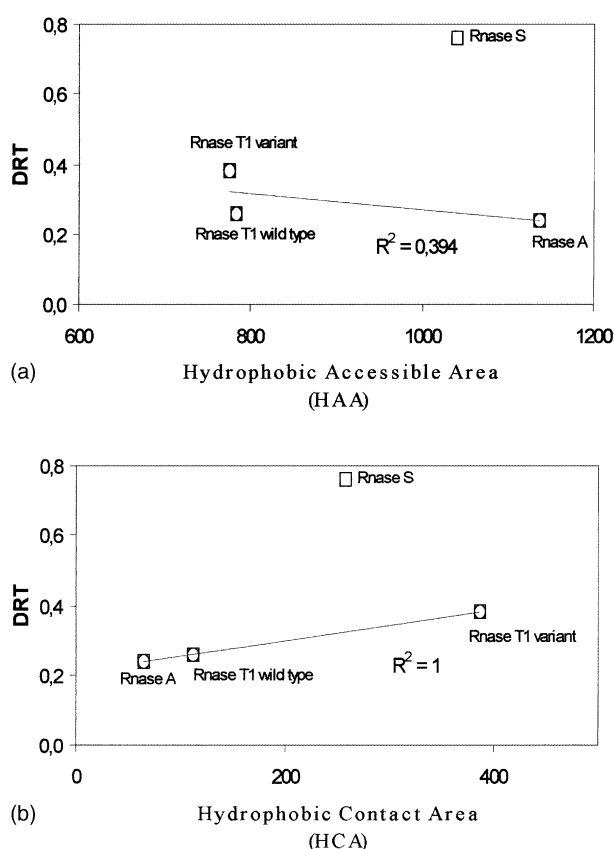

Fig. 5. Correlation level between the dimesionlesst retention time (DRT) of the proteins in gradient elution with butyl-Sepharose and 2 M ammonium sulfate in buffer B and the parameters hydrophobic accessible area, HAA (a) and hydrophobic contact area, HCA (b). Individual points are the experimental data, continuous lines represent the correlation level considering RNase T1 wild type, RNase T1 variant and RNase A.

was so high possibly because of the similar chromatographic conditions (salt and hydrophobic resin) used to obtain both parameters (DRT and HCA). In this way, the proteins should maintain the same structural conformation during the HIC processes (isocratic and gradient elution).

A high value of HCA means that the hydrophobic contact area between the matrix and the protein when attached is big; thus the size of the hydrophobic patch would be important in protein retention in HIC. This statement agrees with the results obtained by Jönsson et al. [13], who showed through computational simulations that the attraction between a polymer and a "simulated" protein is bigger when the protein has larger hydrophobic patches on the surface. The correlation level between DRT and the ratios HCA/HAA and HCA/TAA was very high, too, showing a correlation coefficient equal to 0.998 and 0.999, respectively.

All the correlation coefficients decreased when RNase S was considered. This situation could be explained considering that this protein has an anomalous chromatographic behavior, which the model used is not able to represent. This different behavior can be attributed to a possible conformational change of the protein during the chromatographic
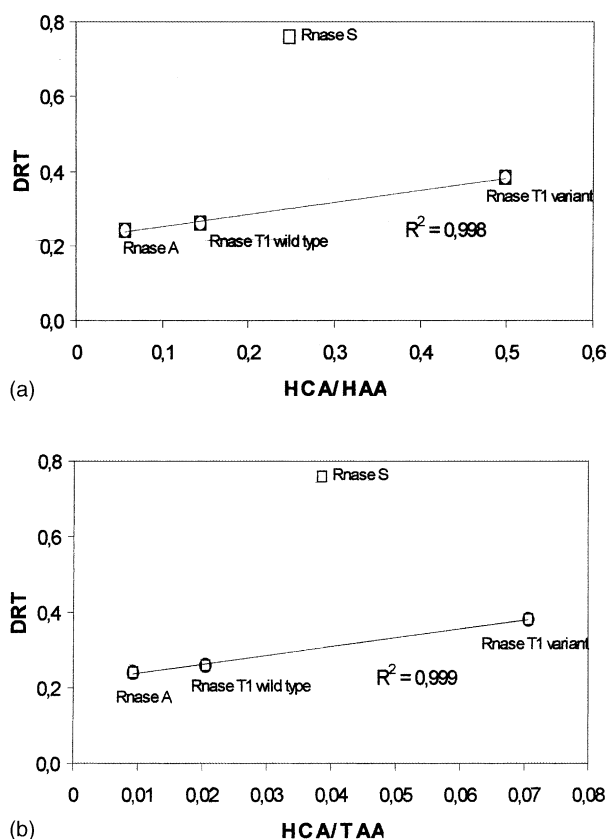
Fig. 6. Correlation level between the dimesionless retention time (DRT) of the proteins in gradient elution with butyl-Sepharose and 2 M ammonium sulfate in buffer B and hydrophobic contact area: hydrophobic accessible area ratio, HCA/HAA (a) and hydrophobic contact area: total accessible area ratio, HCA/TAA (b). Individual points are the experimental data, continuous lines represent the correlation level considering RNase T1 wild type, RNase T1 variant and RNase A.

process. RNase S is a complex that consists of two polipeptidic chains non-covalenly bound by a sulfate molecule, which increases its dynamic flexibility most probably allowing conformational changes and reorientation during the HIC process [25,26].

5. Conclusion

In the present work, it was shown experimentally that the surface hydrophobicity distribution of proteins could have important effect on their retention in hydrophobic interaction chromatography. However, there are other factors that can affect protein retention in HIC, as specific interactions between the protein and the matrix and possible conformational changes during the chromatographic process.

The thermodynamic model for hydrophobic interaction was able to represent the chromatographic behavior of three out of the four proteins studied. The parameter hydrophobic contact area correlated extremely well with the dimensionless retention time of three ribonucleases with similar average surface hydrophobicity but different surface hy-

drophobicity distribution. However, the limited number of proteins used in this study do not allow us to extrapolate our results to other proteins and to different experimental conditions.

It can be expected that the location and the size of the hydrophobic patches can be a very important factor affecting protein retention in HIC. Proteins with a large hydrophobic patch located in a concave zone would have lower retention time than if the patch were located in a convex zone. A big hydrophobic contact area implies a big hydrophobic patch accessible to the hydrophobic matrix and thus a high retention time in HIC would be expected.

The thermodynamic model developed by Melander et al. [14] can be applicable to proteins that present no conformational changes during the process. The previously developed empirical model, based on average surface hydrophobicity, can be applicable to very stable proteins with a relatively homogeneous surface hydrophobicity distribution, as ribonuclease A. The chromatographic behavior of proteins not explained by the empirical model, could probably be explained using the classical thermodynamic model.

References

[1] W. Melander, Cs. Horváth, Arch. Biochem. Biophys. 183 (1977) 200.
[2] J.L. Fausnaugh, L.A. Kennedy, F.E. Regnien, J. Chromatogr. 31 (1984) 141.
[3] A.A. Shukla, K.M. Sunasara, R.G. Rupp, S.M. Cramer, Biotechnol. Bioeng. 68 (2002) 672.
[4] G. Sofer, L. Hagel (Eds.), Handbook of Process Chromatography: A Guide to Optimization, Scale-Up, and Validation, Academic Press, San Diego, CA, 1998, p. 387.
[5] H.P. Jennissen, Int. J. Bio-Chromatogr. 5 (2000) 131.
[6] C. Tanford, J. Am. Chem. Soc. 84 (1962) 4240.
[7] K.-O. Eriksson, in: C. Janson, L. Ryden (Eds.), Protein Purification: Principles, High-Resolution Methods, and Applications, Wiley-Liss, New York, second ed., 1998, p. 283.
[8] M.E. Lienqueo, A.V. Mahn, J.A. Asenjo, J. Chromatogr. A 978 (2002) 71.
[9] M.E. Lienqueo, A. Mahn, L. Vásquez, J.A. Asenjo, J. Chromatogr. A 1009 (2003) 189.
[10] A. Mahn, M.E. Lienqueo, J.A. Asenjo, Biotechnol. Bioeng., submitted for publication.
[11] J.L. Fausnaugh, F.E. Regnier, J. Chromatogr. 359 (1986) 131.
[12] E. Hallgren, F. Kalman, D. Farnan, Cs. Horváth, J. Stahlberg, J. Chromatogr. A 877 (2000) 13.
[13] M. Jönsson, M. Skepö, F. Tjerneld, P. Linse, J. Phys. Chem. B 107 (2003) 5511.
[14] W. Melander, Z. El Rassi, Cs. Horváth, J. Chromatogr. 469 (1989) 3.

[15] G.S. Manning, Q. Rev. Biophys. 11 (1978) 179.

[16] O. Sinanoglu, in: H. Ratajczak, W.J. Thomas-Orville (Eds.), Molecular Interactions, vol. 3, Wiley, New York, 1982, p. 283

[17] H.P. Grunert, A. Zouni, M. Beineke, R. Quaas, Y. Georgallis, W. Saenger, U. Hahn, Eur. J. Biochem. 197 (1991) 203.

[18] J.A. García Dominguez, J.C. Díez-Masa, Pure Appl. Chem. 3 (2001) 969.

[19] K. Berggren, A. Wolf, J.A. Asenjo, B.A. Andrews, F. Tjerneld, Biochim. Biophys. Acta, Protein Struct. M 1596 (2002) 253.

[20] H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, P.E. Bourne, Nucleic Acids Res. 28 (2000) 235.

[21] A. Nicholls, K. Sharp, B. Honing, Proteins Struct. Funct. Genet. 11 (1991) 281.

[22] S. Miyazawa, R. Jernigan, Macromolecules 18 (1985) 534.

[23] A. Katti, Y.F. Maa, Cs. Horváth, Chromatographia 24 (1987) 646.

[24] D. Schubert, G. Schluckebier, J. Backmann, J. Granzin, C. Kisker, H.W. Choe, U. Hahn, W. Pfeil, W. Saenger, Eur. J. Biochem. 220 (1994) 527.

[25] R. Varadarajan, F.M. Richards, Biochemistry 31 (1992) 12315.

[26] P. Blackburn, S. Moore, Enzymes 15 (1982) 317.

[27] B. Lee, F.M. Richards, J. Mol. Biol. 55 (1971) 379.

[28] G. Nadig, G.S. Ratnaparkhi, R. Varadarajan, Protein Sci. 5 (1996) 2104.

[29] D.D. Leonidas, R. Shapiro, L.I. Irons, N. Russo, K.R. Acharya, Biochemistry 36 (1997) 5578.