A Simple Method for the Estimation of Protein Retention in Hydrophobic Interaction Chromatography Under Different Operation Conditions

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Abstract: Protein behavior in Hydrophobic Interaction Chromatography using different chromatographic conditions was investigated. A linear correlation was found between protein retention time on different matrixes and different initial elution salt concentrations. Mathematical correlations between retention times under different chromatographic conditions were obtained and validated, which can be used in process design and scale-up.

Keywords: Hydrophobic interaction chromatography, chromatographic conditions, dimensionless retention time.

INTRODUCTION

Hydrophobic Interaction Chromatography (HIC) is one of the key techniques used for protein purification and also largely used in industrial operations. 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 by decreasing the ionic strength in the mobile phase in a linear gradient [1]. This technique shows a similar capacity to Ion Exchange Chromatography and a similar level of resolution.

HIC is widely used in the downstream processing of proteins, and a huge effort has been done in order to elucidate the way the operating conditions affect protein behaviour in HIC [2 -10]. The main system factors that affect protein retention in HIC are concentration and type of salt [2-6] and type of matrix [2, 7, 8, 11], while the main protein property exploited is surface hydrophobicity [12-15]. Many attempts have been carried out to find the relation between protein hydrophobicity, estimated by different methods, and protein retention time in HIC [17-19]. There are also many studies about the way system variables can affect protein retention in HIC [8, 20-22]. Recently it has been shown that the statistically significant system variables are salt properties (measured as molal surface tension increment of the salt σ_s), ionic strength of the initial eluent and substitution degree of the resin (as given by the manufacturer) [23]. Despite the growing knowledge about system and protein characteristics that affect protein behavior in HIC, it would be very useful to elucidate if any relationship exists between protein behavior using different operating conditions.

In this paper the relationship between the chromatographic behavior of proteins under different chromatographic conditions was studied. Our aim was to find simple correlations that could be used to predict a protein's retention time under certain operational conditions with a reduced number of experiments. These relations could probably be useful to refine a purification process and we think they could help to choose the best operating conditions in a purification process design.

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MATERIALS AND METHODOLOGY

Ten well-characterised proteins were used: conalbumin (con), ribonuclease A (rib), ovalbumin (ova), chymotripsinogen A (chy), lysozyme (lys), α-lactalbumin (lac), myoglobin (myo), α -chymotripsin (chn), concanavalin A (coa), α -amylase (amy), from Sigma Chemical Co. (St Louis, Mo, USA). Water, prepared from a Milli-Q water cleaning system (Millipore, Bedford, MA, USA) and analytical-reagent grade ammonium sulphate and sodium chloride was used in the preparation of the elution buffer (buffer B).

The high-performance liquid chromatography system employed consisted of a FPLC (GE Healthcare, Uppsala, Sweden) equipped with a 200-µl injection loop. The chromatographic matrixes were Phenyl-Sepharose 6 Fast Flow and Butyl-Sepharose Fast Flow (a gift of GE Healthcare, Uppsala, Sweden) packed in 1-mL columns. The experiments were performed at room temperature, using a flow rate equal to 0.75 ml/min and a 10 CV decreasing elution gradient. A decreasing salt gradient was used, with a steepness of 7.5% B/min (a 10 CV gradient). The initial eluent was Bis-Tris 20 mM pH 7.0 plus a maximum salt concentration of 2 or 1M Ammonium sulphate or 4 or 2M Sodium chloride (donated by Amersham Pharmacia Biotech, Uppsala, Sweden). The final eluent was Bis-Tris 20-mM pH 7.0 (Buffer A). All buffers were filtered through 0.22-um Millipore filters after preparation and degassed with helium for 10 min. Protein solutions were prepared to contain approximately 0.5 mg/ml dissolved in the initial eluent. All samples were filtered through 0.22-µm Millipore filters.

Different experiments were performed using different combinations of type of matrix (Butyl or Phenyl-Sepharose), salt type (ammonium sulphate or sodium chloride) and initial salt concentration (varying from 1 to 4 M). The experimental conditions tested were the following: Phenyl-Sepharose-1M Ammonium sulphate, Phenyl-Sepharose-2M Ammonium sulphate, Phenyl-Sepharose-2M Sodium Chloride, Phenyl-Sepharose-4M Sodium Chloride, Butyl-Sepharose-1M Am-

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monium sulphate, Butyl-Sepharose-2M Ammonium sulphate. Retention time of the proteins under the different conditions was related. In order to describe the chromatographic behaviour we used the parameter "Dimensionless Retention Time" (DRT), defined as follows [11, 14]:

$$DRT = \frac{RT - t_0}{t_f - t_o} \quad (1)$$

where RT is the time corresponding to the peak maximum in the chromatogram, t_o is the time corresponding to the start of the elution gradient and t_f is the time corresponding to the end of the salt gradient.

RESULTS AND DISCUSSION

The dimensionless retention times of nine standard proteins on two different matrixes were compared. Fig. **1** shows the relationship between DRT of proteins on Butyl-Sepharose and that on Phenyl-Sepharose with 2M-ammonium sulphate in buffer B (beginning of the elution gradient). The linear relationship obtained has a very high correlation level (r = 0.99) and shows that there is virtually no difference in the elution order of the proteins when using Butyl or Phenyl Sepharose. Phenyl-Sepharose shows a somewhat stronger hydrophobic interaction between proteins and matrix. Retention times are 9-10 % higher on this matrix. The correlation obtained between DRT in Butyl Sepharose and that obtained in Phenyl Sepharose is given by equation (2). Given the high correlation level obtained, this correlation could be used to predict the DRT of a protein in one matrix starting from that obtained using another one.

$$DRT_{\text{Butyl Sepharose}} = 1.09 * DRT_{\text{Phenyl Sepharose}} - 0.14 \quad (2)$$

Fig (1). Comparison of the DRT of nine proteins on different matrixes, Butyl and Phenyl Sepharose. Elution was achieved with a decreasing gradient starting with 2M Ammonium sulphate. $DRT_{BS\text{-}2M}$: dimensionless retention time on Butyl Sepharose, $DRT_{PS\text{-}2M}$: dimensionless retention time on Phenyl Sepharose.

The dimensionless retention times (DRT) of the nine standard proteins with different initial concentration of elution salt were investigated. Fig. **2** shows the relationship between DRT using 1M (PS-1M) and 2M (PS-2M) ammonium sulphate on a Phenyl-Sepharose matrix. Fig. **3** gives the salt concentration at the point of elution of the peak maximum. The correlation between DRT (Fig. **2**) using ammonium sulphate at two different initial concentrations was almost linear, showing an acceptable correlation level (r = 0.95). However two proteins were not retained when 1 M ammonium sulphate was used: ribonuclease A and myoglobin. This situation can be explained by the low surface hydrophobicity of these proteins [24]. The correlation level between salt concentration at elution (SCE) using different ammonium sulphate initial concentrations (Fig. **3**) was high, with r = 0.98. The slope of the linear equation obtained was very close to 1.0 (slope = 0.99) and the intercept was close to 0 (intercept = 0.04), hence the initial ammonium sulphate concentration only affects the number of proteins retained by the matrix, and not the elution order of proteins nor their retention time.

Fig (2). Comparison of DRT of proteins using ammonium sulphate at different initial concentration: 1 M ammonium sulphate v/s 2 M ammonium sulphate on Phenyl Sepharose matrix.

Fig (3). Salt concentration at elution (SCE) of proteins using ammonium sulphate.

Fig. **4** shows the relationship between DRT with 2M (PS-2M NaCl) and 4M (PS-4M NaCl) sodium chloride on Phenyl-Sepharose matrix. Fig. **5** gives the salt concentration at elution peak maximum (SCE). For sodium chloride this correlation was also satisfactory, but three proteins retained with 4 M sodium chloride were not retained when using 2

M: ribonuclease A, myoglobin and concanavalin A. This indicates that ammonium sulphate promotes a much stronger hydrophobic interaction between proteins and hydrophobic resins than sodium chloride, as can be expected from the Hofmeister's lyotropic series [11]. On the other hand, five proteins showed very similar DRT when using 4M sodium chloride; hence this salt is not a good candidate for enhancing hydrophobic interactions in HIC, as it shows lower selectivity. A certain operating condition shows high selectivity if proteins with different physicochemical properties show different DRTs. In this case, proteins that have different surface hydrophobicity showed similar DRT when 4M sodium chloride was used.

As well as with different matrixes, the DRT of a protein using a given initial salt concentration can be predicted starting form the knowledge of DRT with another initial salt concentration. The correction factors for initial salt concentration, which are given by equations (3) and (4), could be used in a purification process design, in order to choose the most appropriate operation conditions for the separation of a target protein.

Ammonium sulphate:

$$DRT_{2M} = 0.60 * DRT_{1M} + 0.38 \quad (3)$$

Sodium chloride:

$$DRT_{4M} = 0.32 * DRT_{2M} + 0.51 \quad (4)$$

Fig. (4). Comparison of DRT of proteins using sodium chloride at different initial concentrations: 2 M Sodium Chloride v/s 4 M Sodium Chloride on Phenyl Sepharose matrix.

The correlation level between DRT using the two salts, ammonium sulphate and sodium chloride, at similar ionic strength, was not high (Fig. **6**). The correlation coefficient (r) was only equal to 0.74. The different behaviour of proteins when using these two different salts in an elution gradient can be interpreted considering the way they interact with the solvent. The effect of salt type in protein retention is related to the molal surface tension increment of the salt, following the Hofmeister [25] series [3, 6]. Salts to the left in the series, which increase the surface tension the most, give strongest hydrophobic interaction. Ammonium sulphate promotes a stronger hydrophobic interaction than sodium chloride, due to its bigger surface tension increment. In addition, it has been reported that the sulphate anion stabilises the native structure of proteins in solution, in contrast to the chloride anion, which shows a destabilising effect [4]. Hence ammonium sulphate allows a higher selectivity than sodium chloride in HIC.

Fig. (5). Salt concentration at elution (SCE) of proteins using sodium chloride in elusion buffer.

Therefore, it would not be possible to correctly estimate DRT using sodium chloride to build the elution gradient, starting from DRT obtained with ammonium sulphate, given the low correlation level observed.

Fig. (6). Comparison of the DRT of nine proteins using different type of salt to build the elution gradient. The matrix used was Phenyl Sepharose.

In order to validate the correlations presented in the previous sections (equations 2 to 4), the DRT of a protein not considered in the previous task (ovalbumin) was estimated under different chromatographic conditions, using equations 2-4. The results are given in Table **1**. The deviation values obtained in all cases were very low, varying from 1.2 % to 3.9 %. This indicates that it would be possible to predict a protein's retention time under certain operating conditions starting from the DRT obtained under another conditions. If this result is considered for a purification process design, experimental work could be minimized in choosing the op-

Table 1. Validation of the Correlations

Operating Conditions			[e]D (%)
Type of Matrix			
[a] DRT $_{\text{Phenyl Sepharose}}$	[a] DRT $_{\text{Butyl Sepharose}}$	[b] Predicted DRT $_{\text{Butyl Sepharose}}$	
0.567	0.498	0.478	3.9
Ammonium Sulphate initial concentration			
[a] DRT $_{1\ M}$	[a] DRT $_{2\ M}$	[c] Predicted DRT $_{2\ M}$	
0.323	0.567	0.574	1.2
Sodium Chloride initial concentration			
[a] DRT $_{2\ M}$	[a] DRT $_{4\ M}$	[d] Predicted DRT $_{4\ M}$	
0.724	0.756	0.742	1.9

[a] DRT of ovalbumin determined experimentally.
[b] DRT estimated using equation (2).
[c] DRT estimated using equation (3).
[d] DRT estimated using equation (4).
[e] $D = Deviation = \frac{\left|DRT_{experimental} - DRT_{prediced}\right|}{DRT_{experimental}} * 100$

timal operating conditions for the separation of a target protein.

CONCLUSIONS

The effect of chromatographic conditions (type of matrix, type and initial concentration of salt) on a proteins' behavior in HIC was studied for Butyl-Sepharose and Phenyl-Sepharose and for ammonium sulphate and sodium chloride. Elution order of proteins was not affected by the type of matrix; retention time is about 10 % higher on Phenyl-Sepharose. Initial salt concentration does not affect the elution order of proteins, showing a good agreement between the DRT obtained with 1M and 2M ammonium sulphate or 2M and 4M sodium chloride in a linear decreasing gradient. Proteins showed a different behaviour when using a different type of salt. Selectivity was reduced when using sodium chloride, and fewer proteins were retained, hence ammonium sulphate should be preferred as an elution salt.

Simple linear correlations were obtained between DRTs under different chromatographic conditions, which could probably be used in a purification process design, in order to reduce the experimental work. Besides, we believe they could help to choose better operating conditions in an established purification process.

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