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Retention behaviour of proteins under conditions of column overload in hydrophobic interaction chromatography

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Abstract

The sample solvent has a significant effect on band profiles in preparative liquid chromatography.This effect has been described by Guiochon and Jandera in non-aqueous reversed-phase high-performance liquid chromatography. Similar behaviour was observed in hydrophobic interaction chromatography of cytochrome *c* and lysozyme under conditions of column overloading. If the sample solvent contains a lower concentration of salt than the starting mobile phase in gradient hydrophobic interaction chromatography, the protein peak will be broken to two peaks. One peak is longer-retarded, another is shorter-retarded by hydrophobic interaction. The latter changes its retention time with the different injection volumes. This results in a loss in the recovery of protein in preparative chromatography. The possible mechanism of the band-splitting was investigated.

Keywords: Retention behaviour; Column overloading; Preparative chromatography; Proteins; Cytochrome *c*; Lysozyme

1. Introduction

In preparative liquid chromatography, the highest production rate and maximum throughput at a defined purity with the lowest cost is the principal consideration. To maximize the throughput, the amount of sample injected should be as large as possible. Theoretical considerations on mass and volume overloading are frequently discussed [1–5]. For solutes with poor solubility in the mobile phase this approach is not applicable. "Dry injection" [6] of solid sample has been used to increase the load amount, although the instrumental prerequisites are complex. For protein samples this method is not applicable. Multiple injections are commonly used particularly in reversed-phase liquid chromatography (RPLC) and hydrophobic interaction chromatography (HIC) [7]. Dissolving the sample in a solvent in which it is more soluble than in the mobile phase is used to overcome this obstacle. However, in many cases the chromatographic elution strength of these solvents is stronger than that of the mobile phases [8]. Jandera and Guiochon [9] pointed out that this situation may cause the deformation and splitting of the band profiles in non-aqueous reversed-phase chromatography. This should be avoided in preparative chromatography, particularly at high sample loading.

HIC has received increasing attention [10–16] because it is very effective for the separation and purification of biopolymers. HIC can be directly linked to ion-exchange chromatography (IEC), size exclusion chromatography (SEC), affinity chromatography (AFC) and other purification steps. Proteins

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and other biopolymers are loaded at high salt concentration (up to 3 *M* ammonium sulphate) and elution is effected by buffers with a low salt concentration. Since some proteins are only poorly soluble in these solutions, they have to be dissolved in buffers with salt concentrations lower than the equilibration buffer. When several chromatographic steps are connected together the protein may be present in low salt buffer from the previous step. Injection of these protein solutions onto a preparative HIC column is not recommended. In analytical applications, the sample in low salt buffer causes only minor problems, because the small sample volume is rapidly diluted with mobile phase. However, in preparative chromatography, the large sample volume can not be thoroughly mixed with the mobile phase within a short time. The strong solvent plug causes a considerable band-profile deformation. Sometimes split peaks are observed, which result in loss of recovery.

In this paper the retention behaviours of cytochrome *c* and lysozyme under overloading conditions in HIC are investigated.

2. Experimental

2.1. Equipment

An LC-6A liquid chromatograph (Shimadzu, Kyoto, Japan) fitted with a Rhendyne injector (loop volume 20–1000 μl) was used, consisting of two pumps (LC-6A), a system controller (SCL-6B), a variable wavelength UV–VIS detector (SPD-6AV).

2.2. Column

A stainless-steel column was packed by an upward slurry technique using a 1224A packing machine (Chemico, Osaka, Japan). A silica-based HIC packing material with ether chain and keto end group described by Chang et al. was used [17,18]. The column size was 50×2 mm I.D.

2.3. Chemicals

All proteins used in this work were purchased from Sigma (St. Louis, MO, USA). Inorganic salts were purchased from the Xi'an Chemical Factory (Xi'an, China).

2.4. Chromatography conditions

Chromatography of proteins was performed using gradients made up from mixtures of 3 *M* ammonium sulphate, 20 m*M* potassium dihydrogen phosphate (starting buffer) and 20 m*M* potassium dihydrogen phosphate (end buffer) with pH values of 7.0. The flow-rate was 0.2 ml/min. The column effluent was monitored at 280 nm.

3. Results and discussion

If a small volume (10 μl) of lysozyme and cytochrome *c* is injected as a very dilute solution, no matter how concentrated the buffer is, the peak is not split. This was observed in analytical applications. If larger samples with good solubility in the mobile phase were injected, the retention times of the bands became smaller, but the protein was still eluted as single peak. Different volumes of cytochrome *c* (30 mg/ml) dissolved in the starting buffer (3.0 *M* ammonium sulphate) were injected into the HIC column. The retention times of the peak maximum decreased with increasing sample volumes (Fig. 1). The shift of the peak maximum is explained by concentration overload. Dilution of the sample with mobile phase during injection depends on the volume. The higher the sample volume the lower the dilution [19].

Fig. 2 shows the results of the experiment with a lower cytochrome *c* concentration (7.5 mg/ml) dissolved in a buffer with lower salt concentration (2.25 *M* ammonium sulphate) than the mobile phase. The retention time of the main band decreased with the increasing injection volume. The shift of the peak is explained by the same effect as mentioned for the experiments shown in Fig. 1. However, when the sample size was further increased (profile 1 and 2), the retention time and area of the peaks did not change, but an additional, nearly non-retarded peak was observed. This peak could not be explained by the sample buffer, which differed from the mobile phase buffer. (1) The additional peak had a red colour and (2) when the fraction collected during the

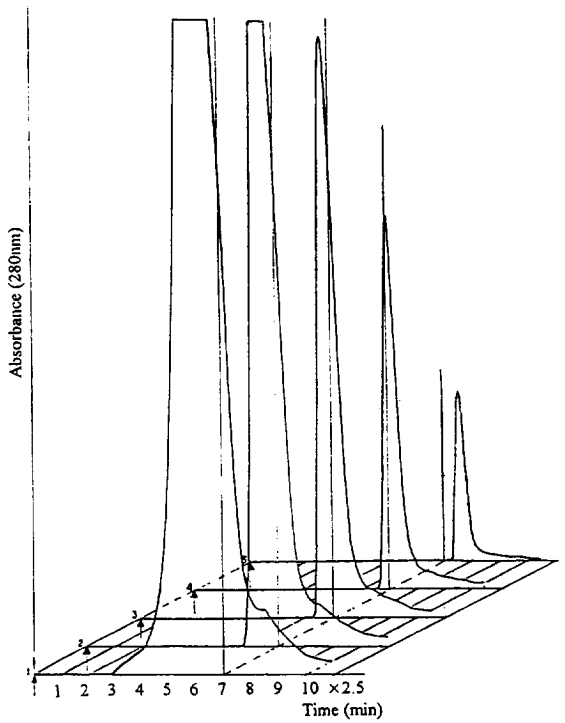

Fig. 1. HIC of cytochrome *c* with different sample volumes. Gradient from 3 *M* $(NH_4)_2SO_4$–20 m*M* K_2HPO_4, pH 7.0 (A) to 20 m*M* K_2HPO_4, pH 7.0 (B) in 25 min. Concentration of cytochrome *c*: 30 mg/ml in solution A. Sample volumes: 1=200 μl; 2=100 μl; 3=50 μl; 4=25 μl; 5=10 μl.

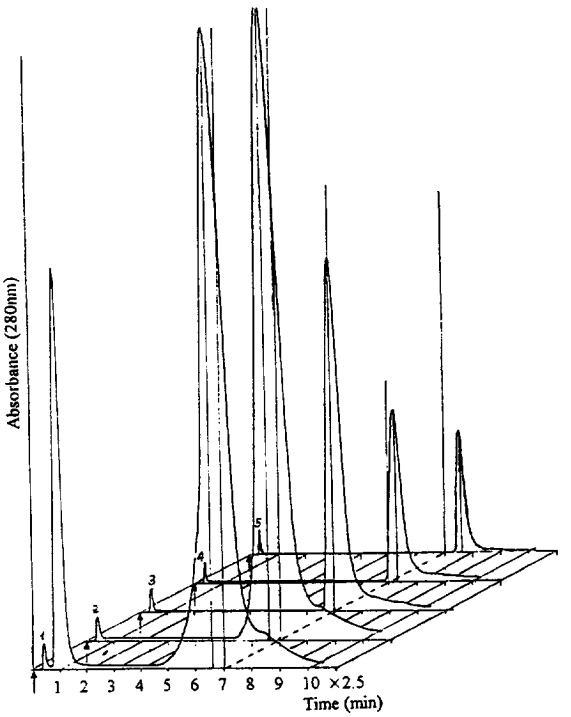

Fig. 2. HIC of cytochrome *c* with different sample volumes. Gradient as in Fig. 1. Concentration of cytochrome *c*: 7.5 mg/ml in a solution of 2.25 *M* $(NH_4)_2SO_4$. Sample volumes: 1=400 μl; 2=300 μl; 3=100 μl; 4=40 μl; 5=10 μl.

elution of this peak was re-injected, its retention time was identical to that of cytochrome *c*; (3) The UV spectra of this fraction were identical to cytochrome *c* spectra in 2.25 *M* ammonium sulphate solution. Later observations proved that the minor peak contained cytochrome *c* and that it was generated by peak splitting.

Although the sample size in profile 1 and 2 (Fig. 2) was different, the area of the retained peak remained nearly constant. The splitting phenomenon suggested that the entire cytochrome *c* could not be retained when the sample volume was higher than 300 μl. The non-retained fraction eluted closely in this case. This volume can be considered as the critical injection volume.

Similar behaviour was observed with more diluted samples (2 mg/ml cytochrome *c*) dissolved in water (Fig. 3). The retained peak increased with increasing sample volume, and the retention times of the peak maximum were identical. At the same time the unretarded band increased with increasing sample size both in band area and in retention time. The major fraction of the applied protein was found in the flow-through.

Bian et al. [20] as well as Staby and Mollerup [21] described the dependence between retention time and salt concentration. They observed that at low salt concentration the retention time decreased with increasing salt concentration. After passing a minimum value it increased again. According to Bian et al. proteins can be strongly retarded in the column under opposite conditions when the salt concentration in the mobile phase is either low or high. When the concentration of salt in the mobile phase is small enough, the retention of proteins decreases with increasing salt concentration, which corresponds to the mechanism of IEC. When the concentration of salt is high, the retention of the proteins increases with increasing salt concentration. This

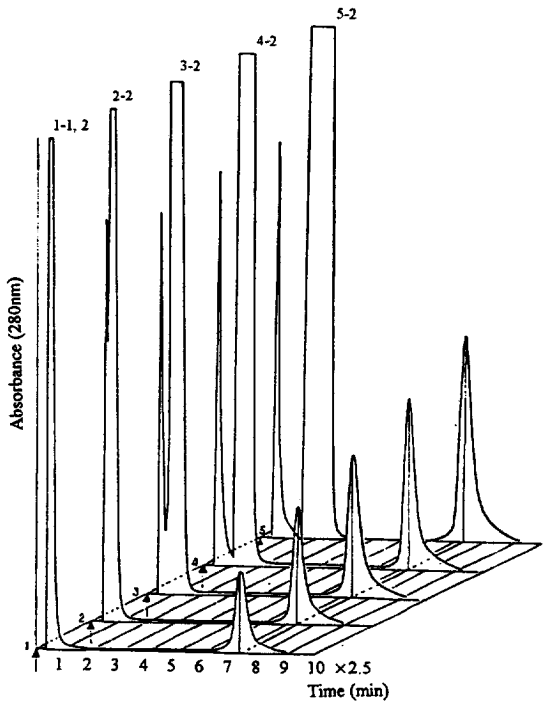

Fig. 3. HIC of cytochrome *c* with water as sample solvent in different sample volumes. Gradient as in Fig. 1. Concentration of cytochrome *c*: 2 mg/ml in water. Sample volumes: 1=100 μl; 2=200 μl; 3=400 μl; 4=600 μl; 5=1000 μl.

behaviour corresponds to the mechanism of HIC. According to Staby and Mollerup, the capacity factor for a protein can be related to the activity of the ion. Activity in relation to the ionic strength has a minimum which is different from the very dilute solution. Thus, a protein can be more retained in a low salt buffer compared to a buffer with slightly higher salt concentration. In small samples the water plug is rapidly eliminated by diffusion into the mobile phase. When a large sample is dissolved in water, a large water plug is present during sample application. The elution strength of this local eluent is stronger than that of the mobile phase. Therefore a certain fraction of the sample is not retarded and is eluted close to the dead time. With increasing sample size, diffusion becomes more and more difficult. The water plug has a very low salt concentration. Ion exchange may take place and a particular fraction of the sample will be retained. This is the reason why the first peak changed its retention time with increasing injection volume (peaks 2, 3, 4, 5 in Fig. 3).

The two cytochrome *c* bands underwent two different chromatographic separation processes. The sample fraction in the water plug was retained by an ion-exchange mechanism. At the front and end of the water plug the corresponding sample fraction was exposed to lower elution strength and it was retained on the stationary phase by hydrophobic interaction. When a larger sample was injected, both the water plug and the boundary zones between the plug and the mobile phase also became larger. More sample component was retarded, in both the first and second peak.

Fig. 4 shows the results for lysozyme. The amount of protein in the sample in these two injections was identical, but the sample volumes were different. The results observed were similar to cytochrome *c* results.

From these results, it could be concluded that the sample solution has a considerable influence on the retention of proteins in HIC. Even small differences in salt concentration between the mobile phase and the sample solution significantly influence the retention behaviour of proteins. In preparative HIC this could result in loss of proteins (Fig. 5).

Cytochrome *c* was dissolved in different concentrations of salt solutions and was injected into the column with the same sample volume and con-

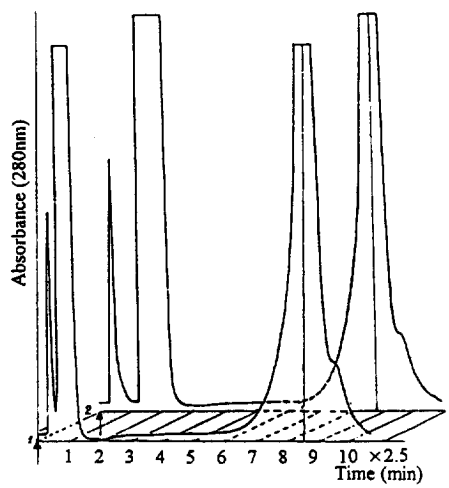

Fig. 4. HIC of lysozyme with same amounts but different concentrations. Gradient as in Fig. 1. Concentration of lysozyme in water: 8 mg/ml for profile 1; 4 mg/ml for profile 2. Sample volumes: 1=350 μl; 2=700 μl.

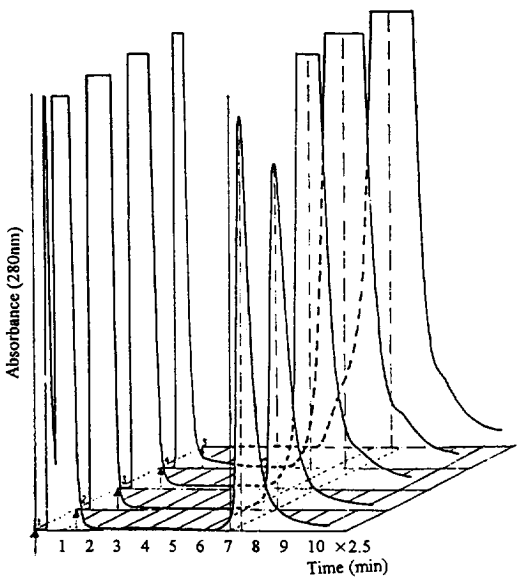

Fig. 5. HIC of cytochrome *c* in the solution of different $(NH_4)_2SO_4$ concentrations. Concentration of cytochrome *c*: 15 mg/ml. Sample volume: 400 μl. Concentration of $(NH_4)_2SO_4$ (in mol/l): 1=0; 2=0.75; 3=1.5; 4=2.25; 5=3.0.

centration. If the sample solution did not contain ammonium sulphate, the retarded peak showed the longest retention, but the amount of retarded cytochrome *c* was smallest. The major fraction of the protein was in flow-through. With increasing salt concentration, the retarded amount of cytochrome *c* increased, but the retention time decreased. If the sample solution had the same salt concentration as the mobile phase, all the cytochrome *c* was retained by the column without any loss in the flow-through. One can conclude that differences in salt concentration between the sample solution and the mobile phase should be avoided in HIC of protein under overloading conditions.

In preparative HIC of protein the sample may be derived from an extraction or from previous purification steps. Before sample application onto the HIC column, the salt concentration of the sample should be carefully examined. If the protein has poor solubility in the buffer, such as 3 *M* ammonium sulphate, it is recommended that another type of salt which has similar elution properties be used.

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