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Journal of Chromatography A, 664 (1994) 27–32

JOURNAL OF CHROMATOGRAPHY A

Hydrophobic interaction chromatography of proteins on an Alkyl-Superose column

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(First received February 2nd, 1993; revised manuscript received November 23rd, 1993)

Abstract

The effects of ammonium sulphate, sodium acetate and sodium citrate on the retention of proteins were investigated under gradient conditions on an Alkyl-Superose column. It was found that the salts exert different effects on the retentions of hydrophobic and hydrophilic proteins. Retention data were evaluated according to the linear solvent strength theory of gradient elution. The calculated ln k–salt molality relationships revealed specific and complex effects of the different salts on the retention behaviour of the proteins having different hydrophobicities.

1. Introduction

Two high-performance chromatographic techniques are available for separating biopolymers by virtue of their differing hydrophobicities, reversed-phase liquid chromatography (RPLC) and hydrophobic interaction chromatography (HIC). In both techniques hydrophobic functional groups are bound to the support, undergoing a London-type dispersion interaction with the hydrophobic regions or patches on the surface of the biopolymers. However, there are considerable differences between the two methods as regards the separation of biologically active substances, *e.g.*, proteins. In contrast to the strongly hydrophobic RPLC stationary phases, in HIC packings the functional groups are more sparsely distributed, producing moderately hydrophobic surfaces and resulting in a mild hydrophobic interaction.

Retention depends on the type of stationary phase (type of ligand, chain length of ligand, ligand density) [1–5] and on the characteristics of the mobile phase such as type of salt, initial salt concentration, gradient time, flow-rate, temperature, pH and addition of the organic modifiers [6–15].

In a previous study [16] we examined the effect of the stationary phase on the retention of proteins by comparing three commercially available HIC columns of different types. It was shown that the effect of the salt type used differed considerably on the various stationary phases. In this paper, further results are given on

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0021-9673/94/$07.00
SSDI 0021-9673(93)E1258-2

the effect of different salts on the separation of proteins on an Alkyl-Superose column.

2. Experimental

2.1. Instrumentation

A fast protein liquid chromatographic (FPLC) system (Pharmacia–LKB, Uppsala, Sweden) was used, consisting of an LCC-500 controller, two P-500 pumps, a UV-M monitor, an MV-7 injector with a 25-μl sample loop and a REC-482 two-channel detector. The chromatograms were acquired, evaluated and stored by a Labchrom chromatographic data station (Labinform, Budapest, Hungary), consisting of a dual-channel interface card and software running on a standard IBM PC/AT computer.

An Alkyl-Superose HR 10/10 column (Pharmacia–LKB) containing neopentyl ligands on an agarose support, with particle size 10 μm, was used.

2.2. Materials

Analytical-reagent grade ammonium sulphate, trisodium citrate, sodium acetate, phosphoric acid and sodium hydroxide were purchased from Reanal (Budapest, Hungary). Distilled water was prepared by double distillation in the laboratory.

The proteins used were the same as studied previously [16]. Cytochrome *c* (CYT), ribonuclease A (RNA), ovalbumin (OVA), lysozyme (LYS) α-chymotrypsinogen A (CHY) were obtained from Sigma (St. Louis, MO, USA). It is worth noting that this set of proteins is routinely used in studies on HIC because their surface hydrophobicities (termed here simply hydrophobicities) differ widely; they are listed above in order of increasing hydrophobicity.

2.3. Procedures

Gradient measurements were carried out with a linear inverse gradient from 2 to 0 *M* salt (0 to 100% B) in 0.05 *M* sodium phosphate buffer with pH adjusted to 7.0 with 0.1 *M* NaOH. With sodium acetate, the starting eluent contained 5 *M* salt in order to achieve retention in the same range as with the other two salts. This initial concentration was determined experimentally and was in good agreement with results in the literature [17,18]. The flow-rate was 0.3–0.5 ml/min. Samples were prepared by dissolving about 1 mg of each protein in 1 ml of doubly distilled water prior to injection. Each sample was chromatographed at four different gradient times (10, 15, 20 and 25 min). All the measurements were repeated at least twice and the average data are used throughout this paper.

3. Results and discussion

First gradient measurements were carried out on the Alkyl-Superose column with three different salts as eluent constituents using the protein samples given above. In order to compare the retention data of different runs, the apparent retention factors, k_g, were calculated for each run according to the literature [19,20]:

$$k_g = (t_g - t_m)/t_m \quad (1)$$

where t_g is the retention time under gradient conditions and t_m is the mobile phase hold-up time.

The data in Table 1 indicate that in most instances not only the absolute but also the relative retention, *i.e.*, the selectivity, is also changed by varying the type of the salt used in the eluent. For better comparison and evaluation of the effect of salt type, the apparent capacity factors, k_g, measured at the same gradient times in the three salt solutions were plotted against each other.

In Fig. 1, the k_g values measured in sodium citrate are plotted against those measured in ammonium sulphate. It can be seen that the retentions of the different proteins did not change similarly with variation in the type of eluent. CHY and LYS have higher retention in ammonium sulphate but RNA and CYT show significantly higher retention in sodium citrate.

Table 1
Apparent retention factors (k_g) of proteins obtained with different eluents

Gradient former[a]	t_G (min)	k_g				
		CHY	LYS	OVA	RNA	CYT
Ammonium sulfate (2–0 *m*)	10	0.891	0.677	0.469	0.384	0.004
	15	1.108	0.869	0.656	0.448	0.005
	20	1.305	1.110	0.768	0.532	0.007
	25	1.518	1.240	0.895	0.576	0.009
Sodium citrate (2–0 *m*)	10	0.727	0.647	0.544	0.552	0.207
	15	0.968	0.943	0.834	0.811	0.305
	20	1.270	1.233	1.083	1.068	0.389
	25	1.554	1.555	1.324	1.304	0.503
Sodium acetate (5–0 *m*)	10	1.038	0.615	0.274	0.109	0.064
	15	1.417	0.847	0.380	0.148	0.073
	20	1.699	1.069	0.463	0.195	0.092
	25	1.978	1.230	0.542	0.230	0.106

For conditions, see text.
[a] *m* = Molality.

As the former are known to have higher surface hydrophobicity and the latter have relatively hydrophilic characteristics, this means that replacing ammonium sulphate with sodium citrate, providing nearly the same average retentions for the samples, decreases the retention of the hydrophobic and increases those of the hydrophilic proteins.

In Fig. 2, the k_g values measured in sodium acetate are plotted against those measured in

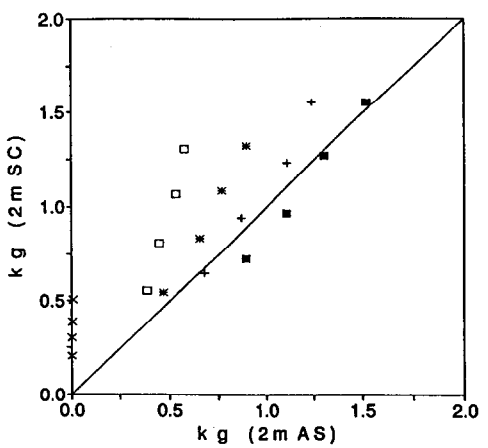

Fig. 1. Plots of apparent retention factors, k_g, of proteins determined in sodium citrate (SC) against those determined in ammonium sulphate (AS). The points represent data obtained at the same gradient time (10, 15, 20 and 25 min). ■ = CHY; + = LYS; * = OVA; □ = RNA; × = CYT.

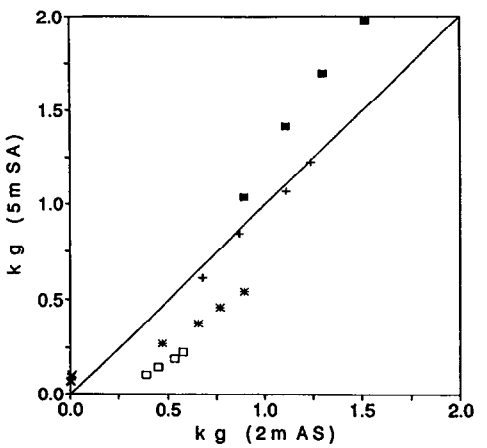

Fig. 2. Plots of apparent retention factors, k_g, of proteins determined in sodium acetate (SA) against those determined in ammonium sulphate (AS). The points represent data obtained at the same gradient time (10, 15, 20 and 25 min). Symbols as in Fig. 1.

ammonium sulphate. Although the comparison shows smaller differences between the hydrophobic and the hydrophilic proteins, the pattern of the data indicates an opposite tendency. In this instance substituting sodium acetate for ammonium sulphate increases the retention of the hydrophobic and decreases those of the hydrophilic proteins when using eluents providing nearly identical average retentions for the components.

It is well known from other studies [6–10] that changing the salt type is one of the most important parameters for modulating retention and selectivity in HIC. It has been demonstrated recently that the effect of the salt type used differs considerably on various stationary phases [16]. In order to characterize an HIC column for a given separation, the effect of salt type should be determined with different salts.

To evaluate retention and selectivity in HIC under different conditions, either isocratic or gradient measurements have been carried out and reported in the literature. As isocratic measurements are laborious and time consuming, we used the gradient data for the determination of the retention factor as a function of salt molality usually determined under isocratic conditions.

In HIC, the retention of the components can be described as

$$\ln k = \ln k_w + Sm \quad (2)$$

where m is the molality of the salt and $\ln k_w$ and S are the intercept and slope of the profile respectively, considered to be constants in a given chromatographic system. When a linear gradient is applied, the characteristic constants of Eq. 2 can be calculated from the results of two runs differing only in the duration of the gradient in accordance with the linear solvent strength (LSS) theory [19,20]. The applicability of the LSS theory under HIC conditions has been proved experimentally in some instances [21].

In addition, under LSS conditions the retention factor at the half of the column is characteristic for each component. This median value k_m determines the resolution with gradient elution just as does k in an isocratic separation. This k_m value corresponds to an average salt molality m_m in each gradient run. This pair of values furnish a good approximation for the description of the ln k–solvent strength relationship even if the retention profile is curved, *i.e.*, Eq. 2 is not valid. These median values can be calculated by using the fundamental equations of gradient elution described by Snyder and co-workers [21–23].

From the data for four different gradient runs, six independent pairs can be formed. If the retention times could be measured without error and the LSS conditions held strictly true, one S and ln k_w and four k_m and m_m values would be obtained for each protein. However, under real conditions the use of four gradient data will result in six S and ln k_w and twelve k_m and m_m values, which makes it possible to explore the error of the calculated values and the validity of the LSS model used, *i.e.*, the linearity of the profiles.

For the calculations, a program was written in C and was compiled and run on the computer used for the data acquisition. Although the applicability of the LSS model under HIC conditions has been demonstrated [21], we checked the predicted retention times in ammonium sulphate under isocratic conditions. The salt concentration of the eluent was varied in the range spanned by the m_m values calculated. As good agreements were found for all the proteins investigated, the retention profiles calculated from the data obtained with the other two salts were accepted without further examination. In Figs. 3 and 4 the results obtained for CHY and LYS are presented. It is seen that the measured value (triangles) fit the calculated profile (solid line) well and the median values (squares) do not indicate any significant curvature.

The characteristic constants of Eq. 2 for the proteins in the different eluents are presented in Table 2. These values are averages of the six values calculated from the independent pairs of retention times. The examination of the predicted profiles reveals that the effect of the salt change is more complex than for gradient measurements. Replacing ammonium sulphate with sodium citrate results in an increase in retention at identical concentration but the relative positions of the components are also altered. All the slope values are increased and the intercepts are

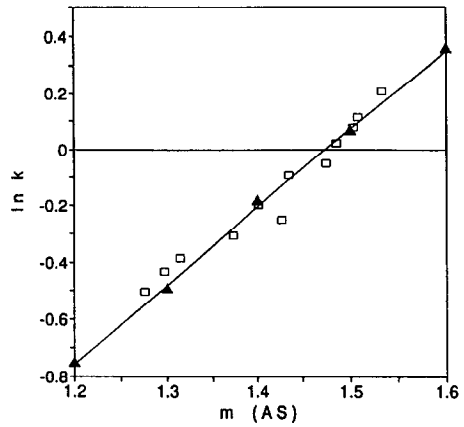

Fig. 3. Retention profile (solid line) of α-chymotrypsinogen A (CHY) calculated from the LSS model of gradient elution. AS = Ammonium sulphate. □ = Characteristic calculated median values; ▲ = retention data obtained experimentally. For details, see text.

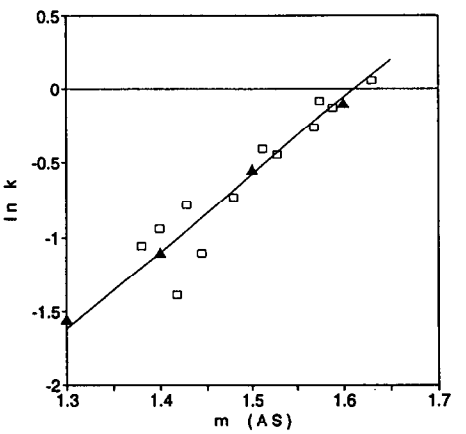

Fig. 4. Retention profile (solid line) of lysozyme (LYS) calculated from the LSS model of gradient elution. AS = Ammonium sulphate. Symbols as in Fig. 3. For details, see text.

decreased. The changes in the parameters are smaller for the hydrophobic proteins (CHY, LYS) and larger for the hydrophilic proteins.

The effect of sodium acetate is different. The retention profiles of the hydrophobic proteins are highly flattened and the intercepts are larger than with ammonium sulphate, but the effect for the hydrophilic proteins is very similar to that with sodium citrate.

4. Conclusions

The effect of the salt type on the retention of standard proteins on an Alkyl-Superose column was investigated by gradient measurements. It was found that the salts have different effects on the retention of the hydrophobic and hydrophilic proteins. At salt concentrations giving nearly the same average retentions of the components, sodium acetate in comparison with ammonium sulphate increased the retention of the hydrophobic and decreased those of the hydrophilic proteins. With sodium citrate the retention of the hydrophilic proteins increased whereas that of the hydrophobic proteins decreased. The relative retentions, *i.e.*, selectivities, were also different in the different salts.

It has been found that the ln k–salt molality relationship, usually determined under isocratic conditions, can be calculated from the data obtained with gradient elution via the linear solvent strength model. Comparison of the calculated retention profiles revealed that a change in salt modifies more specifically the behaviour of the proteins having different hydrophobicities than was shown by the gradient data. Replacing

Table 2
Slope and intercept values of the retention profiles of the proteins calculated from the LSS model

Protein	Ammonium sulphate		Sodium citrate		Sodium acetate	
	S	ln k_w	S	ln k_w	S	ln k_w
CHY	2.770	−4.083	5.980	−4.664	0.736	−0.893
LYS	5.182	−8.263	14.715	−10.171	1.344	−4.109
OVA	7.002	−12.318	33.043	−26.198	2.728	−12.181
RNA	4.391	−8.713	13.223	−12.247	16.517	−77.350
CYT	5.566	−13.481	48.633	−77.596	9.521	−48.522

ammonium sulphate with sodium citrate increased the slope and decreased the intercept of the ln k *vs.* salt molality profiles, but the effect was more pronounced for the hydrophilic proteins. Sodium acetate exerted the opposite effect on the hydrophobic proteins, *i.e.*, the slopes decreased and the intercepts increased, but the same effect on the hydrophilic proteins. This means that varying the salt in the eluent will result in significant changes not only in the overall retention of the proteins but also in the selectivity of the separations.

5. Acknowledgement

We gratefully acknowledge the support given by the Hungarian Academy of Sciences under grant OTKA 1998/91.

6. References

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