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Analysis of pH-dependent protein interactions with gel filtration medium

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ABSTRACT

A prepacked Superose 12 HR 10/30 column was used to study the effects of elution ionic strength and pH on the chromatographic behaviour of a strong hydrophobic *Clostridium thermocellum* endoglucanase (1) and two weak hydrophobic proteins, *Clostridium thermocellum* endoglucanase C and egg white lysozyme. Ion-exclusion or ion-exchange interactions between weakly hydrophobic proteins and the gel matrix were observed at low ionic strength, depending on whether the pH of the elution buffer was higher or lower than the p*I* values of the proteins. These interactions were due to the presence of negatively charged groups on the surface of Superose and could be eliminated at any pH by adding electrolyte at a concentration determined by its chemical identity. The optimum results were observed with sodium sulphate at a concentration of 100 m*M*. The chromatographic behaviour of strong hydrophobic endoglucanase (1) on a Superose column as a function of pH was much more complex because of two interplaying effects, electrostatic and hydrophobic. Ideal size-exclusion chromatography could be achieved only in a narrow range of the conditions: first, the mobile phase must contain a weak salting-out electrolyte such as NaCl, and second, the mobile phase pH must be high enough that hydrophobic interactions between the solute and support are balanced by their electrostatic repulsion. At pH > p*I*, the retardation of endoglucanase (1) gradually increased with decreasing pH as a result of lowering of repulsive electrostatic interactions whether or not the buffer ionic strength was high. At pH < p*I* a drastic increase in the capacity factor k' was observed owing to the additivity of hydrophobic and ion-exchange effects. Overall, the chromatographic behaviour of endoglucanase (1) on a Superose column could be adequately described in terms of the theory of potential barrier chromatography. The explanation presented could obviously be valid for the behaviour of any protein on any gel matrix, as its mechanism was described using lumped physical notions such as hydrophobic and electrostatic interactions, charges and potentials. Virtually all currently known gel filtration media are more or less hydrophobic and are weak cation exchangers.

INTRODUCTION

Non-ideal behaviour of proteins and other high- and low-molecular-weight substances in size-exclusion chromatography (the so-called non-ideal SEC or nSEC) is a well known phenomenon [1–21]. The major reason for such deviation from "pure" SEC is electrostatic interactions between charged solutes and charged support surfaces determined by the presence of ionogenic groups in all of the commercially available SEC packings and hydrophobic interactions. The influence of the mobile phase ionic strength on these interactions is well understood. The electrostatic interactions are considered to be suppressed at high ionic strength and, conversely, the hydrophobic interactions are minor at low ionic strength. The influence of the mobile phase pH at low ionic strength on the retention mechanism has also been well studied [4,8,15,16,19,21]. Depending on the relative values of pH and isoelectric point of a protein, there is either an earlier elution compared with ideal SEC (the so-called "ion-exclusion effect"), or later elution ("ion-exchange effect"), which are due to amphoteric properties of protein molecules. As the residual charges of SEC packings are negative, the ion-exclusion effect occurs at mobile phase pH values above the isoelectric point when protein and matrix are similarly charged, and ion-exchange effect at pH values below p*I*, when protein and matrix are oppositely charged. The only

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exception would be when the pH is too low for the support to be ionized.

The influence of pH in nSEC at high ionic strengths, *i.e.*, under conditions when the hydrophobic interactions are maximized, is far from being understood. All the workers concerned with this question observed increased interactions between the solute and support at low mobile phase pH and a high ionic strength [2,9,13,14], but it was only Holmes *et al.* [14], who suggested the possible cause of this effect, namely the exposure at acidic pH of the key sites of the solute molecules responsible for hydrophobic interactions. As this suggestion is speculative and the purification of proteins at high salt concentrations using unsubstituted gel matrices (termed "salting-out", "hydrophobic salting-out" or "solvophobic" chromatography) has became a widely used technique [6,22–26], the aim of this work was to reinvestigate in more detail the mechanism of the protein–gel matrix interactions under these conditions.

EXPERIMENTAL

Equipment

Chromatographic experiments were performed on a prepacked Superose 12 HR 10/30 column which was part of a complete fast protein liquid chromatographic system (Pharmacia, Uppsala, Sweden).

Materials and reagents

Strongly hydrophobic endoglucanase (1) [EG (1)] of *Clostridium thermocellum* was isolated as described in ref. 27 and non-hydrophobic endoglucanase C (EG C) of the same organism as in ref. 11. Three-times recrystallized egg white lysozyme was purchased from Reakhim (USSR). Elution buffers were prepared using analytical-reagent grade reagents and Milli-Q quality deionized water. Buffers were degassed and passed through a 0.22-μm filter before use. Samples were clarified by centrifugation at 20 000 *g* before application.

The following buffers were used: ethanolamine–HCl (pH 10.0), Tris–HCl (pH 8.5), imidazol–HCl (pH 7.0), histidine–HCl (pH 6.0), sodium succinate (pH 5.5 and 4.0), sodium acetate (pH 5.0 and 4.5) and β-alanine (pH 3.5) at concentrations of 25 m*M*.

Chromatographic procedure

The column was equilibrated with two volumes of elution buffer. A 25-μl loop and MV-7 injector were used to apply the samples. Isocratic elution at a flow-rate of 1 ml/min was used throughout. The protein concentration in the effluent was followed at 280 nm using a UV-1 monitor. The magnitude of protein retardation was expressed by the capacity factor k':

$$k' = (V_R - V^0)/V^0 \quad (1)$$

where V_R is the retention volume and V^0 is the elution volume of an unretained solute. The latter value was calculated from the calibration graph for the Superose column as a function of protein molecular weights, which are 41 000–42 000 dalton for EG (1) [27], 39 000 dalton for EG C [28] and 14 000 dalton for lysozyme [13]. The calibration graph was obtained under the optimum conditions (pH 5.5, $I = 0.38$) under which the interactions between the Superose column and proteins are minimal [8]. Weakly hydrophobic protein standards were used: ferritin (440 000 dalton), aldolase (158 000 dalton), bovine serum albumin (67 000 dalton), ovalbumin (43 000 dalton) and cytochrome *c* (12 300 dalton).

RESULTS AND DISCUSSION

Endoglucanase (1)

Experiments on the non-ideal chromatographic behaviour of proteins and other compounds in gel filtration media have usually been performed at fixed pH values and varying ionic strengths of elution buffers [2,4,8,9,13–16,19,21]. In our opinion, this could hardly provide useful information to explain adequately the pH dependence of solute–support interactions. In this work the elution position of proteins was investigated *vs.* pH at different (but fixed in a given set of experiments) ionic strengths.

The results of experiments with strongly hydrophobic endoglucanase (1) are presented in Fig. 1, where curve 1 shows the dependence of k' on pH at low ionic strength (buffers without any additives), curve 2 shows the dependence of k' on pH at higher ionic strength (buffers with 100 m*M* NaCl added) and curve 3 that at still higher ionic strength (buffers with 100 m*M* sodium sulphate added).

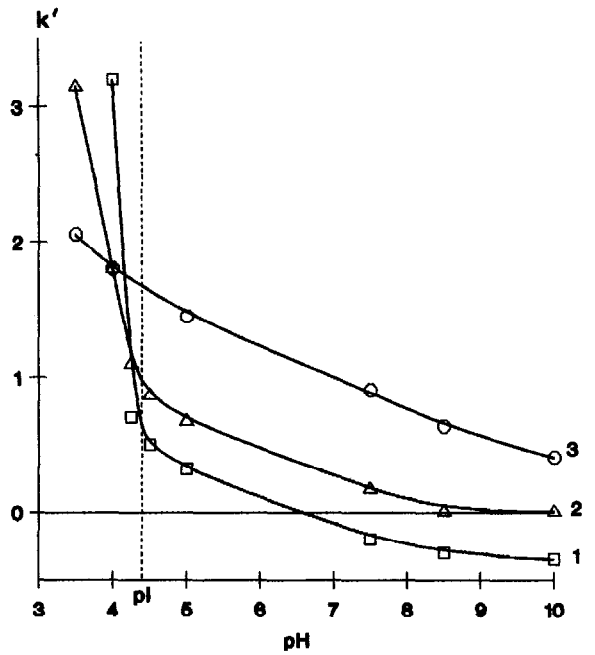

Fig. 1. Effect of pH on capacity factor k' of endoglucanase (1) in buffers of different ionic strength. Curves: 1 = buffers at a concentration of 25 mM with no additional electrolyte; 2 = the same buffers supplemented with 0.1 M NaCl; 3 = the same buffers supplemented with 0.1 M Na_2SO_4.

The results can be properly described and analysed separately for pH regions above and below the isoelectric point of EG (1), which is 4.4 [27]. In Fig. 1 these regions are demarcated by a broken line.

In the pH region above pI, curve 2 is located above curve 1, and curve 3 above curve 2. In earlier work [11] the retarded elution of EG (1) on addition of a salt to the elution buffer was attributed to the hydrophobic interaction of enzyme with the matrix; therefore, the later elution of the enzyme in the presence of NaCl and even more later in the presence of Na_2SO_4 may be associated with stronger salting-out conditions.

The chromatographic process was found to be strongly dependent not only on ionic strength but also on pH. The ideal SEC with $k' = 0$ could be achieved in the presence of NaCl and a mobile phase pH > 8.5 (Fig. 1, curve 2). Under stronger salting-out conditions occurring in the presence of Na_2SO_4 the hydrophobic interactions are superior at any pH tested (Fig. 1, curve 3). Under weaker salting-out conditions occurring without added electrolyte to the buffers the hydrophobic interactions are in excess at pH < 6.5 and the electrostatic repulsion of EG (1) (the "ion-exclusion effect") is in excess at pH > 6.5 (Fig. 1, curve 1).

The true cause of the monotonic increase in the interactions with decreasing pH at all three ionic strengths tested is not easily understood. Intuitively, it is clearly associated with hydrophobic interactions because this effect is absent in the case of non-hydrophobic EG (1) (see Fig. 6). The simplest explanation of this result could be the increased protein hydrophobicity or exposure of hydrophobic sites on the protein surface with decrease in pH, as suggested by other workers for tRNA [14]. Although possible in principle, the suggestion needs to be proved.

However, the pH dependence of interactions can be explained without this speculative supposition. Indeed, Superose contains some carboxylic and sulphate groups that are negatively charged within the working pH range [5,8]. Similarly charged is endoglucanase (1) at pH > pI, its net negative charge being the larger the higher is the pH. In this situation, Hesselink's theory of polyelectrolyte adsorption on charged surfaces [29] and the thermodynamic theory of protein adsorption of Norde and Lyklema [30] predict an increase in sorption with decrease in pH as a result of an increase in the electric contribution to the free energy of sorption. Certainly, neither theory is fully adequate for the experimental results of our work, as they were developed not for the chromatographic process *per se* but just for the adsorption on solid charged surfaces.

A more adequate and comprehensible analysis of the results can be made in terms of the theory of potential barrier chromatography proposed by Rukenstein and Lesins [31]. Below we give some postulates of this theory.

The total potential Φ of the adsorbate–adsorbent interaction is obtained by summing the individual contributions:

$$\Phi = \Phi_{DL} + \Phi_{vdW} + \Phi_B \quad (2)$$

where Φ_{DL} is the electrical double-layer interaction potential, Φ_{vdW} is the London–Van der Waals potential essentially determining the strength of hydrophobic interactions in an aqueous medium and Φ_B is the potential of repulsive short-range Born

interaction. Complex expressions for all three individual potentials and a discussion of their origin, nature and importance have been published [31]. The double-layer interaction arises from the charged surfaces of the adsorbate and the adsorbent. Therefore, its magnitude is readily modified by a pH change of the mobile phase as the degree of ionization of the surface groups is a function of pH. Further, the changes in the ionic strength of the mobile phase also greatly affect the double-layer potential owing to the screening of the surface charges and the binding of the counter ions to the charged surfaces. It should be noted that the chemical identity of the ions will determine the extent to which they bind to the charged surface and consequently reduce the double-layer interaction.

The attractive Van der Waals interaction between the adsorbate and adsorbent originates from the orientation, induction and dispersion interactions and can be altered by the addition of organic solvents to the mobile phase or salts at high concentrations.

The repulsive short-range interaction originates from steric and Born repulsion (the major contribution) and hydration forces and becomes important when the adsorbate approaches the adsorbent within very short distances. Like the Van der Waals potential, the Born potential is not significantly altered by pH or small changes in the ionic strength of the mobile phase.

The general idealized profiles of three individual (curves A, B and C) and total interaction potential (curve D) are illustrated in Fig. 2, taken from ref. 31. Curve A represents the repulsive double-layer interaction, *i.e.*, with a negatively charged matrix such as Superose it holds for pH > p*I* of the protein, where the latter is similarly charged. At short distances between the solute and the support an adsorption energy well (primary minimum) occurs on the total potential profile whereas at intermediate distances a potential barrier to adsorption appears. The solute can move over the potential barrier (if it is not too high) into the primary minimum and subsequently can escape from the adsorption energy well (if it is not too deep) to the bulk solution. The lower the barrier the more readily adsorption occurs, and the deeper the energy well the slower is the process of desorption.

In chromatographic theory, the elution behaviour of a solute is described as repeated adsorption–desorption cycles. The greater the characteristic time for adsorption during the cycle, the slower the solute moves along the column, *i.e.*, the larger is its retention volume, and *vice versa*.

Let us now examine the experimental results in Fig. 1 (region of pH > p*I*) and the potential profiles in Figs. 2–5. First, the analysis provides a simple explanation of increased k' on addition of the salt to the mobile phase. Indeed, the salt decreases the double-layer repulsion by screening the surface charges of the protein and the matrix. Curve A in Fig. 2 shifts to the left whereas the positions of curves B and C do not change significantly, as the Van der Waals potential and, especially, the Born potential are insensitive to small changes in ionic strength of the mobile phase, as mentioned above. As a result, the potential barrier on the total potential profile D will decrease and the potential well will become deeper (Fig. 3). In other words, EG

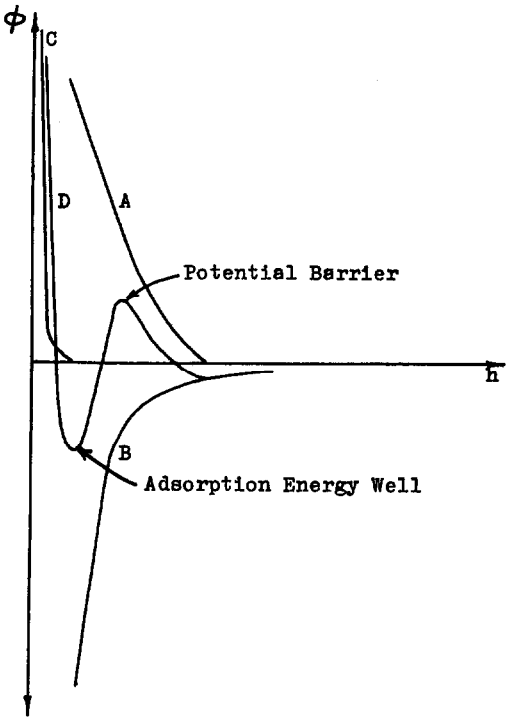

Fig. 2. Schematic profiles of the (A) double-layer, (B) Van der Waals, (C) Born and (D) total interaction potential for negatively charged protein and matrix at low ionic strength [31]. h = Distance between the protein molecule and the matrix.

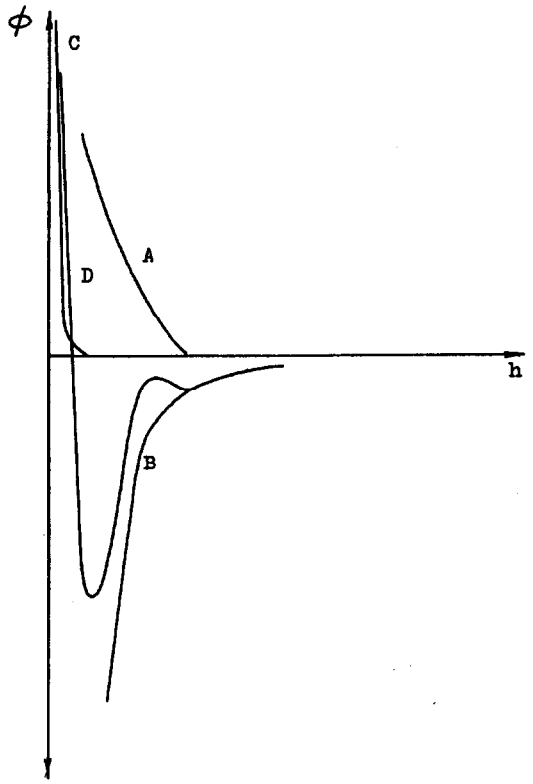

Fig. 3. Schematic representation of the effect of increased ionic strength or decreased pH (but still above the p*I* of the protein) on the individual contributions and the total interaction potential. Curves as in Fig. 2.

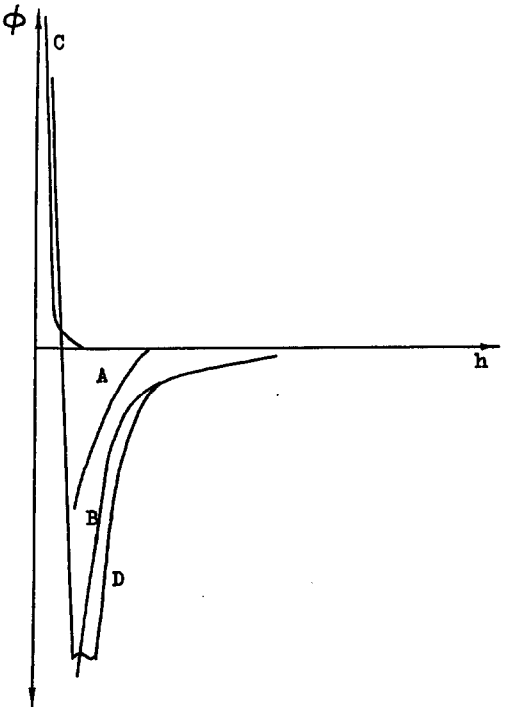

Fig. 4. Schematic potential profiles at pH slightly lower than p*I* of protein and low ionic strength of the mobile phase. Curves as in Fig. 2.

(1) will be more readily adsorbed and more slowly desorbed, which results in an increase in k'. A larger k' in the presence of Na_2SO_4 as compared with an equimolar concentration of NaCl can be explained by the higher ionic strength and stronger salting-out capacity of the former salt (and, consequently, by some enhancement of hydrophobic interactions) and its stronger efficiency in screening of protein and matrix charges.

In much the same manner the potential barrier decreases and the adsorption energy well becomes deeper during the lowering of the pH, which results in an increase in k' (Fig. 1, pH > p*I*). In this instance the double-layer potential is diminished, mainly owing to the decrease in the net negative charge of EG (1), as the degree of ionization of carboxyl groups of Superose is less sensitive to pH in this region, which is far from pK′ values of this group.

Hence the monotonic pH dependence of the sorption of EG (1) on Superose is conditioned by the fact that the potential barrier for sorption is dependent on pH. Therefore, this effect can be observed only when both electrostatic and pronounced hydrophobic interactions take place between the solute and support.

Let us consider now the region of pH < p*I* when the net charge of EG (1) is opposite to that of Superose. The double-layer potential profile (curve A) in this instance tends to go downwards (electrostatic attraction). As a result, the total potential (Fig. 4, curve D) has no barrier for sorption and the potential well becomes deeper. The adsorption–desorption equilibrium sharply changes towards adsorption, which explains the drastic increase in k' at pH < p*I* (the left-hand side of Fig. 1). Interestingly, in this region of pH the relative positions of curves 1, 2 and 3 were inverted as compared with the region of pH > p*I*. This is due to the screening effect of the salt with a consequent attenuation of the

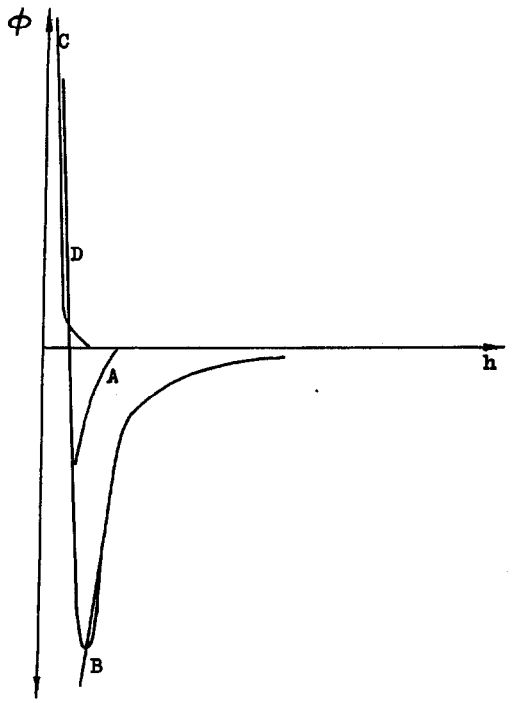

Fig. 5. Schematic representation of the effect of increased ionic strength on the interaction potentials at pH < p*I* of the protein. Curves as in Fig. 2.

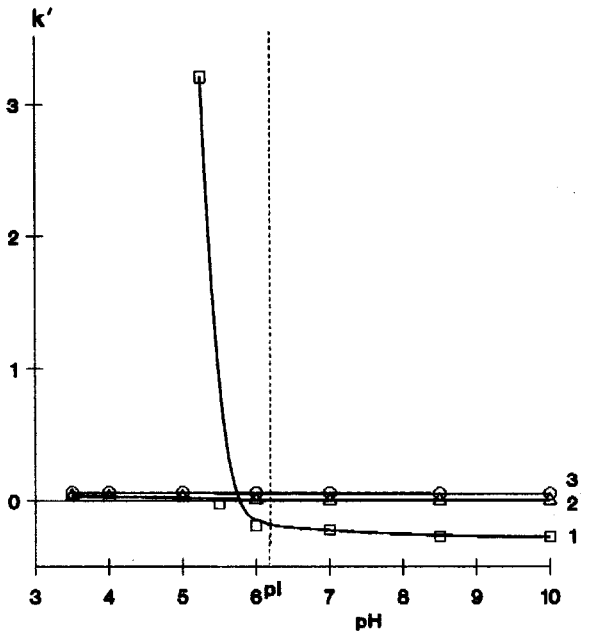

Fig. 6. Effect of pH on the capacity factor *k'* of endoglucanase C in buffers of different ionic strength. Curves: 1 = buffers at a concentration of 25 m*M* with no additional electrolyte; 2 = the same buffers supplemented with 0.1 *M* Na_2SO_4; 3 = the same buffers supplemented with 0.5 *M* Na_2SO_4.

double-layer potential and the decrease in the depth of the potential well (Fig. 5, curve D). The fact that on the left-hand side of Fig. 1 curve 3 proves to be below curve 2 is further evidence for the larger efficiency of Na_2SO_4 in reducing the ionic interactions as compared with NaCl. This conclusion is also supported by the experiments with lysozyme (see below).

Endoglucanase C

The results of chromatographic experiments with non-hydrophobic protein, EG C, are given in Fig. 6. In contrast to the corresponding experiments with EG (1), curve 2 represents the *k'* values in the presence of 100 m*M* Na_2SO_4 and curve 3 in the presence of 500 m*M* Na_2SO_4, *i.e.*, under more salting-out conditions. In spite of this, we found no noticeable hydrophobic interaction of EG C with Superose in the pH range studied. Non-size exclusion of protein from the gel at pH > p*I* and the strong retardation at pH < p*I*, observed at low ionic strength (Fig. 6, curve 1), are apparently due to electrostatic interactions of EG C with the negatively charged matrix because they are eliminated at high ionic strengths (Fig. 6, curves 2 and 3).

Lysozyme

This protein was assayed as the reason for the observed retardation of this and some other proteins on gel-filtration matrices at low pH and high ionic strength [2,9,13] remained unclear. Indeed, our previous results [11] showed that the relative hydrophobicity of lysozyme is closer to that of weakly hydrophobic EG C but not of strongly hydrophobic EG (1). Therefore, the mechanism of the pH-dependent retardation described above for EG (1) is inapplicable for lysozyme.

The results of chromatographic experiments with this protein are shown in Fig. 7. Owing to the very basic properties of the lysozyme (p*I* 11.0), at low ionic strength (buffers with no additional electrolyte) this protein is completely adsorbed on Superose by the ion-exchange mechanism within the pH range investigated, so Fig. 7 lacks a corresponding curve.

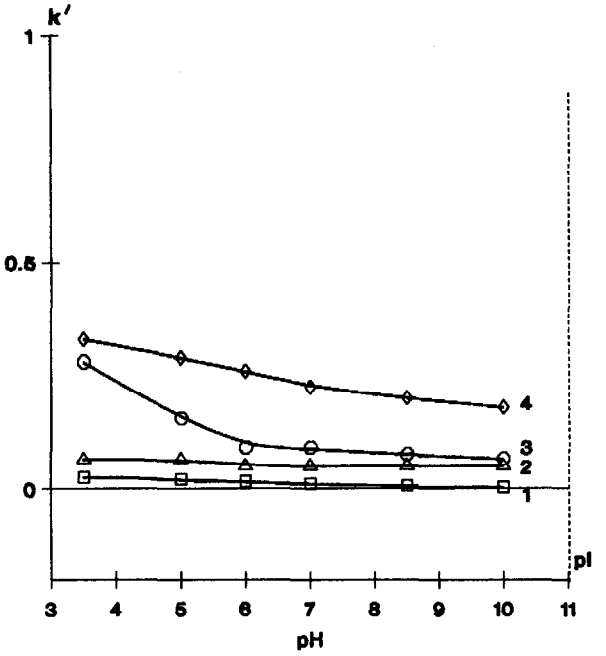

Fig. 7. Effect of pH on the capacity factor k' of lysozyme in buffers of different ionic strength. Curves: 1 = buffers supplemented with 0.1 M Na_2SO_4; 2 = buffers supplemented with 0.5 M Na_2SO_4; 3 = buffers supplemented with 0.5 M NaCl; 4 = buffers supplemented with 0.1 M NaCl.

Four other curves show the pH dependence of k' at increased ionic strengths: in the presence of 100 mM sodium sulphate (curve 1), 500 mM sodium sulphate (curve 2), 500 mM NaCl (curve 3) and 100 mM NaCl (curve 4). In the presence of 100 mM sodium sulphate the chromatographic process approaches the ideal gel filtration, as indicated by $k' = 0$. Sodium sulphate at a concentration of 500 mM evidently stimulates some hydrophobic interaction of lysozyme with the matrix (curve 2). The fact that in the presence of 500 mM NaCl (curve 3), 100 mM NaCl (curve 4) and 500 mM Na_2SO_4 (curve 2) the interaction is inversely dependent on the salting-out strength indicates that in the presence of NaCl some lysozyme adsorption is due not to hydrophobic interactions but to incomplete elimination of the ion-exchange interaction.

As the latter becomes stronger at acidic pH owing to the increased positive charge of lysozyme, it is not surprising that at both NaCl concentrations the retardation of this protein on Superose is enhanced with decrease in pH, as is seen in Fig. 7. Na_2SO_4 effectively eliminates ion-exchange interactions and no pH dependence in the presence of this salt is observed (Fig. 7, curves 1 and 2).

CONCLUSIONS

The aim of this work was mainly to elucidate the cause of the gradual increase of retardation of proteins and some other solutes on gel filtration matrices with decrease in pH, even at high ionic strengths when electrostatic interactions are assumed to be absent or at least negligible.

It should be emphasized that the existing hypothesis on the exposure of hydrophobic sites on the surface of macromolecules at low pH requires justification, whereas the above phenomenon can be well explained from the fact that all known gel filtration matrices are not only weakly hydrophobic but also contain some ionogenic groups that are negatively charged at the working pH.

As a result, at low ionic strengths and pH > pI when proteins are negatively charged, the ion-exclusion effect is observed and at pH < pI the ion-exchange effect. With weakly hydrophobic proteins, such as EG C or lysozyme, these interactions can be easily avoided by adding a salt to the eluent. It should be noted that NaCl, the electrolyte usually used in gel filtration for this purpose, sometimes does not eliminate them totally (*e.g.*, in the case of lysozyme) even at a concentration of 500 mM. Apparently, this is the cause of the increased distribution coefficient (k_D) of lysozyme and other proteins at low pH and high ionic strength observed by some workers [2,9,13]. Nevertheless, 0.1 M sodium sulphate totally suppresses these interactions at any pH tested, which is consistent with the high elution strength of this salt as compared with NaCl [32].

A more complicated situation occurs with strongly hydrophobic proteins such as EG (1). In this instance an ideal SEC can be obtained only under restricted conditions. First, the mobile phase must contain an electrolyte whose concentration and salting-out strength are not high in order to attenuate the electrostatic interactions but not to promote significantly the hydrophobic interactions. Second, the mobile phase pH must be high enough to balance the attractive hydrophobic interactions with the repulsive electrostatic interactions.

As for the true cause of the monotonic increase in the retardation of hydrophobic proteins on gel filtration matrices with decrease in pH, it is most readily explained in terms of the theory of potential barrier chromatography [31] as a result of a gradual decrease in the potential barrier for the sorption of charged solutes on a charged support.

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