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HYDROPHOBIC CHROMATOGRAPHY OF PROTEINS ON SEMI-RIGID GELS: EFFECT OF SALTS AND INTERFERENTS ON THE RETENTION OF PROTEINS BY SPHERON P 300

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SUMMARY

The effect was examined of the concentration of sodium chloride, sodium and ammonium sulphate and potassium bromide on the retention of several globular proteins (lysozyme, chymotrypsinogen, α-chymotrypsin, β-trypsin, DIP-trypsin and -α-chymotrypsin, chymopapain, ribonuclease, basic pancreatic trypsin inhibitor, three bull seminal plasma trypsin inhibitors and cow colostrum trypsin inhibitor) during hydrophobic chromatography on the semi-rigid glycolmethacrylate gel Spheron. It was observed with all proteins except lysozyme that the effect of the individuals salts, if present in concentrations not exceeding 0.5 *M*, can be predicted on the basis of the order of cations and anions in the Hoffmeister series. The concentration dependence of the salting-out effect shows a different course with different salts. The hydrophobic interactions of all the proteins examined are increased most in sodium sulphate whereas the retention of proteins changes only slightly in potassium bromide and even decreases in the case of lysozyme. The effects of several interferents, such as simple alcohols, urea, polyethylene glycol and sucrose, on the hydrophobic interaction of the proteins with the support were compared. It was observed that the concentration dependence for all interferents except sucrose shows the same profile and that the effect of alcohols varies in proportion with the size of the non-polar moiety of their molecules.

INTRODUCTION

The hydrophobic chromatography of proteins[1–11] is an extraordinarily flexible separation method and the separation conditions can be varied over a much wider range than with the other methods. The potential of this method has not been utilized in full, mainly because of the lack of data describing the effect of the individual factors on the separation.

One of the most important factors is the type of separation material. Hydrophobic chromatography is carried out mostly on hydrophilic soft gels of the agarose type with attached flexible non-polar ligands[1–3] or on common reversed-phase col-

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umns, *i.e.*, on rigid porous silica modified by coupling of octadecyl or octyl groups[4,5]. We have shown elsewhere that a macroporous, semi-rigid and highly cross-linked gel with a non-polar backbone can be used for the separation of proteins by hydrophobic chromatography[6–11]. The retention of proteins by this support can differ considerably from that by supports with flexible ligands because the non-polar groups of the proteins, which are exposed on the surface of their molecules, are the only ones which interact[9–11]. A comparison of the retention by both types of gels permits us to judge not only the size of the hydrophobic regions on the surface of protein molecules but also their accessibility, *i.e.*, whether they are exposed on the surface or localized in pockets[9–11]. Hydrophobic chromatography may also serve as a sensitive means of studying conformational changes of proteins, as shown by the investigation of the pH-dependent conformational transitions of human serum albumin[10], of the autoconversion of β-trypsin to α-trypsin[9] and of the formation of complexes of trypsin and chymotrypsin with their inhibitors[9]. Earlier we studied the factors that affect the separation of proteins, *i.e.*, the effects of temperature, pH, flow-rate, eluent and sample loading[6,8,9]. In this work we continued our study of two basic factors that affect the hydrophobic interactions of proteins with supports, *i.e.*, the effects of various cations and anions and of their concentration on the retention of proteins and the effect of several polarity-reducing reagents during the elution.

EXPERIMENTAL

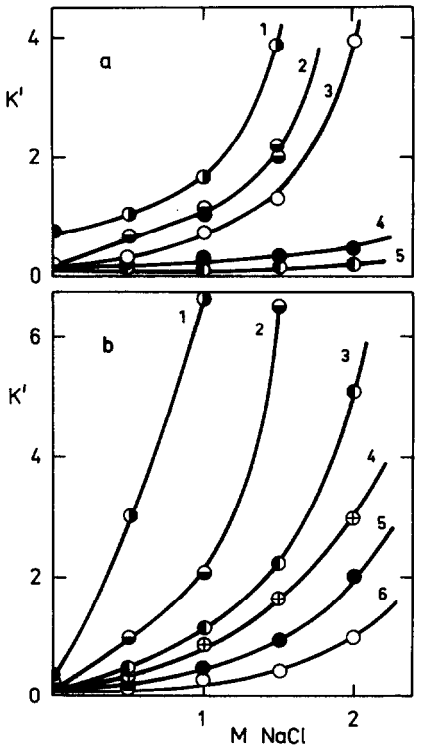
All the chemicals used were of analytical-reagent or spectral grade and most were obtained from Lachema (Brno, Czechloslovakia). *tert.*-Butyl alcohol (analytical-reagent grade) was from Reanal (Budapest, Hungary) and polyethylene glycole (mol. wt. 4000) from Becker (Delft, The Netherlands). The proteins were of the same origin as described elsewhere[6,8–11,13] Spheron P 300[12] (20–40 μm) was obtained from Lachema. Before use, traces of carboxylic groups were removed as described earlier[6].

The equipment was described in an earlier paper[6]. Precision-bore glass columns (420 × 6.18 mm I.D. or 260 × 6.18 mm I.D.) were used and the sample loadings were 0.02–0.2 mg. The pH values were measured with GK 2301C electrode (Radiometer, Copenhagen, Denmark) and were not corrected for high salt concentration or the presence of organic solvents.

The retention volumes were measured under the conditions where the distribution coefficient, K', defined ording to Hamilton *et al.*[14] AS $K' = (V_e - V_0)$. V_t^{-1}, where V_e is the elution volume of the protein, V_0 the elution volume of the protein of equal molecular weight yet not retained by hydrophobic interactions and V_t the total bed volume, is lower than 7 and where (except for HSA) a full recovery of the protein was achieved.

RESULTS

Figs. 1–4 show the retention (expressed as K') of the globular proteins β-trypsin, α-chymotrypsin, chymotrypsinogen, chymopapain, human serum albumin, lysozyme, ribonuclease, basic pancreatic trypsin inhibitor, three bull seminal plasma trypsin inhibitor and cow colostrum trypsin inhibitor[13], as a function of the concentration of NaCl, Na_2SO_4, $(NH_4)_2SO_4$ and KBr. When NaCl is present in the elution



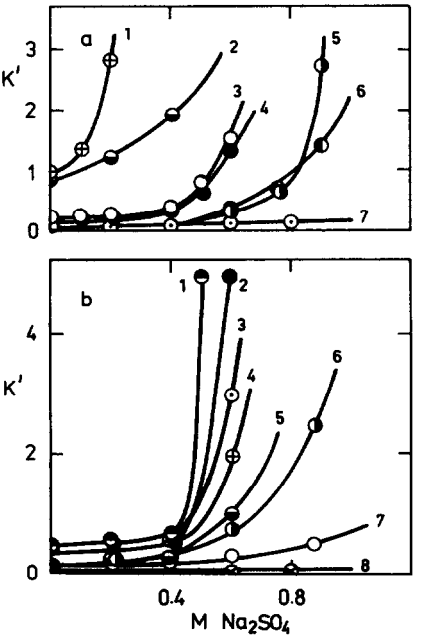


Fig. 1. Dependence of distribution coefficient, K', on NaCl concentration during hydrophobic chromatography of proteins on Spheron P300. (a) 1 = Bull seminal plasma trypsin inhibitor BUSI IA; 2 = basic pancreatic trypsin inhibitor; 3 = bull seminal plasma trypsin isoinhibitor BUSI IB; 4 = cow colostrum trypsin inhibitor; 5 = basic bull seminal plasma trypsin inhibitor BUSI IIA. (b) 1 = Lysozyme; 2 = chymotrypsinogen; 3 = β-trypsin; 4 = DIP-trypsin; 5 = α-chymotrypsin; 6 = DIP-chymotrypsin. Conditions: 20°C, 0.01 *M* phosphate buffer, pH 6.20, flow-rate 150 cm · h^{-1}; for others see Experimental.

Fig. 2. Dependence of distribution coefficient, K', on concentration of Na_2SO_4 during hydrophobic chromatography of proteins on Spheron P300. (a) 1 = Bull seminal plasma trypsin isoinhibitor BUSI IA; 2 = bull seminal plasma trypsin isoinhibitor BUSI IB; 3 = cow colostrum trypsin inhibitor; 4 = basic pancreatic trypsin inhibitor; 5 = human serum albumin; 6 = ribonuclease; 7 = bull seminal plasma basic trypsin inhibitor BUSI IIA. (b) 1 = Chymotrypsinogen; 2 = β-trypsin; 3 = lysozyme; 4 = DIP-trypsin; 5 = α-chymotrypsin; 6 = DIP-chymotrypsin; 7 = chymopapain; 8 = oxidised insulin A chain. For conditions, see Experimental and the legend to Fig. 1.

buffer the bull seminal plasma trypsin isoinhibitor BUSI IA is retained most; less retained are the pancreatic trypsin inhibitors isoinhibitor BUSI IB, and even less the cow colostrum trypsin inhibitor, homologous with the pancreatic trypsin inhibitor[13]. The bull seminal plasma basic inhibitor BUSI IIA shows the weakest interaction with the support in solutions containing all types of salts (Fig. 1a). Of the remaining proteins and their derivatives tested, lysozyme is retained most. The retention then decreases in the series chymotrypsinogen, β-trypsin, DIP-trypsin, α-chymotrypsin, DIP-chymotrypsin. It was observed that the retention of lysozyme increases almost linearly with increasing concentration of NaCl (Fig. 1b). A more rapid increase in retention was observed with all the remaining proteins at NaCl concentrations higher than about 1 *M*.

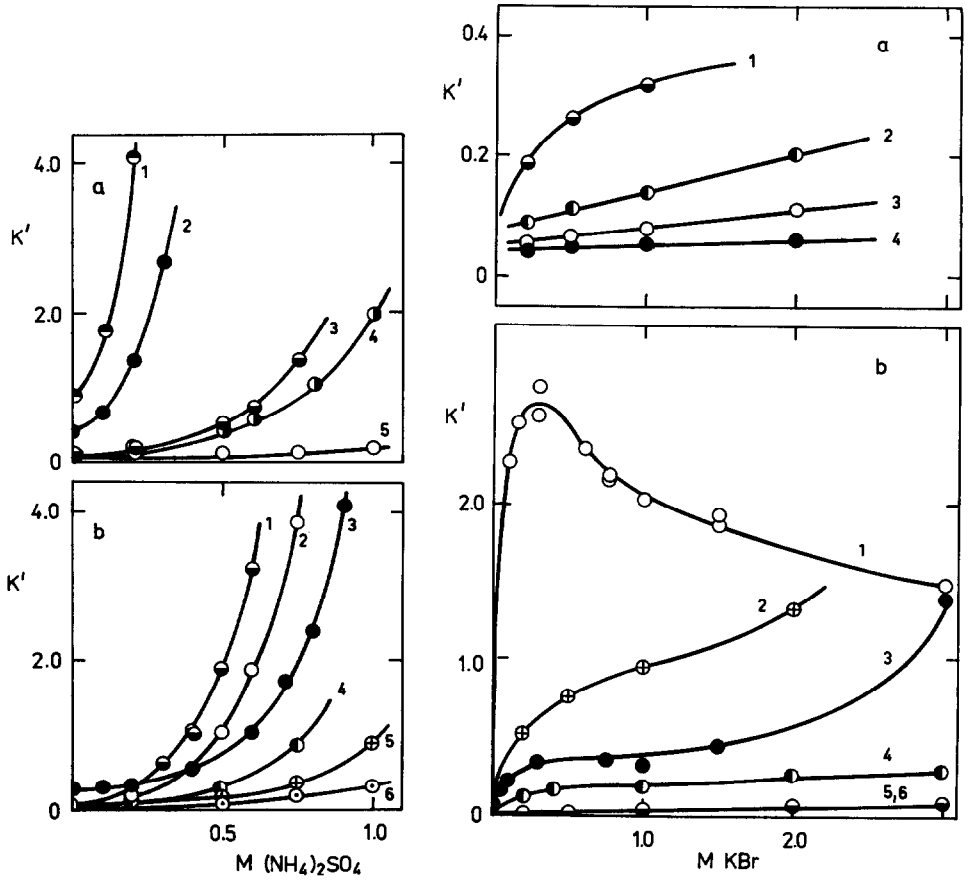


Fig. 3. Dependence of distribution coefficient, K', on concentration of $(NH_4)_2SO_4$ during hydrophobic chromatography of proteins on Spheron P300. (a) 1 = Bull seminal plasma trypsin isoinhibitor BUSI IA; 2 = bull seminal plasma trypsin isoinhibitor BUSI IB; 3 = basic pancreatic trypsin inhibitor; 4 = cow colostrum trypsin inhibitor; 5 = bull seminal plasma basic trypsin inhibitor BUSI IIA. (b) 1 = Chymotrypsinogen; 2 = β-trypsin; 3 = lysozyme; 4 = α-chymotrypsin; 5 = ribonuclease; 6 = chymopapain. For conditions, see Experimental and the legend to Fig. 1.

Fig. 4. Dependence of distribution coefficient, K', on concentration of KBr during hydrophobic chromatography of proteins on Spheron P300. (a) 1 = Bull seminal plasma trypsin isoinhibitor BUSI IA; 2 = bull seminal plasma trypsin isoinhibitor BUSI IB; 3 = bull seminal plasma basic trypsin inhibitor BUSI IIA; 4 = cow colostrum trypsin inhibitor. (b) 1 = Lysozyme; 2 = basic pancreatic trypsin inhibitor; 3 = chymotrypsinogen; 4 = β-trypsin; 5 = ribonuclease; 6 = human serum albumin. For conditions, see Experimental and the legend to Fig. 1.

The effect is even more pronounced with Na_2SO_4, where the K' value increases at concentrations higher than approximately 0.4 M (Fig. 2). The profile of the variation of K' with the concentration of $(NH_4)_2SO_4$ is analogous, a change in slope being observed when the concentration increases above 0.5 M (Fig. 3). The order of the retention of the individual proteins in Na_2SO_4 and $(NH_4)_2SO_4$ is only slightly altered compared with that in NaCl. Isoinhibitor BUSI IA is the most retarded; the retention then decreases in the series isoinhibitor BUSI IB, chymotrypsinogen, β-

trypsin, lysozyme, basic pancreatic trypsin inhibitor, cow colostrum trypsin inhibitor and α-chymotrypsin. Inhibitor BUSI IIA, chymopapain and the A-chain of oxidized insulin show only weak retention. Human serum albumin and ribonuclease are also weakly retarded in the column, but only in concentrations of Na_2SO_4 and $(NH_4)_2SO_4$ lower than 0.8 and 1 *M*, respectively (Figs. 2 and 3).

When we compared the effect of 0.5 *M* solutions of the three salts mentioned above on the retention of the proteins that are retarded more strongly by the support, we observed that the hydrophobic interactions are the highest in Na_2SO_4, weaker in $(NH_4)_2SO_4$ and weakest in NaCl. The interaction in $(NH_4)_2SO_4$, however, is only slightly stronger than in Na_2SO_4 if solutions of low concentrations are used. A stronger retention in NaCl than in Na_2SO_4 and $(NH_4)_2SO_4$ was observed with lysozyme only.

We also investigated the recovery of the individual proteins as a function of increasing K'. The recovery was close to 100%, even at high K' values, with all the proteins except albumin. The amount of albumin eluted decreased with increasing concentration of the salt. The rest was strongly retarded by the column and was eluted only after the ionic strength of the eluent had been decreased. This shows that whereas the conformation of the remaining proteins is stable under the conditions of the measurement, albumin undergoes extensive conformation changes resulting from its hydrophobic interactions with the support and that these changes make large hydrophobic areas of the albumin molecule accessible.

A behaviour markedly different from the elution systems discussed above was observed in solutions of KBr (Fig. 4). The proteins that are strongly retarded in the presence of NaCl, Na_2SO_4 and $(NH_4)_2SO_4$, such as chymotrypsinogen, β-trypsin and inhibitors BUSI IA and BUSI IB, are only weakly retarded in KBr. A considerably different behaviour was observed with lysozyme, whose retention increases strongly with increasing concentration of KBr and is maximal in 0.3 *M* KBr; the retention decreases with further increase in KBr concentration.

Effect of interferents on retention

The effect of various compounds that interfere with the hydrophobic interaction of the solute with the support was investigated with lysozyme and chymotrypsinogen. Fig. 5 shows the dependence of the K' values on the concentration of alcohols, varying in the length of their aliphatic chain, and on the concentration of urea in the eluent. As expected, the retention is more repressed in the presence of alcohols with a larger non-polar moiety of the molecule. The strongest effect was observed with polyethylene glycol, for which, at the same concentration per monomer unit, the increase in K' was greater than with the remaining alcohols, including the less polar *tert.*-butyl alcohol.

Fig. 6 shows the retention of chymotrypsinogen and lysozyme as a function of succrose concentration in the eluting buffer. Both enzymes behave differently with increasing concentration. The retention of chymotrypsinogen increases and the change in K' is very marked starting from sucrose concentrations higher than 10 g per 100 ml. In contrast, the retention of lysozyme starts to decrease even at low sucrose concentrations and the K' value is about 50% lower at a sucrose concentration of 20 g per 100 ml.

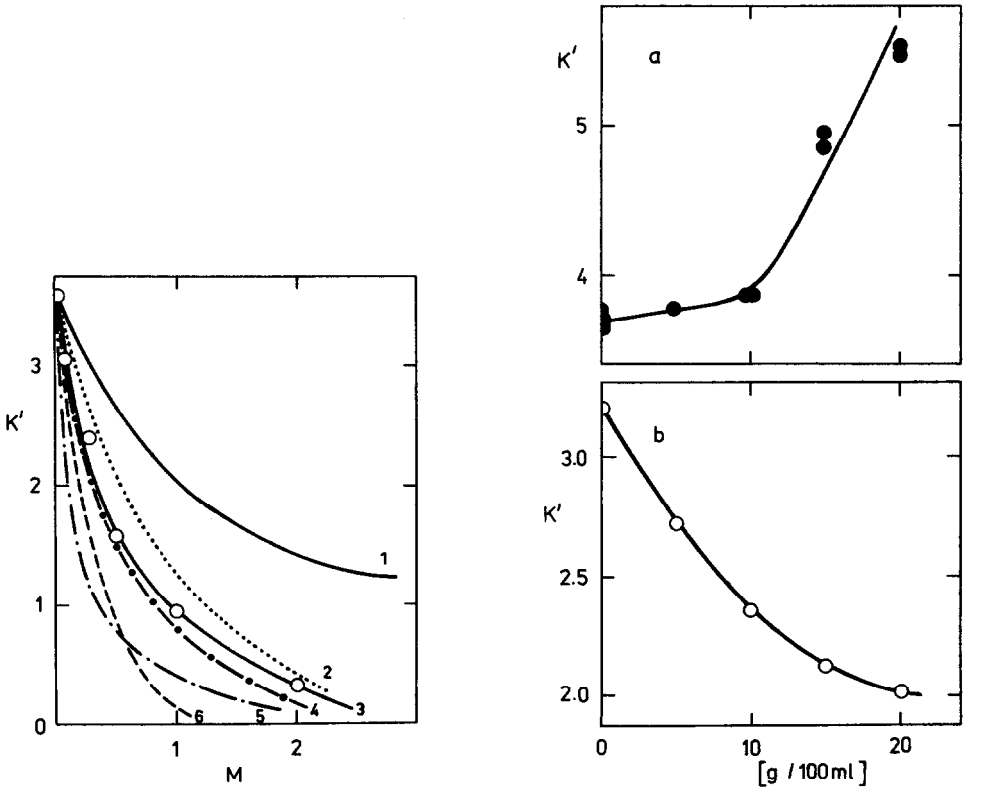


Fig. 5. Dependence of distribution coefficient, K', on concentration of interferent in elution medium during hydrophobic chromatography of chymotrypsinogen on Spheron P300. 1 = Methanol; 2 = ethanol; 3 = urea; 4 = 1-propanol; 5 = polyethylene glycol; 6 = *tert.*-butyl alcohol. Conditions: 0.01 *M* phosphate buffer; 1.5 *M* KCl containing the given concentration of interferent; pH 6.20; for other conditions, see Experimental and the legend to Fig. 1.

Fig. 6. Dependence of distribution coefficient, K', on sucrose concentration during hydrophobic chromatography of (a) chymotrypsinogen and (b) lysozyme on Spheron P300. Conditions: 0.01 *M* phosphate buffer; 1.5 *M* KCl containing the given concentration of sucrose; pH 6.20; for other conditions, see Experimental and the legend to Fig. 1.

DISCUSSION

Effect of salts on the retention of model proteins

The measurements were carried out with several globular proteins of the group of proteolytic enzymes and their inhibitors, and with ribonucleases and lysozyme. All these proteins have relatively low molecular weights (approximately 6000–30,000) and a relatively compact structure[15]. Human serum albumin only is a middle-sized protein (mol. wt. 64,000) and its molecule shows greater flexibility[16]. All of the proteins except lysozyme show the strongest hydrophobic interactions with the support at a 0.5 *M* concentration in $(NH_4)_2SO_4$, and a slightly weaker interaction in Na_2SO_4; the interactions are even weaker in NaCl and weakest in KBr. This finding corre-

sponds to the order that can be predicted from the order of the individual cations and anions in the Hoffmeister series[17]. If the salt concentration increases above 0.5 *M* the retention increases strongly in Na_2SO_4 and is almost the same (or slightly higher) in $(NH_4)_2SO_4$. According to von Hippel's classification, KBr is a neutral salt[17] showing a weak, concentration-dependent salting-out effect. This effect and the water-ordering effect can be observed at KBr concentrations lower than 0.3 *M*. These two effects increase only slightly or are even smaller at higher concentrations of the salt.

Of the ten proteins examined, anomalous behaviour was observed with lysozyme only. Maximal retention in 0.5 *M* solutions was observed in NaCl ($K' = 3$), slightly lower in KBr ($K' = 2.5$), and lowest in Na_2SO_4 and $(NH_4)_2SO_4$ ($K' = 0.8$). The retention of lysozyme at higher concentrations of various salts corresponds to the magnitude of the salting-out effect of the ions present, *i.e.*, to their position in the Hoffmeister series. The anomalous behaviour of lysozyme was also observed in other experiments. Lysozyme shows an opposite temperature dependence of the retention[6] and a decrease in retention after the addition of sucrose to the elution medium (Fig. 6). Lysozyme has the highest isoelectric point of all the proteins tested[18]. As a similar correlation between the behaviour during hydrophobic chromatography and the isoelectric point was not observed with the remaining proteins, it is probable that this anomalous behaviour does not reflect the total net charge under the given conditions. Chymotrypsinogen, the basic pancreatic trypsin inhibitor and the bull seminal plasma basic inhibitor have isoelectric points only slightly lower than that of lysozyme[18], but they behave differently. As the hydrophobic interactions are predominantly short-range interactions[19], the reason for the anomalous behavior of lysozyme will be the result of local structures close to the hydrophobic sites. The largest hydrophobic areas on the surface of lysozyme are formed by the non-polar side-chains of tryptophan residues localized in the closest neighbourhood of the positively charged groups of the arginines and lysines[20]. Lysozyme thus differs from the remaining proteins with known structures examined (chymotrypsinogen, α-chymotrypsin, β-trypsin, ribonuclease, basic pancreatic trypsin inhibitor). The positively charged ionic groups may affect the local ion distribution in the neighbourhood of hydrophobic domains and thus also the hydrophobic interactions of lysozyme and its retention.

Effect of polarity-reducing reagents

A plot of the decrease in retention as a function of concentration of various short-chain alcohols, polyethylene glycol and urea shows the same profile. If the concentration of alcohol is the same the efficiency of alcohols with varying non-polar moieties in the molecule (ethanol, 1-propanol, 2-methoxyethanol and *tert.*-butyl alcohol) is proportional to the size of the non-polar moiety of the alcohol molecule (Fig. 7). The effect of methanol is weaker. Polyethylene glycol, especially at low concentrations, is a more efficient desorbent than the alcohols tested, even though the non-polar part of the surface of the monomer unit is smaller than with ethanol. This is probably due to the fact that because of even weak interactions with the support or with the protein the effective local concentration of polyethylene glycol may be higher in the neighbourhood of the protein or of the support than in the bulk of the solution. At the same time this indicates that a complete separation of poly-

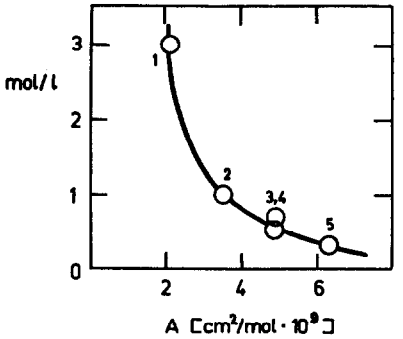


Fig. 7. Relationship between the efficiency of alcohols and the size of the non-polar moiety of their molecules. Plot of alcohol concentration in elution medium for $K' = 1.5$ and surface size of non-polar moiety of the molecule of the individual alcohols. The non-polar surface areas were calculated[21] from the individual contributions without correction for crowding. 1 = Methanol; 2 = ethanol; 3 = 2-methoxyethanol; 4 = 1-propanol; 5 = *tert.*butyl alcohol. For conditions, see Experimental and the legend to Fig. 5.

ethylene glycol from the protein or the regeneration of the support could be problematic. We have tested the long-term effect of the above alcohols on the activity of β-trypsin, α-chymotrypsin and bull acrosin. The most favourable results were obtained with *tert.*-butyl alcohol[22], which was therefore used for the isolation of acrosin on Spheron P300[23].

CONCLUSIONS

It has been demonstrated that proteins can be retarded in the presence of various salts on a hydroxyalkyl methacrylate gels and quantitatively eluted by the appropriate concentration of salt or an interfering agent. The retention of proteins increases with increasing salt concentration; the slope of the dependence profile is a function of the type of the salt, its concentration and the protein. The order in which the proteins are retarded is not the same with all types of salts. These differences permit a higher specificity of separation to be achieved on the adsorbent described by using an appropriate gradient or a combination of two salts or a combination of a salt and an interferent.

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