Journal of Chromatography, 216 (1981) 169–174
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,062

RESOLUTION IN HIGH-PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY

DEPENDENCE ON ELUITE DIFFUSION INTO THE STATIONARY PHASE

V. KASCHE*
Biology Department, University of Bremen, D-28 Bremen 33 (G.F.R.)
K. BUCHHOLZ
Dechema Institute, Frankfurt, D-6000 Frankfurt 97 (G.F.R.)
and
B. GALUNSKY
Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia (Bulgaria)
(Received June 1st, 1981)

SUMMARY

The resolution in high-performance liquid affinity chromatography was studied using proteases as eluites and soy bean trypsin inhibitor immobilized on aminosilanized LiChrospher as a biospecific adsorbent. The plate height was found to be much larger (≈ 1 cm) due to:

(i) lower rate constants for eluite dissociation from the immobilized inhibitor

(ii) flow-rate dependent transfer of the slowly diffusing eluites (biopolymers) into the solid phase and inhomogeneous adsorbent sites. The column was used more than 100 times without marked change in the separation behaviour.

INTRODUCTION

High-performance liquid affinity chromatography (HPLAC) combines the advantages of HPLC, *e.g.*, rapid and automated analysis, with the extremely specific interactions of biomolecules used in affinity chromatography[1]. The technique is capable of separating eluites having diffusion coefficients an order of magnitude lower than those of the eluites usually separated in HPLC[2]. For HPLC, theoretical analysis has been made of the situations where the eluites can penetrate the whole stationary phase during the chromatographic procedure, and where the adsorbent sites all have the same association constant[2,3]. The dissociation rate constants for adsorbed eluites are of the order of 10 sec^{-1}. In HPLAC the corresponding constants are much lower due to the more specific interactions between the immobilized ligands and the eluites[4]. This should increase the plate height compared with HPLC at similar mass distribution ratios and velocities. The velocities, 0.1–1 cm/sec, normally used in HPLC may also be too high for the complete transfer of the biopolymers by diffusion from

0021-9673/81/0000–0000/$02.50 © 1981 Elsevier Scientific Publishing Company

the mobile phase to the whole stationary phase in HPLAC. Thus, in HPLAC the eluite penetration into the stationary phase increases with decreasing flow-rates. If the adsorption sites are not homogeneous, *i.e.*, have different association constants[5], more sites with lower association constants should be involved in sorption–desorption cycles when the flow-rate decreases. This may result in an increase in plate height with decreasing flow-rates at much higher flow-rates than those that give larger plate heights due to axial diffusion dispersion in the mobile phase[2].

The effects of

(i) lower dissociation rate constants than for eluites used in HPLC

(ii) flow-rate dependent penetration distance into the stationary phase and a non-uniform distribution of association constants

on resolution have not yet been studied for HPLAC. They are investigated here using proteases as eluites and an immobilized protease inhibitor as a biospecific adsorbent.

EXPERIMENTAL

Materials

Bovine trypsin (E.C. 3.4.4.4.; Merck 24579), bovine α-chymotrypsin (E.C. 3.4.4.5.; Worthington CD I), bovine chymotrypsinogen A (Worthington CGC) and soy bean trypsin inhibitor (STI, Worthington SI) were used as purchased. Aminosilanized LiChrospher 500 NH_2 (research sample) (pore diameter 50 nm, diameter 10 μm) was a gift from E. Merck (Darmstadt, G.F.R.). All other chemicals were of reagent grade.

Preparation of the biospecific adsorbents

STI was immobilized on aminosilanized LiChrospher by the glutardialdehyde method[6]. To 2.5 g LiChrospher were added 65 ml 2.5% glutardialdehyde in phosphate buffer, pH 7.0 (I = 0.05). The suspension was shaken at room temperature for 1 h. Then the LiChrospher was poured on a glass filter and washed siveral times with distilled water. The modified carrier was suspended in 250 ml STI solution (0.85 mg/ml) in phosphate buffer, pH 8 (I = 0.05), and shaken at room temperature for 4 h. The STI–LiChrospher was washed until the eluate had negligible absorption at 280 nm, and modified to constant inhibitory activity with α-chymotrypsin (CT) for 4 days as described in refs. 5 and 7. The binding yield from the mass balance was 50%. The stationary capacity of the active bound inhibitor and the association constant for the bound inhibitor–free enzyme interaction were determined from equilibrium binding experiments as described in ref. 5.

Apparatus

A Spectra-Physics liquid chromatograph equipped with a SP 8700 solvent delivery system, a SP 8750 organizer, a Rheodyne 7125 injector and a SP 8400 UV/vis detector was used. Chromatograms were recorded with a Philips PM 8252 strip chart recorder. A column (120 × 4.6 mm) from Knauer (103.03) was packed with the biospecific adsorbent using a Knauer packing apparatus.

Column procedure

The column was either run at constant pH or with a gradient using 0.1 M KH_2PO_4 solutions at different pH. The flow-rate was checked gravimetrically, and

the pH of the eluate by measuring the pH in fractions collected in a fraction collector (Gibson Microcol TDC 80). The interstitial flow-rate was calculated from the pump flow-rate assuming a value of 0.4 for the interstitial porosity.

RESULTS AND DISCUSSION

Stationary binding properties of STI–LiChrosphere

In Fig. 1 a Scatchard plot is given for the binding of α-chymotrypsin to STI–LiChrospher. The curve indicates that non-specific adsorption is negligible. The adsorbent sites are, however, not homogeneous as the curve is non-linear. All bound STI molecules retain the property to bind protease. The average apparent association constant, $\bar{K}_{app} = 3 \cdot 10^6\ M^{-1}$, determined from the intercepts is lower than the corresponding value, $1 \cdot 10^7\ M^{-1}$, for the interaction between the free components but similar to values for the low pressure affinity adsorbent STI–Sepharose[5]. This indicates that the immobilization does not markedly change the specificity of the ligand, and that all bound ligands retain their biospecific function. The association constants for the most and least specific adsorbent sites differed by about a factor of 10, as estimated from the limiting slopes.

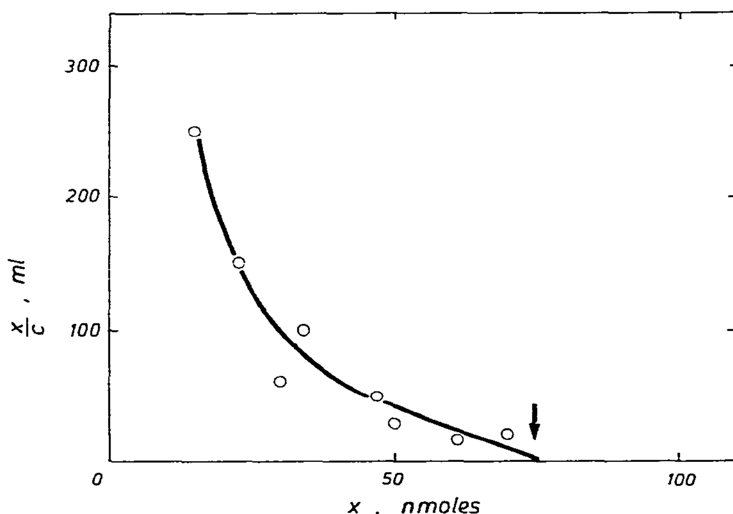

Fig. 1. Scatchard plot for the interaction between STI–LiChrospher and α-chymotrypsin. Particles (36 mg LiChrospher containing 75 nmoles STI) were incubated with various amounts of free α-chymotrypsin for 60 min at pH 8 [Tris–HCl (I = 0.05), 0.2 M NaCl] and 25°C. The particles were kept suspended by agitation. After equilibration the bound amount of enzyme, x, was determined from the added amount and the free enzyme content, c, in the filtrate of the suspension. The arrow gives the amount of STI bound in the particles.

HPLAC

Fig. 2 shows elution diagrams for the HPLAC of proteases with and without gradient elution. The resolution or peak width depends markedly on the flow-rate. For elutions at constant pH the plate height was determined from the peak dispersion using standard procedures[3]. The mass distribution ratio, k', was calculated from the

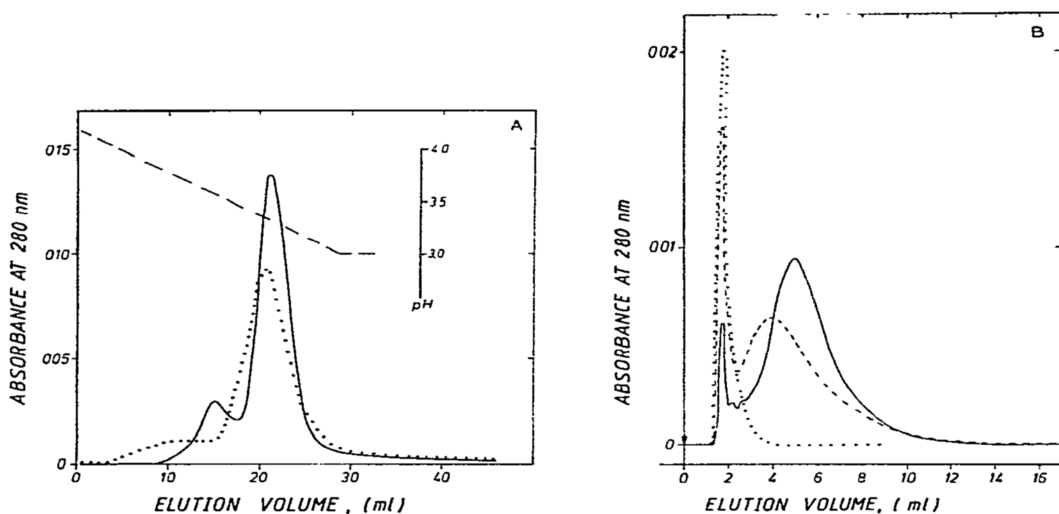

Fig. 2. Influence of flow-rate on the resolution in HPLAC of proteases. Column: 120 × 4.6 mm, packed with aminosilanized LiChrospher (pore size 50 nm, particle diameter 10 μm) in which 42 mg/g soy bean trypsin inhibitor was immobilized with glutardialdehyde. Elution buffer: 0.1 M KH_2PO_4 of different pH. A, Separation of α- and β-trypsin with a pH gradient. Flow-rate: ...., 5 ml/min; —, 2 ml/min. α- and β-trypsin form the first and second peaks, respectively. B, Separation of α-chymotrypsin and chymotrypsinogen at constant pH (4.0). Sample size: 10 μl with 50 μg protein. ...., Chymotrypsinogen, flow-rate 6 ml/min; — — —, α-chymotrypsin, flow-rate 6 ml/min; ———, α-chymotrypsin, flow-rate 1 ml/min.

elution volumes of the sorbed, V_e, and non-sorbed peaks, V_0, respectively, using the formula[2,3]:

$$k' = (V_e - V_0)/V_0 \quad (1)$$

The flow-rate dependence of the plate height and the distribution ratio is given in Fig. 3. At high flow-rates the plate height increases approximately linearly with flow-rate, and at lower flow-rates a minimum is observed. At the highest flow-rates some of the active enzyme passed the column without being adsorbed. This indicates that the time of passage of the mobile phase through the column is too small for diffusion of all eluites into the stationary phase. At the highest flow-rate this time, $\tau = L/u_e \approx 6$ sec where L and u_e are column length and interstitial flow-rate, respectively. During this time the enzymes diffuse over a distance $l = \sqrt{D_{CT}\,\tau}$ where D_{CT} is the diffusion coefficient of the enzyme (in free solution: $\approx 10^{-6}$ cm^2/sec (ref. 8); the value may be lower inside the porous carrier). Then $l \approx 24$ μm is of the order of magnitude of the distance between the particles in the column. When the flow-rate is decreased all the eluites may diffuse into the stationary phase and be adsorbed there.

From the linear part of the curves of H vs. u_e and from k', the dissociation rate constant, k_d, for the eluite desorption can be estimated as described in ref. 2. Here it is assumed that the plate height at high flow-rates depends mainly on the non-equilibrium term that is proportional to

$$2\,k'\,u_e/(1 + k')^2\,k_d \quad (2)$$

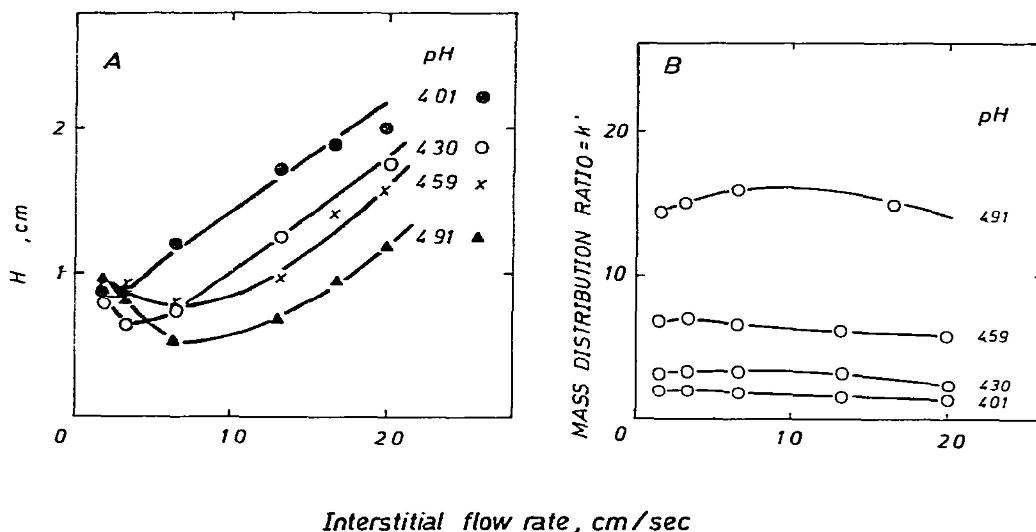

Fig. 3. The flow-rate and pH dependence of (A) the effective plate height, H, and (B) the mass distribution ratio, k', in HPLAC of α-chymotrypsin on a STI–LiChrospher column (120 × 4.6 mm) eluted at constant pH and room temperature.

where $k' = \Phi K_{app}$, Φ is the phase ratio and K_{app} the apparent association constant for the protease–inhibitor interaction. At high flow-rates k' decreases (Fig. 3B) therefore H increases. The phase ratio also decreases as less of the stationary phase is penetrated by the eluites.

At high flow-rates H_{exp} is approximated by eqn. 2; k_d can then be estimated from the experimental data[2]. At pH 4.9 a value of $k_d \approx 0.1$ sec^{-1} is obtained that is much smaller, by a factor of 100, than the corresponding values obtained in HPLC of low-molecular-weight eluites[2]. The plate height is not markedly reduced when compared with similar data for normal affinity chromatography[7]. At pH 4.8 H_{exp} was found to be 1.5 cm when $u_e = 10^{-2}$ cm/sec for CT separated with STI–Sepharose.

At lower flow-rates k' also decreases as more adsorbent sites with lower association constants are involved in sorption–desorption cycles. From Fig. 3A it follows that this effect more than balances the reduction in plate height due to the decrease in u_e, and the plate height increases. This occurs at higher flow-rates with increasing pH, where $\bar{K}_{app}$ increases[5].

When compared with HPLC of small eluites ($H_{exp} \leqslant 0.1$ cm)[3], these results show that the plate height in HPLAC is much larger (≈ 1 cm) due to:

(i) low dissociation rate constants

(ii) flow-rate dependent transfer of the slowly diffusing eluites (biopolymers) into the solid phase and inhomogeneous adsorbent sites.

The peak broadening due to (i) can be decreased at lower flow-rates or with less specific adsorbents (larger k_d). The latter may, however, reduce the specificity. At low flow-rates (ii) increases H and this counteracts the flow-rate dependence of (i). Smaller particles or those in which the biospecific adsorbents are only on the exterior should reduce the effect due to (ii), when this gives a more homogeneous distribution in K_{app}. A pH gradient also improves the resolution (Fig. 2A).

The column used in these experiments has undergone more than 100 cycles without marked change in the separation behaviour. This is considerable more than the optimum number of cycles, ≈ 10–30, observed in normal affinity chromatography using the same eluite–ligand system[9]. Therefore HPLAC seems to be a very promising analytical tool. Optimal resolution and short analysis times require, however, that the factors (i) and (ii) can be controlled as outlined above.

When existing theories describing the plate broadening due to sorption of low-molecular-weight eluites is extended to include the particle penetration distance (ii) then HPLAC can also be used to analyse the physico-chemical phenomena underlying the chromatographic process, and to obtain interaction data.

ACKNOWLEDGEMENTS

This work was supported by grants from the Deutscher Akademischer Austauschdienst (B.G.), Fonds der Chemischen Industrie (V.K.) and Bundesministerium für Forschung und Technologie (K.B.).

REFERENCES

1 S. Ohlson, L. Hansson, P.-O. Larsson and K. Mosbach, *FEBS Lett.*, 93 (1978) 5–9.
2 Cs. Horváth and H.-J. Lin, *J. Chromatogr.*, 149 (1978) 43–70.
3 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979.
4 A. Fersht, *Enzyme Structure and Mechanism*, W. H. Freeman, Reading, 1977, pp. 130–131.
5 V. Kasche, *Stud. Biophys.*, 35 (1973) 45–56.
6 H. H. Weetal, *Methods Enzymol.*, 44 (1976) 140.
7 V. Kasche, *Acta Univ. Uppsaliensis*, 2 (1971) 1–132.
8 G. W. Schwert and S. Kaufmann, *J. Biol. Chem.*, 190 (1951) 807.
9 V. Kasche, in K. Buchholz (Editor), *DECHEMA Monograph*, Vol. 84, Verlag Chemie, Weinheim, 1979, p. 374.