Journal of Chromatography, 376 (1986) 245—257
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2972

APPLICATION OF 1,1'-CARBONYLDIIMIDAZOLE-ACTIVATED MATRICES FOR THE PURIFICATION OF PROTEINS

IX*. DYNAMIC MULTIZONING EFFECTS IN BIOSPECIFIC AFFINITY CHROMATOGRAPHY ON POROUS SUPPORTS: EVALUATION OF ACTIVATION AND LIGAND COUPLING EFFECTS WITH DIFFERENT SUPPORT MATERIALS

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SUMMARY

The activation of several porous polymeric gels and chemically bonded glycerylpropyl-silica-based packing materials with 1,1'-carbonyldiimidazole has been further investigated. The ligand coupling characteristics of these matrices following activation at low and high levels have been determined and the properties of the derived affinity chromatographic supports examined. In addition the dynamic capacities of several immobilised-ligand systems have been compared. The use of these different affinity chromatographic supports in assessing flow-rate and column residency effects is described.

INTRODUCTION

It is now well recognised that the interplay of slow diffusion and slow adsorption or desorption kinetics can lead to multizoning effects and peak asymmetry when proteins are separated on heterogeneous interactive surfaces such as those commonly found with open column or high-performance reversed-phase, ion-exchange or biospecific affinity chromatographic media [1—6]. For example, the fraction of a particular protein which can be bound and then desorbed in biologically active state from a specified hydrophobic or biospecific ligand immobilised onto an inert matrix has for some time been known from experimental studies to be dependent on the flow-rate, the sample volume, the ligand concentration and the ligand—protein association constant

*For Part VIII, see ref. 15.

0378-4347/86/$03.50

(see for example refs. 5—11). Other factors, such as the time of residence of the protein in the chromatographic system and the spatial distribution of the ligand on the support surface have also been implicated but these effects in quantitative terms have been less well documented.

With biospecific affinity chromatographic adsorbents and other interactive media such as reversed-phase support materials, the free energy changes on solute interaction with the stationary phase surface are mainly derived from unfolding/refolding processes and associated reorientational changes in microscopic regions of the solvated structure of the protein solute at the stationary phase surface, or in the intraparticulate spaces of the pore chamber. When the relaxation times (τ_n) for such protein conformational interconversions are similar to the times (t_m) for mass transference along a section Δx of the chromatographic bed, and in particular in the limiting situation when $t_m/\tau_n \approx 1$, then the elution profile will be characterised [5] by peak degeneracy which takes the form of asymmetric or shouldering multiple peaks of large bandwidth. In contrast, when the magnitude of t_m/τ_n is very large (i.e. $\gg 1000$) or very small (i.e. $\ll 1/1000$), kinetic effects associated with such protein conformational interconversions essentially vanish with the chromatographic profile reflecting the average macroscopic behaviour of all the participating conformational forms at their apparent equilibrium concentrations. In these circumstances either a single sharp peak zone or alternatively two (or more) sharp peak zones of significantly different relative retention may be observed with the later eluting components typically representing unfolded or inactivated species. As the stationary phase surface itself becomes more heterogeneous in an interactive sense (for example when the ligand is immobilised in two different microscopic forms with non-uniform surface distribution) or when the residence and contact times for protein migration and protein—ligand interaction are of similar magnitude then further increases in bandwidths, decreased mass recovery and loss of biological activity of the interacting protein species may occur. An additional case of zone dispersion, typified by the split peak or break-through peak phenomenon, seen for example in immunoaffinity chromatography [12], can arise with porous support materials when at inappropriately chosen flow-rates the rate-limiting step associated with protein retention depends more on solute diffusion through the stagnant mobile phase pools within the pore chambers of the support material rather than the adsorption kinetics of the protein—ligand system per se.

During the course of our studies on the immobilisation of different low-molecular-weight ligands as well as different proteins to 1,1′-carbonyldiimidazole (CDI)-activated matrices, we observed variation in the overall quality or performance (in terms of peak bandwidth and solute loadability) of the various support materials in subsequent biospecific affinity chromatographic separations when the activation level, ligand coupling time, solute contact time and solute residence time during elution were changed. In the context used here, the solute contact time refers to the time the protein solute is adsorbed at specific ligand sites whilst the solute residence time refers to the total time for the solute molecules to move through and out of the exit of the chromatographic bed. Similar observations are common to most interactive chromatographic systems and arise due to the composite interplay of different

phenomena such as non-linear adsorption isotherm effects, conformational re-orientational effects and aberrant diffusional or kinetic behaviour. In order to distinguish between the origins of these phenomena, detailed chromatographic characterisation of different activated matrices and the derived immobilised-ligand systems are required. In this paper, the preparation and properties of a variety of different immobilised-ligand chromatographic supports is described as a prelude to an examination of the importance of matrix and CDI-activation effects on zone broadening or multizoning phenomena in biospecific affinity chromatography.

EXPERIMENTAL

Chemicals

1,1′-Carbonyldiimidazole was purchased from Pierce (Rockford, IL, U.S.A.) or Fluka (Buchs, Switzerland). Analytical-reagent grade acetone was purchased from BDH (Poole, U.K.), distilled from potassium permanganate, dried over anhydrous calcium sulphate, filtered, redistilled and stored over 4-Å molecular sieves. Sepharose CL-6B, Fractogel HW-65 and Trisacryl GF-2000 were purchased from Pharmacia (Uppsala, Sweden), E. Merck (Darmstadt, F.R.G.) and Industrie Biologique Française (Villeneuve-La-Garenne, France), respectively. LiChrospher Si 500 (10 μm particle size) was purchased from E. Merck. γ-Glycidoxypropyltrimethoxysilane was obtained from Dow Chemicals (Midland, MI, U.S.A.). Equine liver alcohol dehydrogenase, lactate dehydrogenase, TPCK-treated bovine trypsin, soya bean trypsin inhibitor, *p*-aminobenzamidine hydrochloride, protein A, concanavalin A, adenosine 5′-monophosphate (AMP), nicotinamide-adenine dinucleotide (NAD), aminohexanoic acid and 1,6-diaminohexane were all commercial preparations obtained from Boehringer-Mannheim (Mannheim, F.R.G.), Worthington Biochemicals (Freehold, NJ, U.S.A.) or Sigma (St. Louis, MO, U.S.A.), respectively. Monoclonal anti-human thyrotropin antibodies and polyclonal human immunoglobulin G were laboratory preparations characterised by established methods. The glycerylpropyl-bonded LiChrospher Si 500 was prepared either by reaction of the thermally activated silica (200°C for 10 h at reduced pressure, 10^{-3} bar) with γ-glycidoxypropyltrimethoxysilane in anhydrous dichloromethane containing 2,6-dimethylpyridine [13] under reflux conditions for 24 h with a slow argon purge followed by hydrolysis in 10 m*M* hydrochloric acid at 85–90°C for 2 h or alternatively by reaction of the parent silica suspended in 100 m*M* sodium acetate, pH 5.5, with γ-glycidoxypropyltrimethoxysilane for 8 h at 85–90°C. The glycerylpropyl-bonded silicas were washed consecutively with water, methanol and diethyl ether and vacuum-dried. The ligand densities established from elemental analysis and periodate oxidation of the glycerylpropyl silicas prepared under anhydrous conditions and under aqueous conditions were 3.1 and 4.8 μmol/m^2 of nominal surface area and equivalent to ca. 85 and 140 μmol/g of silica matrix, respectively.

Activation procedures

The activation of the soft polymeric gels, which had been previously solvent exchanged from an aqueous suspension, or the glycerylpropyl silicas (2 g dry

weight gel or its moist cake equivalent) with CDI (2 and 25 mmol) was carried out in anhydrous acetone for 30—60 min at room temperature. The very highly activated matrices (super-activated gels) were prepared by three sequential additions of CDI (10—25 mmol depending on the matrix and extent of cross-linkage required) to partially activated gels in anhydrous acetone. The various products were washed with the same solvent and stored until required for ligand coupling experiments under anhydrous acetone.

Analysis of carbonylated and coupled ligand matrices

Aliquots of the freshly prepared CDI-activated soft gel matrices were suspended in 150 m*M* sodium hydroxide in a stoppered volumetric flask and the contents gently agitated for 4 h at 20°C. The supernatant liquid was recovered by centrifugation and titrated under nitrogen using a Radiometer TTT-2 autotitrator with 1 *M* hydrochloric acid over the pH range 3—9 to determine the amount of imidazole and carbon dioxide derived from the imidazoyl-carbonate and cyclic carbonate groups (see activation scheme, Fig. 1). Aliquots of the silica-based activated matrices were hydrolysed at pH 3 for 4 h at 20°C and the imidazole groups present again determined by autotitration of the supernatant under nitrogen. The coupled 1,6-diaminohexyl or 6-aminohexanoic leash matrices were similarly analysed by titration of the amino or carboxyl end-groups of the leash using as appropriate 200 m*M* sodium hydroxide or 200 m*M* hydrochloric acid. Immobilised-protein contents were determined by amino acid analysis or by quantitative analysis with the bicinchoninic acid (BCA) reagent based on the procedure of Smith et al. [14]. Partially hydrolysed CDI-activated matrices where prepared by back-hydrolysis using 150 m*M* sodium carbonate, 500 m*M* sodium chloride—sodium hydroxide, pH 10.0, for contact times up to 30 h.

Preparation of biospecific adsorbents

Immobilisation methods for proteins and low-molecular-weight ligands were based on procedures described previously [15—18] using ca. 5 g of CDI-activated gel matrices suspended in degassed buffers (100 m*M* sodium carbonate—500 m*M* sodium chloride, pH 8.5) at 4—6°C with reaction times up to 150 h. In some experiments coupling reactions were also carried out at pH 7.2 using a potassium phosphate buffer or at pH 4.5 using a sodium acetate buffer. The various biospecific affinity adsorbents prepared from soft-gel matrices were slurry-packed into glass columns (50—150 mm × 10 mm I.D.) or in the case of the silica-based packing materials into stainless-steel columns (20—150 mm × 4.0 mm I.D.) at a constant flow-rate. Chromatographic experiments in glass columns were carried out using either Pharmacia P3 peristaltic pumps or P500 pumps programmed with an LCC 500 controller whilst chromatographic experiments with stainless-steel columns were carried out using a DuPont Series 8800 gradient controller and pump, a Rheodyne 7125 injection valve with 25-μl sample volume and a Waters Assoc. Model 450 variable-wavelength detector. Column capacities (loadability as μmol accessible ligand per g of biospecific affinity adsorbent) were determined by frontal analysis of the break-through curve generated by continuously passaging a dilute solution typically 0.5 mg/ml individual proteins at a flow-rate of 0.3

ml/min through the column. In each case the column capacity was derived from the $V_{0.5}$ value of the break-through volume.

RESULTS AND DISCUSSION

Effect of activation level and coupling conditions on the immobilisation of ligands

Biospecific affinity chromatographic purification of enzymes usually requires, as the initial step, the immobilisation of low-molecular-weight ligands such as substrates or inhibitors. It is commonly assumed that the overall process for a (monovalent) interaction between a protein and an immobilised specific ligand can be represented by

$$E + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EL$$

Under such conditions the relationship between the dissociation constant (K_d) of the complex (EL), the rate constants for adsorption k_1 and desorption k_{-1}, the column phase ratio, Φ, of the system and the mass distribution ratio or capacity factor is given by

$$k' = \frac{[\text{solute bound to stationary phase}]}{[\text{solute in mobile phase}]} = \Phi k_1/k_{-1} = \Phi K_d$$

The observed k' and the apparent K_d, k_1 and k_{-1} values for such monovalent ligand–protein interaction in immobilised-ligand chromatographic systems represents the average behaviour of the interacting species. Central to the issue of column quality and performance is the requirement that a stable homogeneous binding environment is created following immobilisation of the ligand onto the surface of an inert matrix. In order to immobilise the ligand, the inert matrix must usually be prepared in activated form. To avoid secondary effects, the activation procedure per se should ideally lead to a uniform surface modification without introduction of additional charged or hydrophobic groups. When mixed populations of ligand binding sites are obtained owing, for example, to the chemical heterogeneity of the activated matrix surface, entropic restriction of the pore chambers arising from ligand crowding or stacking at high matrix activation levels, or large distributions of pore diameter and surface contour features, then these microscopic differences in ligand characteristics will be reflected for notionally equivalent support materials in macroscopic changes in peak efficiency and peak asymmetry, in different patterns of column loadability as a function of flow-rate and also in changes in the mean retention time and apparent K_d for columns of the same dimensions. Although various quantitative studies with biospecific affinity supports prepared by the immobilisation of low-molecular-weight ligands onto cyanogen bromide-activated agaroses have suggested a relatively uniform ligand density throughout the porous bead structure, with the consequent ability of the ligand binding units to function in a uniform manner, random distribution of pores with variable diameters within the essentially open network structure of the agarose supports have nevertheless been observed [7]. With silica-based

support materials, even larger pore diameter distribution may occur with commercially available silicas which, in some cases, may exhibit up to 25% of their pore volume due to pores of diameter greater than twice the mean pore diameter [19].

One consequence of these disparate pore diameter distribution and surface contour effects is that the concentration of a low-molecular-weight ligand accessible to an interacting protein may be significantly lower than the total concentration of the ligand covalently immobilised onto the matrix. In such situations both the apparent dissociation constant and the apparent kinetic rate constants will differ significantly from the corresponding values for the same parameters determined in bulk solution for the free ligand–protein complex. For example, Liu et al. [20] have found that only ca. 2% of immobilised Reactive blue 2 on Sepharose CL-6B was functionally accessible for rabbit muscle lactate dehydrogenase whilst Nilsson and Larsson [21] have observed large discrepancies between the dissociation constants of adenine nucleosides for alcohol dehydrogenase determined with the enzyme in the free state and immobilised onto silica. Divergencies between K_d values, obtained from chromatographic measurements and other methods of determination in the bulk state, of two to three orders of magnitude have been found with affinity chromatographic supports prepared by other activation procedures, for example, cyanogen bromide or tresyl activation, when a significant proportion of the bound ligand is not accessible to protein solutes. Because weakly

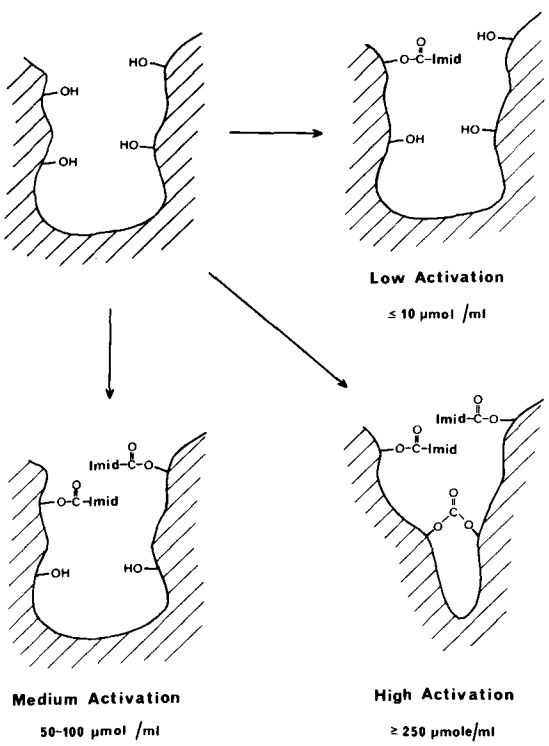

Fig. 1. Scheme showing reaction possibilities for carbonyldiimidazole activation of polyhydroxylic matrices.

activated through to very highly activated matrices can be prepared with CDI and related carbonylating reagents [17, 22] it is experimentally straightforward to evaluate the effect of different activation levels and ligand coupling capacities on the subsequent performance of the derived affinity support. Various product possibilities for CDI activation of polyhydroxylic matrices are shown in Fig. 1. At low activation levels the predominant activated form involves only imidazoylcarbonate groups whilst at very high activation levels a composite mixture of imidazoylcarbonate and cyclic carbonate groups are anticipated. The relative distribution of these different activated sites depends on the initial concentration of the CDI as well as the chemical nature of the gel matrix and, in particular, the partial disposition of the hydroxyl groups accessible to the solvent. At very high activation levels, i.e. at superactivation levels above 200 μmol/ml of gel, significant changes in pore volume of soft gel matrices will thus arise owing to cross-linkage of adjacent and non-contiguous hydroxyl groups. In the present investigation weak activation through the superactivation conditions were examined.

Table I shows typical data for three soft gel supports and two silica-based supports activated at two different levels. At the low activation level, all three soft gel supports generated CDI-activated matrices in which the content of vicinal cyclic carbonate and hindered bridged active groups was low (as assessed from the ratio of released carbon dioxide to imidazole on hydrolysis).

TABLE I

CHARACTERISTICS OF VARIOUS MATRICES FOLLOWING ACTIVATION WITH 1,1′-CARBONYLDIIMIDAZOLE

Matrix	Activation level I*		Activation level II**	
	Active groups per g matrix*** (μmol)	Hindered groups per g matrix § (μmol)	Active groups per g matrix*** (μmol)	Hindered groups per g matrix § (μmol)
Sepharose CL-6B	600	20—25	2400	340—350
Fractogel HW-65	450	10—20	2600	480—500
Trisacryl GF-2000	400	10—20	1800	220—250
LiChrospher Si 500 diol I §§	50	5	80	10
LiChrospher Si 500 diol II §§§	80	10—15	125	18—20

*Matrices were activated using 2 mmol CDI to 2 g dry weight or moist cake equivalent.
**Matrices were activated using 25 mmol CDI to 2 g dry weight or moist cake equivalent.
***Total active group content determined by titration analysis after hydrolysis using 150 m*M* sodium hydroxide or hydrochloric acid for 4 h at 20° C and expressed as μmol active groups per g dry weight of gel matrix.
§Residual active group content as carbon dioxide and imidazole determined after back-hydrolysis of activated matrix using 150 m*M* sodium carbonate—500 m*M* sodium chloride, pH 10.0 for 30 h at 20° C.
§§Glycerylpropyl-bonded LiChrospher Si 500 prepared under anhydrous reaction conditions with a phase density of 85 μmol diol groups per g silica.
§§§Glycerylpropyl-bonded LiChrospher Si 500 prepared under aqueous reaction conditions with a phase density of 140 μmol diol groups per g silica.

However, at the higher activation level a significant proportion of the active groups were due to cyclic carbonates. As reported previously [17], matrix activation with CDI in the presence of tertiary amines such as triethylamine or 2,6-dimethylpyridine resulted in a higher proportion of cyclic carbonates. Concomitant with the formation of the more hindered active group structure at these higher activation levels was a decrease in pore volume (ca. 15–20%) and associated gel bed shrinkage. Since the theoretical maximum activation level (i.e. ca. 5000 μmol per g of dry matrix) of cross-linked agarose is approximately twice that obtained under the second activation condition used here, even higher substitution is possible although considerable gel shrinkage would be anticipated to occur at these levels. The lower level of activation obtained with the glycerylpropyl-modified LiChrospher Si 500 prepared under base-catalysed anhydrous conditions is consistent with a monolayer structure for the glycerylpropyl groups. Under aqueous conditions polymerisation of the

TABLE II

COUPLING PERFORMANCE OF DIFFERENT LOW-MOLECULAR-WEIGHT LIGANDS AND PROTEINS USING VARIOUS MATRICES WITH LOW LEVEL CDI ACTIVATION

Ligand	Coupling time (h)	Coupling yield (μmol ligand per g matrix)				
		Sepharose CL-6B*	Fractogel HW-65*	Trisacryl GF-2000*	LiChrospher Si 500 diol I**	LiChrospher Si 500 diol II**
6-Aminohexanoic acid	4	10.0	14.0	14.0	0.4	0.8
	16	14.0	20.0	22.0	1.2	1.8
	64	28.0	22.0	26.0	1.8	2.2
1,6-Diaminohexane	4	12.0	20.0	20.0	1.0	1.2
	16	16.0	22.0	26.0	1.6	1.6
	64	30.0	24.0	28.0	2.0	2.6
p-Aminobenzamidine	4	6.0	8.0	6.0	0.4	0.4
	16	9.0	11.0	14.0	2.0	2.4
	64	20.0	12.0	18.5	2.2	3.6
N^6-(Aminohexyl)-AMP	4	20.0	24.0	20.0	3.0	4.0
	16	35.0	36.0	36.0	5.0	6.0
	64	40.0	40.0	41.0	6.0	6.2
NAD	4	20.0	24.0	18.0	1.0	1.5
	16	26.0	28.0	32.0	3.5	5.0
	64	36.0	32.0	40.0	5.0	5.5
Soya bean trypsin inhibitor	4	0.6	0.8	1.0	0.3	0.4
	16	3.1	2.9	3.6	1.9	2.1
	64	6.2	4.2	4.8	2.3	2.5
Protein A	4	0.2	0.2	0.4	0.05	0.2
	16	2.6	3.2	3.0	0.2	0.25
	64	4.2	3.8	4.0	0.25	0.35
Concanavalin A	4	0.2	0.8	1.0	0.2	0.2
	16	1.8	2.0	2.5	0.3	0.5
	64	3.1	2.2	2.8	0.6	0.6
Anti-human thyrotropin monoclonal antibodies	4	0.2	0.4	0.4	0.05	0.1
	16	0.8	0.8	1.0	0.1	0.1
	64	1.8	1.6	1.6	0.15	0.2

*Coupling reactions were carried out using 150 m*M* sodium carbonate—500 m*M* sodium chloride buffer using a fixed weight ratio of ligand to gel matrix.

**Coupling reactions were carried out using 100 m*M* potassium phosphate—1 *M* sodium chloride, pH 7.2 buffer.

trimethoxyorganosilane is known to occur [23] leading to multimolecular layers of polymerised organosilane and thus an apparently greater surface coverage. Based on the determined diol phase density of 85 μmol diol groups per g silica and 140 μmol diol groups per g silica, respectively, for the two phases in both cases an apparently near stoichiometric hydroxyl group activation was obtained with the high activation conditions. However, as can be seen from the data in Table I, activation of the glycerylpropyl LiChrospher Si 500 phase II with CDI at both the low and high level resulted in a significantly higher level of hindered active groups per g of silica. It is also evident from these data that the higher level of activation leads to a greater percentage of hindered active groups with both surface-modified porous silicas. At an activation level of 12.5 mmol CDI per g of matrix, approx. 15% of the active groups are in this form with these porous silica-based adsorbents.

Since hindered active groups such as cyclic carbonates are much less reactive towards N-nucleophiles, activated supports with higher carbonate content tend to couple biphasically and slower with ligands. The presence of hindered active

TABLE III

COUPLING PERFORMANCE OF DIFFERENT LOW-MOLECULAR-WEIGHT LIGANDS AND PROTEINS USING VARIOUS MATRICES WITH HIGH-LEVEL CDI ACTIVATION

Ligand	Coupling time (h)	Coupling yield (μmol ligand per g matrix)				
		Sepharose CL-6B	Fractogel HW-65	Trisacryl GF-2000	LiChrospher Si 500 diol I	LiChrospher Si 500 diol II
6-Aminohexanoic acid	4	12.0	10.0	14.0	3.0	4.0
	16	23.0	18.0	20.0	6.0	9.0
	64	48.0	30.0	40.0	12.5	16.0
1,6-Diaminohexane	4	14.0	20.0	7.0	2.0	2.0
	16	38.0	29.0	18.0	5.0	10.0
	64	50.0	36.0	50.0	9.0	14.0
p-Aminobenzamidine	4	16.0	14.0	16.0	2.0	2.0
	16	20.0	26.0	24.0	3.5	6.0
	64	30.0	29.0	26.0	6.0	8.0
N^6-(Aminohexyl)-AMP	4	12.0	14.0	14.0	2.0	3.0
	16	48.0	40.0	38.0	13.0	17.0
	64	51.0	54.0	46.0	17.5	21.0
NAD	4	14.0	14.0	3.0	2.0	3.0
	16	30.0	40.0	36.0	15.0	18.0
	64	58.0	50.0	42.0	19.0	23.0
Soya bean trypsin inhibitor	4	0.8	1.0	1.2	1.3	1.2
	16	4.1	3.7	3.1	2.8	2.9
	64	8.0	5.1	5.0	3.1	3.4
Protein A	4	0.2	0.2	0.4	0.1	0.2
	16	1.9	2.0	1.8	0.4	0.9
	64	5.2	4.6	4.0	1.2	1.6
Concanavalin A	4	0.4	0.6	0.9	0.1	0.2
	16	3.1	3.0	2.9	0.4	0.7
	64	4.8	4.6	3.6	1.1	1.4
Anti-human thyrotropin monoclonal antibodies	4	0.3	0.2	0.5	0.1	0.2
	16	0.6	1.4	1.0	0.15	0.25
	64	3.6	2.2	2.0	0.3	0.4

TABLE IV

ADSORPTION CAPACITIES DETERMINED BY FRONTAL ANALYSIS OF DIFFERENT AFFINITY SUPPORTS

Matrices were packed into columns of identical configuration and eluted at a fixed flow-rate (0.3 ml/min) as indicated in Experimental.

Protein*	Ligand	Ligand densities** and dynamic capacities*** for different column matrices									
		Sepharose CL-6B		Fractogel HW-65		Trisacryl GF-2000		LiChrospher Si 500 diol I		LiChrospher Si 500 diol II	
LDH	N^6-(Aminohexyl)-AMP	20.0	2.7	24.0	2.2	20.0	1.9	3.0	0.25	4.0	0.6
		40.0	4.8	40.0	3.9	41.0	4.0	6.0	0.7	6.2	1.2
		170.0	8.0	74.0	5.7	66.0	4.6	17.5	1.3	21.0	1.5
ADH	NAD	20.0	3.2	24.0	3.1	18.0	2.4	1.0	0.3	1.5	0.4
		36.0	4.1	32.0	4.3	40.0	4.0	5.0	0.5	5.5	0.8
		160.0	7.9	80.0	6.1	82.0	4.6	19.0	0.9	23.0	1.3
Trypsin	*p*-Aminobenzamidine	6.0	1.2	8.0	1.8	6.0	1.4	0.4	0.1	0.4	0.2
		20.0	2.5	11.0	2.0	14.0	1.8	2.0	0.8	2.4	1.1
		130.0	6.8	29.0	2.2	26.0	2.1	6.0	1.6	8.0	1.7
Trypsin	Soya bean inhibitor	0.6	0.1	0.8	0.2	1.0	0.1	0.3	0.1	0.4	0.1
		3.1	0.6	2.9	0.8	3.6	0.7	2.3	0.4	2.5	0.35
		8.0	0.7	5.1	1.0	5.0	0.8	3.1	0.5	3.4	0.6
Immunoglobulin	Protein A	0.2	0.1	0.2	0.1	0.4	0.2	0.05	0.03	0.2	0.1
		4.2	1.2	3.8	0.7	4.0	1.0	0.25	0.1	0.35	0.1
		5.2	1.4	4.6	1.2	4.0	1.0	1.2	0.4	1.6	0.6

*Protein solutions were made up in the equilibration buffer and applied directly to the specific columns.
**Ligand densities, expressed as μmol ligand per g of matrix, are shown in each matrix case as the first column of data.
***Dynamic capacity for each matrix expressed in terms of μmol ligand per g matrix accessible to the specific protein was calculated from the V_e, 0.5 value as determined by frontal elution by continuous application of the protein solution at a concentration of 0.5 mg protein per ml. The dynamic capacities are shown in the second column of data in each matrix case.

groups thus necessitates either longer coupling times, greater free ligand concentrations or both. Typical of this behaviour are the results for the immobilisation of several low-molecular-weight ligands and proteins to the different activated matrices (Tables II and III). As noted in previous studies [16–18] on the immobilisation of ligands with CDI-activated supports, higher levels of activation can lead to high ligand coverage for both soft gels and silica-based supports. Ligand coverages of 1—2 μmol/m^2 for the low-molecular-weight species down to 25 nmol/m^2 for the high-molecular-weight proteins could be achieved. These levels equate with ligand loadings in the range 15–70 mg ligand per ml of gel support. For all cases, the final coupling yield ranged between 85—95% of the weight of protein (or low-molecular-weight ligand) used per g of matrix. Furthermore, it was evident that protein coupling yields invariably improved when coupling times were greater than 16 h. Previous procedures with protein immobilisation to CDI-activated supports have employed overnight conditions (i.e. coupling times ranging from 12—16 h) but it is evident from Tables II and III and associated data on coupling yield that with the larger, more bulky, proteins such as immunoglobulins coupling times in the range 60—70 h can lead to nearly quantitative immobilisation yields.

The trend is also evident from Tables II and III that higher immobilised-ligand densities can be obtained with Sepharose CL-6B than with either of the other two soft polymeric gels. This difference, in part, may be due to the more open pore structure of the agarose-based gel following activation. It has been our experience that in some specific applications, for example, in the purification [24] of plasminogen activator or prekallikrein, the combination of these lower levels of ligand density and their greater mechanical stability to pressure deformation exhibited by Fractogel HW-65 and Trisacryl GF-2000 polymeric matrices result in improved purification factors and throughput rates. The incremental coupling rates for the different gels suggest that some ligands, i.e. *p*-aminobenzamidine or 1,6-diaminohexane couple more rapidly with Trisacryl GF-2000 than with Sepharose CL-6B at similar levels of activation whilst the opposite occurs with other ligands. Finally, it is clear from the above data that high substitution with low-molecular-weight ligands as well as with proteins can be affected with the different gel supports thus offering the potential for multisite attachment and higher loading capacities in larger scale preparative purifications of solute proteins.

Matrix capacity and loadability

As part of concurrent investigations on the effect of flow-rate on column loadability, the matrix adsorption capacities for several immobilised-ligand systems was determined using frontal analysis chromatographic procedures. Table IV summarises the results for the dynamic capacities of different affinity chromatographic adsorbents for lactate dehydrogenase, alcohol dehydrogenase, trypsin and human immunoglobulin. It is clear from these data that in all cases the dynamic capacity of the various affinity chromatographic supports (as assessed in terms of μmol of accessible ligand per g of support material) was considerably less than the theoretical capacity based solely on the ligand coverage. Similar behaviour has been documented [25, 26] for affinity chromatographic matrices derived from cyanogen bromide or other activation

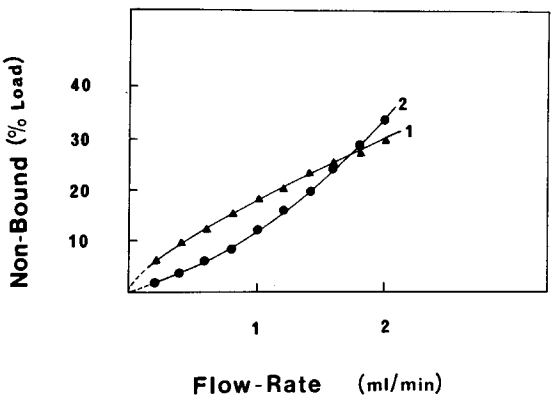

Fig. 2. Plot of amount of trypsin not bound to soya bean trypsin inhibitor immobilised onto two different glycerylpropyl LiChrospher Si 500 supports as a function of flow-rate. A fixed amount of trypsin (100 U) was injected at flow-rates from 0.2—2.0 ml/min onto columns of identical dimensions (50 mm × 4.0 mm I.D.) and the break-through peak assayed for trypsin activity. Column 1 was prepared by bonding γ-glycidoxypropyltrimethoxysilane to LiChrospher Si 500 under anhydrous reaction conditions whilst column 2 was prepared using aqueous bonding conditions. The soya bean trypsin inhibitor coverages of the affinity supports used were 3.1 and 3.4 μmol soya bean trypsin inhibitor per g support material for column 1 and 2, respectively. All experiments were carried out at 20°C.

methods. It is also evident from Table IV that as the ligand density increases, the dynamic capacity does not increase proportionately. With support materials of relatively high coverage, geometrical constraints owing to the close packing of the ligand and the physical phenomena of a reduction in pore volume combine to limit access of the solute to the ligand. These effects are also manifested in the dependency of the dynamic capacity on the flow-rate as well as in differences in the average association constant as determined by frontal elution techniques. For example, Fig. 2 illustrates the effect of flow-rate on the amount of trypsin adsorbed to soya bean inhibitor immobilised onto glyceryl-propyl LiChrospher Si 500 supports (diol I and diol II phases) of similar ligand densities (3.1 and 3.4 μmol soya bean trypsin inhibitor per g support, respectively). As the pore diameter of the parent silica is common to both systems, then either different diffusion or adsorption kinetics must operate even with these notionally similar biospecific affinity supports. Similar dependencies between flow-rate and break-through behaviour were also evidence from comparison of similar experimental data with the other matrices. In the above cases, as the major difference between the two silica-based support materials relates to the nature of the glycerylpropyl-bonded phase (monomeric layer versus partial polymeric layer) the most likely explanation for the lack of synonomy in the plots of percentage solute bound versus eluent flow-rate involves adsorption processes of different surface kinetics rather than different solute diffusion rates. Whether these differences are due to the manner in which the protein itself interacts or to the nature of the ligand immobilised at the surface in dissimilar conformations is the subject of an on-going investigation.

ACKNOWLEDGEMENTS

This investigation was supported in part by the National Health and Medical Research Council of Australia and the Medical Research Council of New Zealand. The stimulating discussion and technical assistance in the initial stages of this project of E.J. Harris and S.J. Su are also acknowledged.

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