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Study of affinity supports based on reactive polymers immobilized on silica: affinity constant determination from isocratic zonal elution

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ABSTRACT

Polymers bearing benzamidine moieties have been prepared from reactive copolymer containing chloroformate functions and deposited on porous silica matrices. These high-performance affinity chromatography supports were characterized by quantitative methods, which analyse the zonal elution behaviour of trypsin in the presence of a soluble competitor (L-arginine). The column loading capacity for trypsin was measured by the zonal elution method in mass overload conditions. On the basis of a Langmuir isotherm, the influence of the protein capacity and the concentration of the soluble ligand on the elution volume was studied for the determination of the binding constants. The plate heights determined for silica supports of various porosities and particle diameters show that the strong affinity interactions between trypsin and *p*-aminobenzamidine are mainly responsible for the low efficiencies observed.

INTRODUCTION

Affinity chromatography is not only a powerful method for purifying biomolecules but is also suitable to measure molecular interactions [1]. It is based on the ability of biologically active substances to interact specifically and reversibly with ligands. The affinity support is generally prepared by covalently attaching the ligand to an inert matrix. This matrix must interact weakly with the biopolymers to minimize non-specific adsorption but must possess chemical groups that can be activated to permit the covalent linkage of a variety of ligands.

Until recently, nearly all solid matrices were soft gels, derivatives of polysaccharides, polystyrene or polyacrylamide gels. Because of their poor mechanical stability, their use is mainly limited to conventional affinity chromatography.

High-performance affinity chromatography (HPAC) [2–4] combines the advantages of the conventional technique (high selectivity) with those of high-performance liquid chromatography (HPLC): speed of operation and sensitive detection. The matrices used are mainly silica, but non-specific interactions may occur if the support is not totally passive. The active groups generally used for ligand immobilization are epoxide or carbonyldiimidazole bonded to silica [5].

The aim of the present work is to describe new supports for HPAC where a reactive copolymer is adsorbed on silica. It has already been shown that HPLC silica supports coated with ion-exchange copolymers are excellent supports for protein separations [6–10]. The affinity chromatographic phase is a random copolymer of N-vinylpyrrolidone and vinyl chloroformate [11]. The reactive group is chloroformate, which im-

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mobilizes ligands with an amino group. The stationary phase is deposited on silica according to the polymer-adsorbed technology.

To illustrate the potential of the method, an affinity support with *p*-aminobenzamidine (pAB) was prepared. This ligand is well known as a low-molecular-mass serine protease inhibitor, and many quantitative studies have been performed on this type of affinity support [12,13]. The role of the silica matrix is important and often determines the efficiency and the protein capacity. Therefore, we discuss the application to HPAC of several silica supports with different porosities and particle diameters.

EXPERIMENTAL

Chemicals

The reagents used for copolymer synthesis were N-vinylpyrrolidone (VP) (Fluka, Buchs, Switzerland), vinyl chloroformate (CF) (Aldrich, Milwaukee, WI, USA) and azobisisobutyronitrile (AIBN) (Eastman Kodak, Rochester, NY, USA). Dichloromethane and all the other organic solvents were from SDS (Peypin, France). Ethylenediamine was provided by Prolabo (Paris, France). L-Arginine and the chemicals used for the preparation of phosphate and glycine–HCl buffers were obtained from Fluka (Buchs, Switzerland). *p*-Aminobenzamidine (pAB) was from Sigma (St. Louis, MO, USA).

Two types of porcine trypsin (EC 3.4.21.4) (Sigma) were used for the chromatographic studies: type IX (T0134), the purest grade available, and type II (T8128), which is *ca.* 10–20 times less active. The chromogenic substrate for trypsin (CBS 40.17) was purchased from Diagnostica Stago (Asnières, France) and the protein assay reagent (BCA) using bicinchoninic acid came from Pierce (Rockford, IL, USA).

The characteristics of the different silica supports studied are reported in Table I. The silica IBF was a gift from IBF (Villeneuve-la Garenne, France). LiChrospher was obtained from Merck (Darmstadt, Germany). The porous silicas Nucleosil and Polygosil were purchased from Macherey-Nagel (Düren, Germany). For covalent polymer immobilization we used Polygosil silica modified with an alkylsilane with free amine moieties (NH_2-Polygosil, 0.3 mequiv./g Si). This support was a gift from M. G. Félix (Laboratoire de Chimie Organique et Organo-Métallique, URA, Bordeaux, France).

Copolymerization of VP–CF

The radicalar copolymerization of VP and CF has been described in detail [11]. VP (5 g) and CF (16.2 g), previously distilled, were diluted in 153 ml of dichloromethane (dried with a 4-Å molecular sieve) and polymerized with 153 mg of AIBN at 35°C under a nitrogen atmosphere for 48 h. The copolymer was precipitated in diethyl

TABLE I

CHARACTERISTICS OF SILICA SUPPORTS

Support[a]	Silica type	Porosity (Å)	Particle diameter (μm)	Specific area (m^2/g)	Fixation of polymer	Quantity of deposited polymer[b] (mg/m^2)	Protein capacity, Q_X (mg/g Si)
LAB	LiChrospher	4000	10	10	Adsorption	2.5	0.7
IAB	IBF	1200	15–25	25–35	Adsorption	1.7	1.17
NAB	Nucleosil	300	5	100	Adsorption	1.6	2.35
PAB	Polygosil	300	20	100	Adsorption	1.3	2.91
FAB	NH_2-Polygosil	300	20	100	Covalent binding	1.6	2.58

[a] LAB = *p*-Aminobenzamidine copolymer adsorbed on LiChrospher; IAB = *p*-Aminobenzamidine copolymer adsorbed on IBF; NAB = *p*-Aminobenzamidine copolymer adsorbed on Nucleosil; PAB = *p*-Aminobenzamidine copolymer adsorbed on Polygosil; FAB = *p*-Aminobenzamidine copolymer grafted to Polygosil.

[b] Determined by elemental analysis of carbon.

ether, collected by filtration under a nitrogen atmosphere and vacuum-dried for 12 h. The copolymer was stored at −30°C under vacuum. The chemical composition in molar fractions was determined by elemental analysis of carbon, chlorine and nitrogen. The copolymer used for the preparation of chromatographic supports contains 50% of each comonomer. The average molecular mass of *ca.* 30 000 was measured with a high-speed membrane osmometer (Mechrolab 501) in dichloroethane at 20°C.

Copolymer coating procedure. The adsorption method at saturation was used to deposit the maximum amount of copolymer that could be adsorbed. Silica (1 g) was previously heated under vacuum at 250°C for 20 h. It was added to 10 ml of 5% (w/v) copolymer solution in dichloromethane. The suspension was sonicated for 2 min in an ice-bath (35 kHz; Bioblock, 88155) and gently stirred for 4 h at room temperature. The coated support was washed with the solvent and filtered under a nitrogen atmosphere and dried under vacuum.

The other method consisted of grafting the copolymer onto NH_2-Polygosil. A fraction of chloroformate reacted with the amino groups grafted on the silica: 200 mg of copolymer were dissolved in 10 ml of dichloromethane. A sample of NH_2-Polygosil (1 g) was added to this solution. The suspension was sonicated for 2 min and gently mixed for 2 h at room temperature. The support was washed with the solvent and filtered under a nitrogen atmosphere and dried under vacuum. The amount of deposited copolymer was determined by the elemental analysis of carbon (Table I).

p-Aminobenzamidine ligand immobilization (Fig. 1). pAB (4 g) was diluted in 200 ml of dimethylformamide (DMF) with 8 ml of triethylamine. A sample of coated silica (1 g) was added. The suspension was gently shaken for 20 h at room temperature. The support was washed successively with DMF and dichloromethane, filtered and dried under vacuum.

Chromatographic equipment

The affinity supports were slurry-packed into 5 cm × 0.46 cm I.D. columns. The temperature was maintained at 20°C during the experiments, using a temperature-controlled water-bath. The liquid chromatographic apparatus consisted of a pump (Model 420, Kontron Instruments, Zurich, Switzerland), a UV detector (Spectra 100, Spectra-Physics, San Jose, CA, USA) operating at 280 nm and a sample injector (Model 7125, Rheodyne, Berkeley, CA, USA). For chromatographic elution studies a 0.02-ml sample loop was used. The absorbance output was connected to a digital voltmeter (Model 3497, Hewlett-Packard, Palo Alto, CA, USA). The data stored on a floppy disk were processed with a personal computer (Model 386/20e, Compak Deskpro, Houston, TX, USA) equipped with an arithmetic coprocessor.

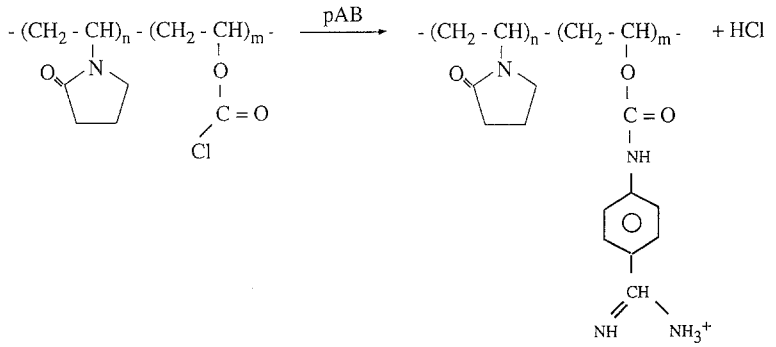

Fig. 1. Chemical reaction of ligand immobilization.

Evaluation of the non-specific interactions of the matrix

In order to prove that the matrix does not interact by non-specific interactions with proteins, we synthesized and tested a chromatographic support with free amino groups. It is made of a (50:50) copolymer VP–CF adsorbed on Nucleosil silica, with ethylenediamine grafted onto the chloroformate units using the following method. Ethylenediamine (0.374 ml) was solubilized in dichloromethane. The coated silica (2 g) was suspended in this solution and gently stirred for 3 h at 35°C. The support was filtered, washed with the solvent and dried under vacuum. It was packed into a column, which was tested by injecting trypsin and other basic proteins in a 67 m*M* KH_2PO_4 buffer (pH 7.4). This support does not retain trypsin and the basic proteins, and this shows that the copolymer is well adsorbed and that the interactions between proteins and the OH groups of the silica surface are negligible at this pH.

Biochemical characterization

For these tests, a 1-ml sample loop was used. A 5 g/l trypsin (T8128) solution was injected into the column. The protein concentration of the collected fractions was measured by BCA reagent from a standard curve determined with the purest trypsin (T0134, 96% of protein). The amidase activity was obtained by using the chromogenic trypsin substrate: BzD-Ala-Ala-Arg-pNA. The samples (0.005–0.1 ml) were diluted to 0.8 ml with Tris–imidazole buffer (pH 7.4) and 0.2 ml of substrate was added. The reaction mixture was incubated at 37°C. The absorbance variation was recorded by a UV spectrophotometer (Model 8452A, Hewlett-Packard). One activity unit corresponds to the enzyme activity that releases 1 μmol of substrate per minute. Specific activity is defined as the ratio of enzyme units to the amount of protein measured by protein reagent assay.

RESULTS AND DISCUSSION

Trypsin purification

A typical affinity elution pattern is shown in Fig. 2, and demonstrates that trypsin is bound successfully to the polymeric adsorbed stationary phase. In these experiments, the amount of protein injected did not exceed the protein capacity, and no trypsin activity was detected in the first peak. The active enzyme was then recovered from the column by elution with 50 m*M* glycine–HCl, 50 m*M* NaCl buffer (pH 2.8) (peak 2). The amount of protein in peak 2 represents *ca.* 30% of the injected sample. The specific activity of the injected sample is 70 U/mg of protein. It is about three times as large in peak 2. The initial amount of protein and amidase activity were entirely recovered during all the chromatographic process. There were no significant differences between the various supports studied. Thus this new pAB affinity support may be used for trypsin purification.

Quantitative affinity chromatography

To evaluate the chromatographic properties of the affinity support we studied the interactions between the solute (trypsin) and the stationary phase by the zonal elution method. The reversible protein elution was obtained by adding a soluble

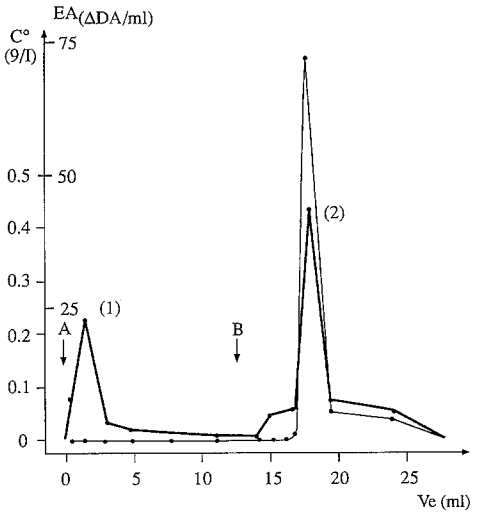

Fig. 2. Trypsin purification on pAB grafted onto VP–CF copolymer adsorbed on Nucleosil (NAB support). Eluents: A, 15 m*M* KH_2PO_4 (pH 7.4); B, 50 m*M* glycine–HCl with 50 m*M* NaCl (pH 2.8). Sample size, 5 mg; flow-rate, 1.0 ml/min. The thinner line shows the enzymic activity, and the thicker line shows the protein concentration. 1 = Non-retained peak; 2 = peak observed after elution by solution B.

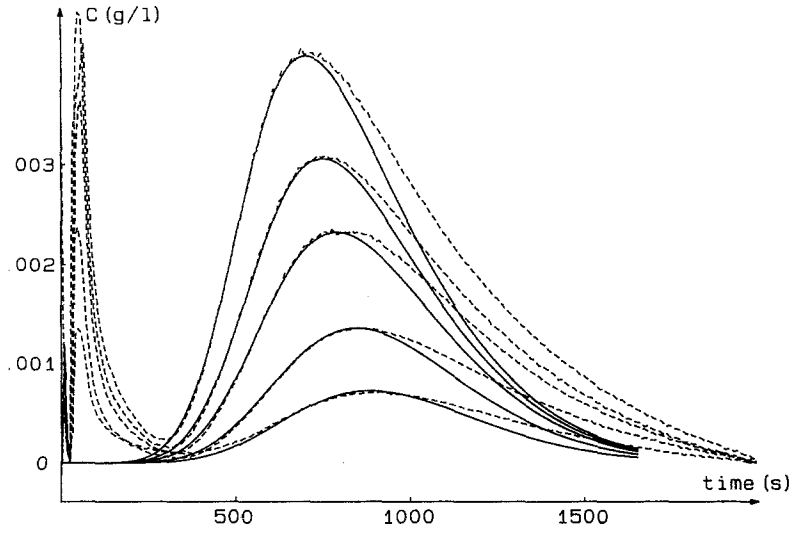

Fig. 3. Zonal elution HPAC of trypsin as a function of sample size, on the NAB support. Sample size, 25–150 μg; flow-rate 1.0 ml/min; eluent A, 15 m*M* KH_2PO_4 (pH 7.4)–0.05 *M* L-arginine. (—) Theoretical values; (---) experimental values.

competitor in the eluent (L-arginine). The peak retention and its shape contain all the information needed to determine the magnitude of the physico-chemical and kinetic constants.

Column loading capacity determination. The protein capacity is usually determined from the equilibrium isotherm using frontal analysis [13]. In the present work the capacity of the support was determined from peak-shape analysis on the basis of a Langmuir isotherm. The interaction of the protein P with the immobilized ligand (X = pAB) and the soluble one (L = arginine) is described by the following pair of equations:

$$P + X \overset{K_{PX}}{\rightleftarrows} PX$$

$$P + L \overset{K_{PL}}{\rightleftarrows} PL$$

The resulting partition isotherm is of the Langmuir type and may be determined by fitting the theoretical elution profiles to the experimental ones. The equation of the elution peak is the solution of a system of differential equations describing the solute migration through the column and the Langmuir kinetic law [14,15].

The changes in the elution profiles were studied by injecting increasing amounts of trypsin into the column. The decrease of the retention time with increasing sample size shows that a saturation effect intervenes. The analysis of the experimental curve enables the capacity of the column for the protein to be determined (Fig. 3). The theoretical model (solid line) does not entirely fit the total experimental profile, and the peak tailing end is not well described. This may be due to the support heterogeneity. The values of the capacity Q_X determined for the various supports are listed in Table I.

Equilibrium measurements. The retention volumes V_z at infinite dilution is equal to the slope of the equilibrium isotherm at the origin:

$$V_z - V_0 = K_{PX}Q_X/[1+(L)K_{PL}] \quad (1)$$

where V_0 is the interstitial volume. It was determined by chromatography of trypsin under non-retention conditions (pH 2.8). This equation permits the binding constants K_{PL} and K_{PX} to be determined [1].

As shown in Fig. 4, the elution volume of trypsin is inversely proportional to the concentration of the soluble ligand, L-arginine. The ratio of slope to intercept gives the value of the binding constant $K_{PL} = 1.7 \cdot 10^2$ l/mol.

The elution volume was linearly dependent on the amount of immobilized ligand (Q_X) as pre-

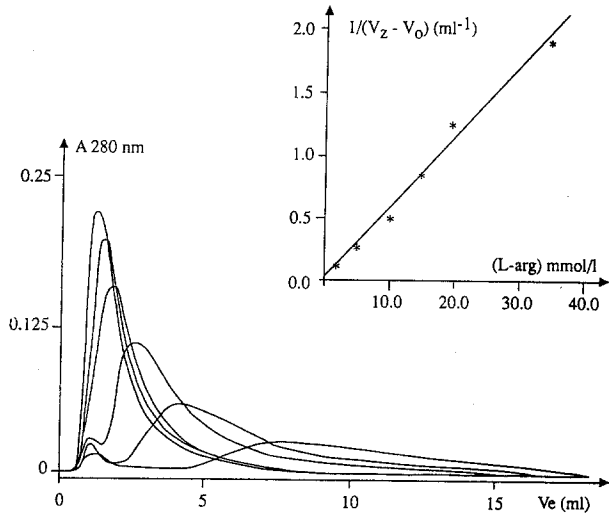

Fig. 4. Dependence of the trypsin elution volume on the concentration of L-arginine on the NAB support. Sample size, 20 μg; flow-rate, 1.0 ml/min; support, LAB; eluent A, 15 m*M* KH_2PO_4 (pH 7.4) plus 0.2–35 m*M* L-arginine. Inset: a plot of $1/V_e - V_0$ *versus* the concentration of L-arginine in solution.

dicted by eqn. 1. For all the experiments, the concentration of the soluble ligand was maintained at 0.1 *M*. The trypsin elution volume was plotted *versus* Q_X determined for every support (Fig. 5). In agreement with eqn. 1, the variation of $V_z - V_0$ as a function of Q_X is a straight line that passes through the origin. The binding constant, K_{PX}, is deduced from its slope: $K_{PX} = 2.6 \cdot 10^6$ l/mol. This result is in good agreement with the values given in literature and measured by analytical affinity chromatography [1,12].

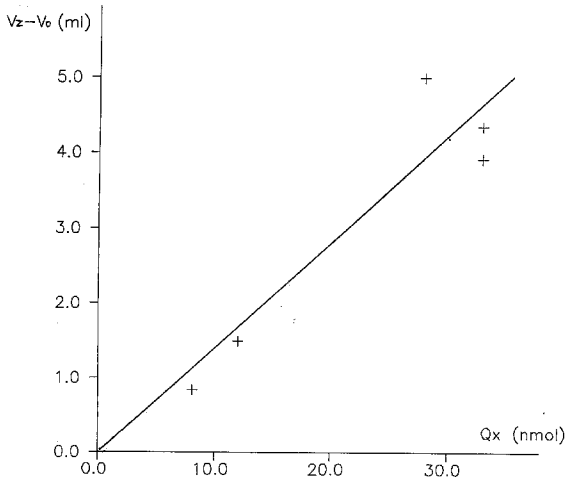

Fig. 5. Dependence of the trypsin elution volume on the protein capacity. Sample size, 20 μg; flow-rate, 1.0 ml/min; eluent A, 15 m*M* KH_2PO_4 (pH 7.4)–0.1 *M* L-arginine.

Comparison of the column efficiencies

The band broadening of the elution peak was characterized from the variation of the plate height (*H*) *versus* the flow-rate. The plate height is the sum of several terms [16]: axial dispersion, eddy diffusion, diffusion into the pores and kinetic mass transfer between the mobile phase and the stationary phase. The last two contributions are proportional to the linear velocity, *u*. The increment due to diffusion into the pores may be evaluated from the variation of the particle size or the porosity. The plate-height variation against the linear velocity is given in Fig. 6 for the various supports. The injected amount of protein was sufficiently small not to overload the columns (linear part of the adsorption isotherm). Values of *H* were determined from peak-width measurements at 0.6 of peak height.

The plate height were found to be very large (0.5–1.5 cm). These values are comparable with

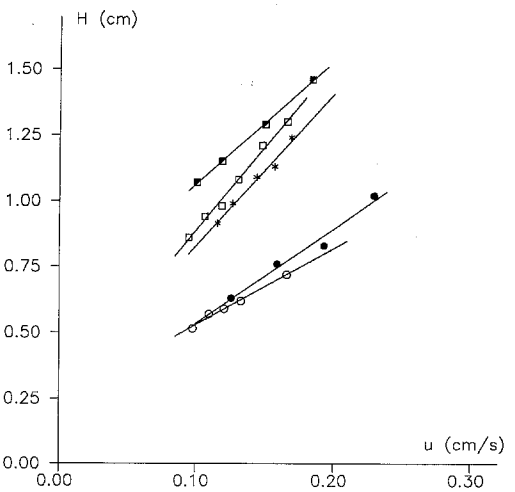

Fig. 6. Plate height (*H*) of trypsin elution peak as a function of the linear velocity (*u*). Sample size, 20 μg; flow-rate,1.0 ml/min; eluent A, 15 m*M* KH_2PO_4 (pH 7.4) with L-arginine (L) added to obtain $k' = 10$. (◩) FAB support, [L] = 0.05 *M*; (□) PAB support, [L] = 0.08 *M*; (∗) IAB support, [L] = 0.05 *M*; (●) NAB support, [L] = 0.1 *M*; (○) LAB support, [L] = 0.02 *M*.

the results obtained by HPAC by other authors, who studied the elution of trypsin on soybean trypsin inhibitor immobilized on silica [17]. The poor efficiencies in HPAC are mainly due to two different contributions: the low-rate dissociation constants and the restricted diffusion into the pores. By comparing porous and non-porous supports of identical affinity stationary phase, Anspach *et al.* [18] have shown that the chromatographic performance, in terms of speed and protein recovery, is better with non-porous ones. With these supports, no resistance to pore diffusion is possible. For supports with the same particle size, but differing in porosity (IAB 300 Å, 20 μm and PAB 1200 Å, 20 μm) the efficiencies are quite similar. Larger plate heights were obtained with silica of larger particle diameter (20 μm). The plate-height curve determined with the 4000 support (LAB 4000 Å, 5 μm) is close to that obtained with the 300 support (NAB 300 Å, 10 μm), although the particle diameter is lower for the first. This reveals that the limiting mass transfer kinetic exchange is not diffusion into the pores but chemical desorption of the protein from the pAB stationary phase.

As shown in Fig. 6, the efficiency of the column is not significantly affected by the mode of the copolymer fixation either by adsorption (PAB support) or by covalent links to silica (FAB support).

This study shows that the slow desorption kinetics are mainly responsible for the poor efficiencies observed in the affinity system studied.

CONCLUSION

The (VP–CF) reactive copolymer was successfully used as a stationary phase for affinity chromatography. The chloroformate units react vigorously with the nucleophilic groups, and the activation method is well suited for the covalent linkage of a ligand in an organic medium. By this technique a *p*-aminobenzamidine affinity support was synthesized and can be used for trypsin specific retention. In reversible conditions (L-arginine in the eluent), the comparison between the various supports studied shows that the large band broadenings observed for trypsin elution peak are mainly due to the strong affinity interactions. The contribution from the restricted diffusion into the pores is small.

The column loading capacity for trypsin was measured from peak-shape studies as a function of the amount injected. A linear dependence was observed with the retention volume extrapolated to zero sample size and measured for a given concentration of arginine in the eluent. From this variation, the binding constant between trypsin and the immobilized ligand was determined and was in good agreement with values from previous affinity studies. This demonstrates the validity of applying the zonal elution method in mass overload conditions for measuring the column loading capacity.

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