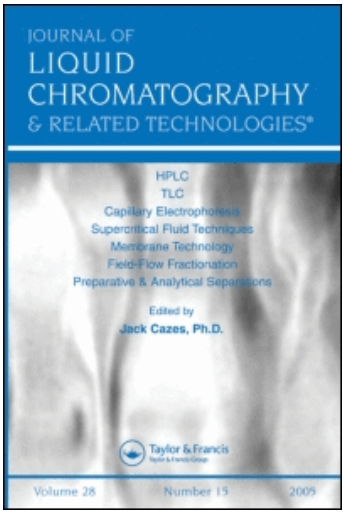

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PREPARATION AND CHARACTERIZATION OF CELLULOSE BASED ADSORBENTS FOR LARGE SCALE HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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ABSTRACT

The hydrophobic packings for preparative low pressure hydrophobic interaction chromatography (HIC) on the new granulated cellulose matrix Granocel were prepared by alkylation with phenyl glycidyl ether in the presence of sodium hydroxide. The ligand concentration up to 600 μmol/g was achieved with high reproducibility. The relative amount of eluted model protein at the fixed set of conditions was used as a criterion for the evaluation of the relative hydrophobicity of adsorbents. The relative hydrophobicity was found to depend on accessible, rather than total, amount of coupled ligand as well as on the nature of matrix.

The chromatographic behaviour of adsorbents both in laboratory and in large scale was evaluated by the enrichment of recombinant α_2-interferon from Pseudomonas putida VG-84.

INTRODUCTION

Hydrophobic interaction chromatography is known as a suitable method for protein isolation and purification with regard to the preservation of biological activity. Since salt fractionation is a very common early step in protein fractionation schemes, it is convenient to take the active fraction at high ionic-strength, to adsorb it on a hydrophobic column and after washing develop the column with decreasing gradient of salt.

The development of biotechnology has increased the importance of preparative large scale low pressure HIC. Large scale chromatography requires improved, tailored and cheap support materials, flexible with respect to process optimization. In order to purify the product as effectively as possible, a wide assortment of hydrophobic packings is desirable. If packings with the optimal pore size are used, fractionation of proteins according to their hydrophobicity as well as to their molecular size may be achieved. The ligand concentration at the surface of packings and their accessibility to sorbates plays a great role in optimization of adsorption-desorption process.

However, commercially available assortment of packings for preparative HIC is quite limited. The most of widely used adsorbents are based on Sepharose matrix. Their regeneration with organic solvents is complicated in large scale process.

In this paper the preparation of packings for HIC on the matrix Granocel is reported. Granocel is trade-mark of semi-rigid cellulose gels recently developed in our laboratory. Gels exhibit high mechanical properties in a wide range of pore size and are cheap to produce, therefore are suitable for large scale chromatography. The hydrophobic packings Phenyl-Granocel were prepared by alkylation of cellulose matrix with phenyl glycidyl ether. The effect of ligand concentration and pore size on binding strength and capacity of model proteins was investigated. The suitability of Phenyl-Granocel for the enrichment of α_2-interferon in large scale process was also studied.

EXPERIMENTAL

Equipment

A liquid chromatograph (KOVO, Czechoslovakia) consisting of HPP 5001 precision electromechanical pump, RIDK 102 differential refractometer, LCD

2563 UV-VIS detector and TZ 4620 linear recorder was used for inverse gel-permeation chromatography and for the chromatography of model proteins.

The chromatography of α_2-interferon in laboratory and in pilot scale was performed on a low pressure liquid chromatograph (Pharmacia-LKB, Sweden) constisting of Microperpex S and Minipuls 2 (Gilson, France) peristaltic pumps, gradient mixer GM-1, UV-monitor UV-2 with analitical or industrial flow cell, columns C10/70, XK 50/60, K 100/45, BP 113, fraction collector FRAC-300 and two-channel recorder REC-482, for large scale chromatography was used Bioprocess I (Pharmacia, Sweden).

A Milton Roy spectrophotometer Spectronic 1201 with a 1-cm cell and multichannel spectrophotometer Multiskan MCC (Flow Laboratories) were used for spectrophotometric measurements.

Materials

Cellulose matrix Granocel was synthesized in our laboratory. Phenyl-Sepharose CL-4B was purchased from Pharmacia (Uppsala, Sweden), Phenyl-Silochrom C-80 was from "Fermentas" (Lithuania). Dextran kits were purchased from Pharmacia and Serva (Germany). Phenyl glycidyl ether (PGE) was obtained from Reachim (U.S.S.R.) and distilled at 109°C/5mm Hg. Bovine serum albumin (BSA), human serum albumin (HSA) and horse hemoglobin (Hb) were obtained from Reanal (Hungary).

All other chemicals were of analytical or reagent grade.

Methods

Preparation of cellulose matrix Granocel

Cellulose matrix Granocel was prepared by the saponification of acetylcellulose in solution (patent pending). The gel obtained was mechanically cut and the fraction of 100-200 μm was used for the further modification. The matrixes with exclusion limit of $2x10^6$ (Granocel-2000), $5x10^5$ (Granocel-500) and $3x10^5$ (Granocel-300) for dextrans were used at the present work.

The cross-linking of cellulose matrixes was performed by reaction with epichlorohydrin in 1M NaOH at 50°C for 2h.

Coupling of phenyl glycidyl ether

30 g of suction dried cellulose matrix was suspended in 37 ml of 1 M NaOH and 0.76-4.0 ml of phenyl glycidyl ether was added. The slurry was stirred

at 60°C for 5 h. The product was washed thoroughly with acetone and distilled water.

Determination of the degree of substitution

The dried gel (50 mg) was hydrolyzed at room temperature with 2 ml of 9.2 M H_2SO_4 for 15 h. The hydrolyzed gel was diluted to 50 ml with water and the absorbance at 270.5 nm was registered. The absorbance of acid hydrolysed unsubstituted gel is approx. 0.

The solutions of phenol in the concentration range 0.1-1 mM in 0.37 M H_2SO_4 were used for the determination of the molar absorbtivity (ε). The molar absorbtivity 1.18×10^3 M^{-1} cm^{-1} was used in the calculation of the degree of substitution.

Evaluation of relative hydrophobicity and adsorption capacity of adsorbents

The chromatography of model proteins for evaluation of relative hydrophobicity of adsorbents was performed. A 15 x 1 cm I.D. glass column was packed with the gel to obtain a bed volume of 7 ml. The column was equilibrated with 0.05 M sodium phosphate buffer (pH 7.0) containing 1.5 M $(NH_4)_2SO_4$. The column was charged with 50 mg a protein dissolved in 5 ml of the salt-buffer solution at a flow rate of 30 cm/h and washed with 2 bed volumes of the same buffer. The experiments were performed at a room temperature. The adsorbed protein was eluted from the column in two desorption steps: the first with 2-3 bed volumes of 0.05 M sodium phosphate buffer (pH 7.0), and the second with 2-3 bed volumes of 30% (v/v) isopropanol in the same buffer. The concentration of eluted protein in each fraction was determined according to Bradford[1]. The relative hydrophobicity of adsorbents was expressed by the amount of protein desorbed from the column on the first step of elution, calculated relatively to the total amount of adsorbed protein. Elution with 30% (v/v) isopropanol was found to be sufficient to regenerate the adsorbents.

The same chromatographic process for evaluation of adsorption capacity of packings was performed except that the bed volume of column was 2 ml and only one step elution with 30% isopropanol was used. The adsorption capacity was calculated from the amount of protein adsorbed on the column.

Evaluation of porous structure of adsorbents by inverse gel permeation chromatography

Gel filtration of dextran standards in 10 % (v/v) ethanol solution was performed to evaluate the porous structure of adsorbents. The solutions of

dextrans (2.0 mg/ml) were applied to the column (30 x 0.6 cm I.D.) at a flow rate of 25 cm/h. The results were interpreted according to the method described by A.A. Gorbunov et al[2].

Chromatography of α_2-interferon

HIC of α_2-interferon was performed using solutions after cell disruption, centrifugation and precipitation by ammonium sulfate. Precipitated proteins were dissolved in 0.025 M phosphate buffer, pH 7.2. The conductivity of the solutions was adjusted to 90-100 $mScm^{-1}$ with sodium chloride. The solutions of α_2-interferon were prepared in such way that protein concentration was 2 mg/ml (determined according to Lowry[3]) and interferon activity by ELISA - $3x10^7$ IU/ml. The quantity of α_2 -interferon loaded on adsorbent was $2x10^8$ IU for 1 ml of adsorbent. Equilibration of column, load of the sample and desorption were performed at 50 cm/h. The column was equilibrated and washed after adsorption with 6-8 bed volumes of 0.025 M phosphate buffer, pH 7.2, with 3 M sodium chloride (conductivity - 110 $mScm^{-1}$). α_2-interferon was desorbed step wise with 9-13 bed volumes of 0.025 M phosphate buffer, pH 7.2, without sodium chloride. After desorption adsorbent was regenerated without repacking it in the column with 0.1 M NaOH solution. The solution was put into the column from the bottom to the top at a flow rate of 80 cm/h.

The adsorbent of 10-12 ml, packed in 1 cm I.D. column was used for analytical purposes. The column K45/100 or BP/113 of 2-5 l of adsorbent was used for large scale process.

The recovery of α_2-interferon was calculated from the quantity of α_2-interferon remained in the column after washing.

RESULTS AND DISCUSSION

Synthesis of adsorbents

At a present work synthesis of hydrophobic adsorbents was performed by coupling of phenyl glycidyl ether (PGE) on the cellulose matrix in the 1M NaOH solution. The routine procedure was described above, the results obtained are shown in Fig.1.

A linear relationship and a high reproducibility is observed for the ligand concentration up to 600 μmol/g dry gel. So, in this range of the degree of substitution an uniform distribution of the ligands on the gel may be expected[4,5].

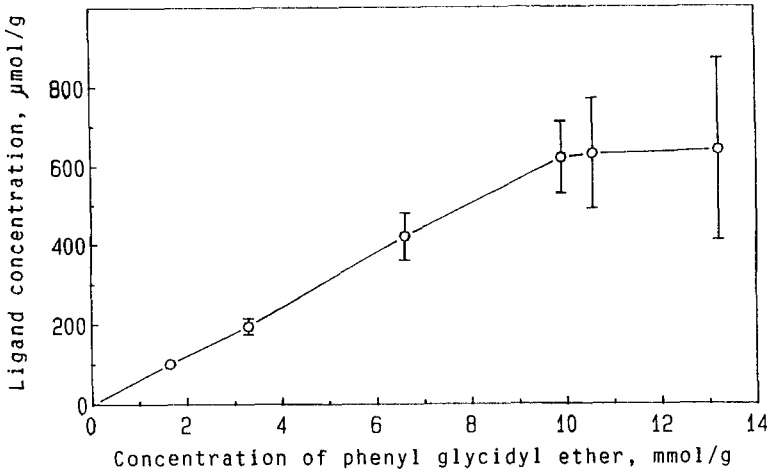

FIGURE 1

Relationship between the amount of phenyl glycidyl ether used during coupling and the degree of substitution. Each synthesis was performed at least five times and standard deviation was calculated (vertical bars).

When the amount of added PGE was more than 10 μmol/g dry matrix, the ligand concentration up to 800 μmol/g was achieved, but the reproducibility of process was unsatisfactory. The same degree of substitution was obtained when matrixes Granocel-2000 and Granocel-300 were used (data not shown).

The coupling yield was found to be low (approx. 6 %). This may be a direct consequence of the low solubility of PGE in the aqueous alkali medium and hydrolysis of oxirane groups in the presence of water. It was found that this serious disadvantage of the method cannot be diminished by the optimization of the synthetic route. It should be emphasized, however, that synthesis of commercially available Phenyl-Sepharose CL-4B, performed in a essentially water-free medium gives a coupling yield at best 50 % and includes tedious procedure of exchanging of the water with an organic solvent[6]. The methods of synthesis of hydrophobic adsorbents suggested later[5,7] are complicated enough too, because the step of activation of the matrix is required.

The flow-resistance of Phenyl-Granocel, synthesized on various matrixes, and Phenyl-Sepharose CL-4B for comparison is shown in Fig. 2. The results

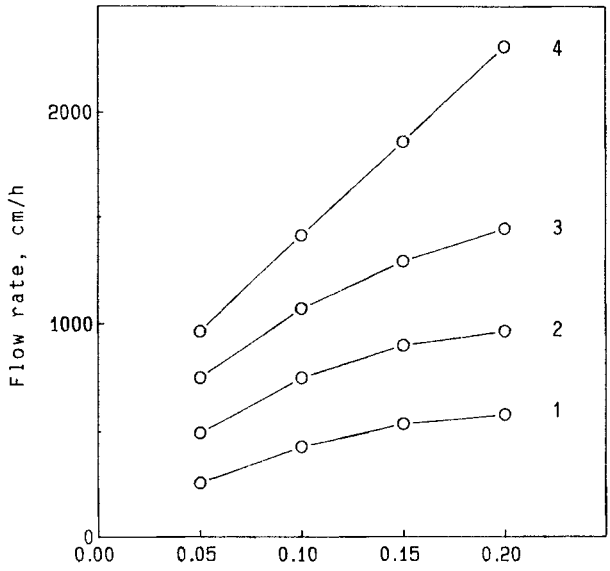

FIGURE 2

Flow characteristics of hydrophobic adsorbents. 1 - Phenyl-Sepharose CL-4B, 2 - Phenyl-Granocel-2000, 3 - Phenyl-Granocel-500, 4 - Phenyl-Granocel-300.

suggest that new-synthesized adsorbents are suitable for low pressure chromatography.

Relative hydrophobicity of Phenyl-Granocel

It is well known, that extent and strength of binding of proteins by the hydrophobic adsorbents depend on the concentration of hydrophobic ligand and therefore the degree of substitution is widely used as a criterion of the hydrophobicity of adsorbents. But there are more factors, such as chemical and physical structure of matrix, an accessibility of ligands to the sorbate molecules, that also affect the strength of hydrophobic interaction. Thus, if the different matrix and (or) different synthetic routes are used, the nearly equally substituted with the same ligand adsorbents may significantly differ in ability to bind proteins.

More criteria are suggested for evaluation of the hydrophobicity of adsorbents: the sorption capacity of hydrophobic probes[8], the partition coefficient

of the amphiphilic probe in the system amphiphilic sorbent-water[9], the energy of interaction between the methylene group and the surface of an adsorbent[10] and some others. However, the evaluation of these criteria is rather tedious.

One of the features of the hydrophobic interaction is that the proteins adsorbed to highly substituted gels are more difficult to elute than they are from gels with lower degrees of substitution[5,11]. This means, that the "difficulty" of elution of model proteins reflects the strength of their binding and so, the amount of eluted protein may be used for comparison of hydrophobicity of various adsorbents.

At the present work the BSA, HSA and Hb were used as a model sorbates. The proteins were adsorbed and eluted at a fixed set of conditions (see Experimental) and the relative amount of bound protein displaced by eluent was determined. Although the values obtained for different proteins differ and depend on the hydrophobicity of protein used (see Table 1), but the same general trend is observed, i.e. the higher ligand concentration leads to higher strength of binding of proteins (the amount of eluted protein decreases). Secondly, it is seen from data in the Table 1, that the protein binding strength depends on the peculiarities of the matrix of adsorbent. Note, that the values of the amount of eluted protein obtained for the adsorbents synthesized on the matrix cross-linked with epichlorohydrin (Phenyl-Granocel CL-500) are appreciably higher than those obtained for the noncross-linked adsorbents with the same ligand concentration and nearly equal pore size. The similar results were obtained on the adsorbents, cross-linked with glutaraldehyde and 2,3-dibrompropanol-1 (data not shown). This decrease of relative hydrophobicity of adsorbents is probably caused by the decrease of accessibility of hydrophobic ligands as a result of cross-linking.

For comparison the results obtained on Phenyl-Sepharose CL-4B and Phenyl-Silochrom C-80 are also given. It is evident, that the relative hydrophobicity of Phenyl-Granocel-500 with substitution degree of 440 μmol/g is very similar to that of Phenyl-Sepharose CL-4B. The relative hydrophobicity of Phenyl-Silochrome C-80 was found to be significantly higher, probably due to the hydrophobic nature of the matrix. So, this rapid and simple test may be used for the qualitative comparison of various commercial and new-synthesized hydrophobic adsorbents.

The dependence of adsorption capacity and the strength of binding of HSA upon the ligand concentration of Phenyl-Granocel-500 is shown in Fig.3.

TABLE 1

Model proteins elution from hydrophobic adsorbents

Adsorbent	Ligand concentration		Relative amount of bound protein displaced by eluent*, %		
	μmol/g	μmol/ml	BSA	HSA	Hb
1. Phenyl-Granocel-500	200	19	98	96	82
2. Phenyl-Granocel-500	380	36	95	82	68
3. Phenyl-Granocel-500	450	44	85	75	53
4. Phenyl-Granocel-500	800	81	79	60	10
5. Phenyl-Granocel CL-500	410	40	97	90	78
6. Phenyl-Granocel CL-500	700	70	95	85	70
7. Phenyl-Sepharose CL-4B	860	40	83	71	56
8. Phenyl-Silochrom C-80	82	27	55	45	36

* 0.05 M sodium phosphate buffer, pH 7.0

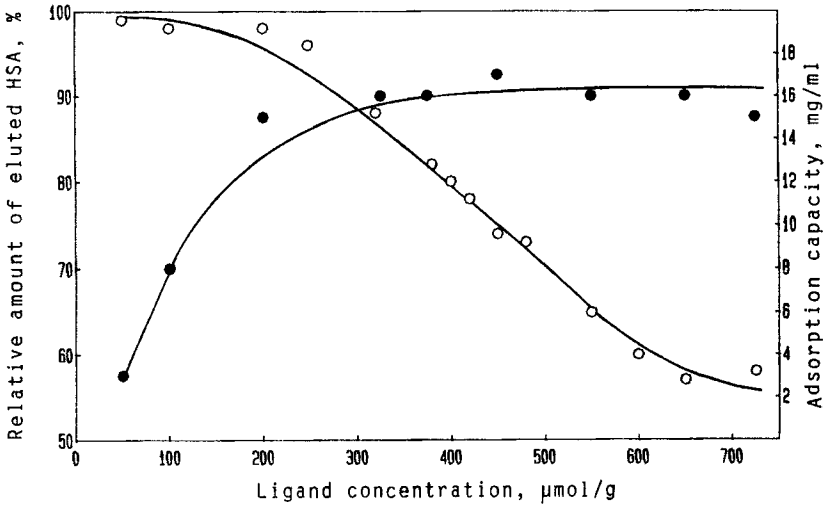

FIGURE 3

Influence of ligand concentration on adsorption capacity (●) of Phenyl-Granocel-500 and relative amount of eluted HSA (o).

It can be seen that the binding capacity is directly proportional to ligand concentration up to 200 μmol/g. At higher ligand concentrations the extent of binding is constant and equal to approx. 16 mg/ml. Otherwise, the relative amount of eluted HSA decreases gradually with increase of ligand concentration within the range of 200-600 μmol/g. So, the dependence of relative hydrophobicity of Phenyl-Granocel on the ligand concentration may be defined exactly in the conditions used.

Chromatography of α_2-interferon on Phenyl-Granocel

In order to study the chromatographic behaviour of synthesized adsorbents and their suitability for use in large scale processes, chromatography of α_2-interferon on the series of Phenyl-Granocel was performed. HIC on Phenyl-Silochrom C-80 or on Phenyl-Sepharose CL-4B is used as one of the first chromatographic step in the scheme of the purification of recombinant α_2-interferon from Pseudomonas putida VG-84[12]. Protein sorption is performed in 0.025 M phosphate buffer solution (pH 7,2) in the presence of 3 M NaCl and the same buffer without salt is used for elution. It should be noted, that the optimum conditions for the use of Phenyl-Granocel were not determined in the present work - both adsorption and elution were performed under condition identical to those in large scale process on Phenyl-Silochrom C-80 or Phenyl-Sepharose CL-4B.

The characteristics of adsorbents used and data of chromatography of α_2-interferon are summarized in Table 2.

As expected, the chromatographic performance is sensitive to relative hydrophobicity of adsorbents. Using columns packed with the samples 1 and 8, which exhibit low relative hydrophobicity, the significant amount of α_2-interferon was detected in unadsorbed fractions. In addition, due to low binding strength of adsorbed protein on these adsorbents, part of adsorbed α_2-interferon was lost by the subsequent washing of the column, therefore the values of recovery are low. The low recovery from the sample 6 with high relative hydrophobicity is obtained too. But in this case the binding strength is seen to be too high to elute the protein with buffer solution. This would suggest, that desorption of α_2-interferon from such adsorbent requires more strong eluent.The influence of pore size of adsorbents on binding capacity of the column was found to be little, except when the adsorbent with average pore diameter of 17 nm,

TABLE 2

Chromatography of α_2-interferon on hydrophobic adsorbents

Adsorbent	Characteristics of adsorbents					Chromatography of α_2-interferon		
	Pore size, nm	Poli-disper-sity	Ligand concen-tration, μmol/g	Relative amount of eluted HSA, %	Adsorption capacity for HSA, mg/ml	Unadsorbed interferon, %	Recovery of inter-feron, %	Enrich-ment, fold
1. Phenyl-Granocel-500	25	1.0	380	82	16	48	44	1.0
2. Phenyl-Granocel-2000	32	1.1	420	78	18	3	79	2.8
3. Phenyl-Granocel-500	23	1.0	450	74	17	7	99	3.2
4. Phenyl-Granocel-500	23	1.0	550	65	16	2	83	3.3
						9	75	3.5*
5. Phenyl-Granocel-2000	28	1.0	600	60	18	0	90	3.8
6. Phenyl-Granocel-500	22	1.0	720	60	15	1	67	3.1
7. Phenyl-Granocel-500	17	1.0	830	55	5	25	79	3.1
8. Phenyl-Granocel CL-2000	28	1.0	680	85	16	28	55	1.8
9. Phenyl-Sepharose CL-4B	-	-	860	83	20	0	96	3.6
10. Phenyl-Silochrom C-80	60	-	82	45	21	2	67	3.2

* The results are obtained in large-scale process after three times of regeneration in column

see sample 7, was used. The adsorption capacities for α_2-interferon and for HSA on sample 7 are significantly lower that these on the other samples despite higher ligand concentration. Results suggest that pore size of 17 nm is not enough to penetrate proteins used.

The enrichment of α_2-interferon in large scale was performed in the column K 45/100 on Phenyl-Granocel-500 (ligand concentration 560 μmol/g). Adsorbent volume - 2.5 l, flow rate 30-60 cm/h. A 3.5-fold enrichment with good recovery was achieved (Table 2). The results obtained are similar to these in laboratory scale.

The gels did not compress noticeably, when packed in the column and used over several runs. The adsorbents have also shown a high resistance to physical attrition which means that production of fines does not occur on repeated handling or mechanical stirring. Consequently the adsorbents can be re-used many times. Another advantage observed for the Phenyl-Granocel adsorbents was their excellent resistance to 1M sodium hydroxide solutions. This feature enables to perform the regeneration process with sodium hydroxide solutions in column without loss in adsorption capacity, recovery and degree of enrichment of α_2-interferon.

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