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DIRECT LIQUID CHROMATOGRAPHIC SEPARATION OF ENANTIOMERS ON IMMOBILIZED PROTEIN STATIONARY PHASES

VII. SORBENTS OBTAINED BY ENTRAPMENT OF CROSS-LINKED BOVINE SERUM ALBUMIN IN SILICA

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SUMMARY

Chiral sorbents have been prepared by physical immobilization of bovine serum albumin (BSA), cross-linked with glutaraldehyde, into silica pores. BSA is first loaded into the silica and then entrapped by the cross-linking reaction. Sorbents of this kind were prepared even from 60-Å silica without loss of BSA content due to limited access by size exclusion. The cross-linker contributes to a large extent to the increased hydrophobicity, leading to significantly larger capacity factors for hydrophobic solutes. Treatment with organic solvents leads only to a minor irreversible reduction of enantioselectivity.

INTRODUCTION

The successful use of biopolymers, such as proteins, as chiral stationary phases in liquid chromatography[1] has led to an interest in understanding how the performance of sorbents of this kind is influenced by the method used for immobilization. One of the most relevant questions has been whether or not it is necessary for the protein to reside in its native structure. Related to this problem is the possible denaturing effect that may be caused by the presence of large amounts of organic cosolvents in the mobile phase. Since the availability of the enantiomer-discriminating binding sites is of primary importance, one might assume that steric effects arising from inappropriate immobilization techniques would be reflected in decreased separation factors.

Two recent publications by Aubel and Rogers[2,3] are of great interest in this respect. They found that a glutaraldehyde-bound bovine serum albumin (BSA)–silica sorbent may show increased performance after pretreatment with organic solvents followed by 4 *M* aqueous urea.

For the last couple of years, we have been using BSA–silica sorbents based exclusively upon physical immobilization of BSA within the silica pores by glutar-

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aldehyde cross-linking. We therefore feel that a comparison of results is justified. The present paper describes our method of immobilization and the properties of the chiral sorbent, and also presents a discussion on the rôle of the silica matrix as well as the effects of the hydrophobicity of the material obtained.

EXPERIMENTAL

Chemicals

The amino acids were from Sigma (St. Louis, MO, U.S.A.). The N-benzoyl derivatives were prepared using benzoyl chloride by a standard method[4]. The preparation of the N-(2,4-dinitrophenyl) (DNP) amino acids was performed as described previously[5]. (±)-Benzoin was from Fluka (Buchs, Switzerland). The glutaraldehyde, 50% in water from Eastman Kodak (Rochester, NY, U.S.A.), was diluted in water to give a 5% solution before use.

BSA was a "pure" grade quality (Cat. No. 11930) from Serva (Heidelberg, F.R.G.). The silica used was from Macherey-Nagel (Düren, F.R.G.) and was either spherical (Nucleosil) or irregular (Polygosil). Different pore sizes and particle sizes were used in the experiments. Information concerning the pore diameters and particle sizes was obtained from the suppliers of the materials.

All other chemicals and solvents were of analytical grade.

Preparation of the BSA–silica sorbent

BSA (2 g) was added to 15 ml of an ammonium dihydrogenphosphate buffer (100 m*M*, pH 4.4) and placed in an ultrasonic bath for 3 min. Silica (4 g) was added to the BSA solution in small portions and then ultrasonicated for 10 min. The slurry was slowly shaken for 24 h at room temperature. Then, 250 μl of 5% glutaraldehyde were added to the mixture and the shaking continued for 2 h.

The slurry was filtered on a Millipore membrane (RA, 1.2 μm) and the silica washed twice with 20 ml of the buffer. It was then resuspended in 20 ml of the buffer and 600 μl of the glutaraldehyde solution were added. The slurry was shaken at 37°C overnight and the silica sorbent isolated by filtration. It was washed with phosphate buffer (50 m*M*, pH 7.0) and thereafter with 40% 1-propanol in the same buffer.

In the case of larger batches, the filtration steps were replaced by centrifugation at 1600 g for 5 min.

The BSA content of the packing material was determined by elemental analysis of nitrogen and sulphur. The analyses were performed by Mikro Kemi (Uppsala, Sweden).

Column packing

The columns were packed by the ascending slurry packing method[6]. As slurry and packing medium a 50 m*M* phosphate buffer, pH 7.0, containing 40% 1-propanol, was used. A stainless-steel tube of volume *ca.* 18 ml was used as a slurry reservoir and the packing was performed using a pressure of 300 bar.

Liquid chromatography

The chromatographic system consisted of a Model 2150 pump (LKB, Bromma, Sweden), a 7125 sample injector (Rheodyne, Cotati, CA, U.S.A.) equipped with a

20-μl loop and a Model V[4] variable wavelength UV absorbance detector (ISCO, Lincoln, NE, U.S.A.). The high-performance liquid chromatographic (HPLC) columns were 150 mm × 4.6 mm or 200 mm × 2.6 mm packed with the BSA–silica prepared as described. For comparative experiments a 150 mm × 4.0 mm Resolvosil-BSA column from Macherey-Nagel was used.

The mobile phase was a phosphate buffer containing 0–10% of 1-propanol. All experiments were made under isocratic conditions. Retention times and peak areas were obtained by an electronic integrator, Model 740; (Waters, Milford, MA, U.S.A.) interfaced with the detector.

Conditioning with organic solvent

The packing material was generally used without any further conditioning after being washed with 40% 1-propanol solution. In two cases, however, a conditioning of the packing material with organic solvents was performed. The first case entailed rinsing with water, acetone and then hexane. In the second case the conditioning sequence described by Aubel and Rogers[2] was employed: water, methanol, ethanol, hexane, hexane–dichloromethane (80:20)

RESULTS AND DISCUSSION

Column performance

The performance of a column packed with physically immobilized, cross-linked BSA is shown in Fig. 1 for the resolution of (±)-benzoin. The capacity factors (k') obtained under the conditions used are 9.3 and 25.3, respectively, giving an α value of 2.7. The resolution factor, R_s, calculated is 7.9. This and many other chromatograms obtained with this type of sorbent show that the polymeric nature of the chiral stationary phase does not give rise to peak broadening due to slow exchange and, in the majority of cases, does not negatively affect the α values.

Ultrasonic treatment of the silica–BSA mixture during the preparation of the

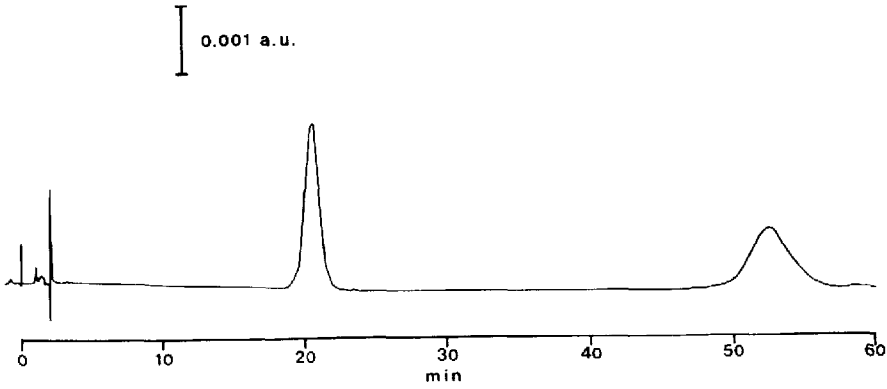

Fig. 1. Enantiomers of (±)-benzoin separated on entrapped, glutaraldehyde cross-linked BSA. The silica used was Nucleosil 100-7 which was packed in a 150 mm × 4.6 mm column. Phosphate buffer (50 mM, pH 8.0) + 2% 1-propanol. Flow-rate 1.0 ml/min UV detection at 247 nm. A 50 μM solution was injected.

sorbents was used to degas the silica and hasten the solubilization of BSA. We have prepared sorbents both with and without ultrasonic treatment, and found no difference in column performance.

Effect of silica properties on BSA content

Sulphur and nitrogen analyses of the dried sorbent showed considerable variation in the amount of entrapped BSA, depending on the pore diameter of the silica material (Table I). The BSA molecule is an ellipsoid with axes of 140.9 Å × 41.6 Å (ref. 7). The low amount of BSA in the 50-Å silica is thus probably caused by restricted diffusion. On the other hand, in the case of the 300-Å material, the poor entrapment may be caused by the rinsing step. The method described here is based on addition of the bifunctional glutaraldehyde reagent after the BSA; it is essential that the cross-linking reaction is carried out in two steps to avoid the formation of external polymer and agglomeration of the silica particles. Therefore, a washing step is included in order to eliminate BSA from the exterior of the silica. The second addition of glutaraldehyde will then yield an isoluble polymer structure, well anchored within the silica pores.

TABLE I

BSA CONTENT BASED ON NITROGEN AND SULPHUR ANALYSIS OF BSA–SILICA SORBENT MATERIAL (7 μm SILICA PARTICLES)

Pore diameter Å	*Amount of BSA (mg/g)*
50	131
100	215
300	78

The column performance is a reflection of the amount of entrapped BSA. The k' values and the separation factors, α, for (±)-benzoin were found to be dependent on the amount of entrapped BSA (Table II). Both the resolution and the retention of the enantiomers on the columns increase with increasing amount of entrapped BSA. Also from the asymmetry factors, η, the 100-Å material (highest BSA content) has a higher column load threshold than those of the 50- and 300-Å materials.

A comparison between a sorbent prepared with the method described here and a commercially available Resolvosil-BSA column shows that the latter is significantly less hydrophobic and thus gives lower k' values. This is well illustrated by Fig. 2 which shows chromatograms of N-DNP-aspartic acid on the two types of columns under otherwise identical conditions. While the elution is more rapid, there is a certain decrease in resolution. A comparison of data is given in Table III. The Resolvosil-BSA columns are prepared by an immobilization technique other than the one described in this paper, and contain *ca.* 100 mg BSA per gram silica.

Effect of treatment with organic solvents

The washing procedure, using 40% 1-propanol in buffer, gave a stable packing material that showed no signs of leakage. A portion of this sorbent was washed with

TABLE II

COMPARISON OF THE RESOLUTION OF (±)-BENZOIN ON SILICA WITH DIFFERENT PORE DIAMETERS

Mobile phase: phosphate buffer (50 m*M*, pH 7.9) + 6% 1-propanol. UV detection at 247 nm. Solute: (±)-benzoin.

Injected quantity (nmol)	k'_1	k'_2	η_1	η_2	α	R_s
Nucleosil: 50 Å, 7 μm						
2	3.5	4.5	0.9	0.9	1.3	1.7
10	3.5	4.2	1.1	1.5	1.2	1.3
50	3.4	4.0	1.4	1.7	1.2	0.8
Nucleosil: 100 Å, 7 μm						
2	5.9	8.6	0.8	0.8	1.5	2.0
10	5.5	7.5	0.8	1.0	1.4	1.6
50	5.0	6.5	0.9	1.0	1.3	1.0
Nucleosil: 300 Å, 7 μm						
2	2.3	3.0	1.1	1.4	1.3	1.7
10	2.3	2.8	1.1	1.6	1.2	1.2
50	2.1	2.4	1.8	2.0	1.2	0.6

water, acetone and finally hexane. A column was then prepared using hexane as the liquid for packing. Another column that had been packed in the normal way (in aqueous buffer) was rinsed in turn with water, acetone and hexane. These columns were then tested with the use of 2–6% 1-propanol in hexane as the mobile phase. Although (±)-benzoin was retained on the columns, no separation of the enantio-

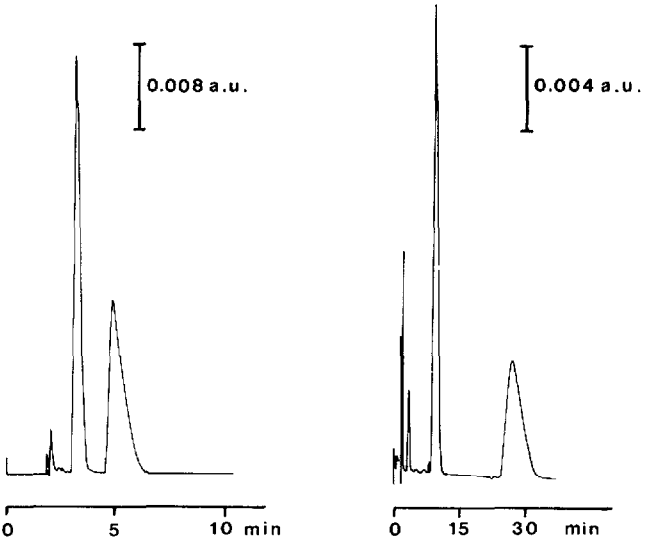

Fig. 2. Optical resolution of N-DNP-aspartic acid on Resolvosil-BSA (left) and on entrapped, glutaraldehyde cross-linked BSA (right). Phosphate buffer (50 m*M*, pH 7.7), 3% 1-propanol. Flow-rate 1.0 ml/min UV detection at 340 nm. Solute concentration: (left) 100, (right) 200 μ*M*.

TABLE III

COMPARISON OF CHROMATOGRAPHIC DATA OBTAINED WITH THE TWO DIFFERENT BSA COLUMNS

(a) Solute: (±)-benzoin. Mobile phase: phosphate buffer (50 m*M*, pH 8.0) + 2% 1-propanol; 1.0 ml/min; UV 247 nm. (b) Solute: N-(2,4-dinitrophenyl)-D,L-aspartic acid. Mobile phase: phosphate buffer (50 m*M*, pH 7.7) + 3% 1-propanol; flow-rate 1.0 ml/min. UV detection at 240 nm.

Column		k'_1	k'_2	α	R_s
BSA-nucleosil 100-7	a	9.9	24.7	2.5	7.6
	b	4.0	14.0	3.5	4.2
Resolvosil-BSA	a	5.6	6.9	1.2	1.3
	b	0.7	1.7	2.4	1.9

mers was achieved. The columns were rerinsed with acetone and water, equilibrated with the aqueous mobile phase and (±)-benzoin was again readily resolved. These data are shown in Table IV. However, the separation factor was found to be somewhat lower than that before the organic solvent treatment (Fig. 3).

Aubel and Rogers[2] tested two N-benzoylamino acids on their BSA–silica columns and found that organic conditioning of the column increased the separation factors for these compounds. Since this was contrary to our results with benzoin, one of our columns was conditioned according to their procedure and tested with the use of three N-benzoylamino acids. The results are given in Table V. While the organic conditioning decreases the R_s values for the least hydrophobic compounds, *i.e.*, the serine and alanine derivatives, an opposite, but small, effect was observed for the phenylalanine derivative. There seemed to be no general trend in the effect on the capacity factors, however.

On the other hand, a general effect caused by the organic conditioning of the column seems to be a significant improvement of peak symmetry (Table V). However, this effect was not associated with any gain in column efficiency in terms of plate number. The reason for this change in chromatographic behaviour is not yet known.

These results resemble those of Aubel and Rogers[3] insofar as they indicate that changes created in the protein conformation by treatment with organic solvents can be almost completely restored after prolonged treatment with the initial buffer. The

TABLE IV

RESOLUTION OF (±)-BENZOIN BEFORE (a) AND AFTER (b) TREATMENT OF THE COLUMN WITH ORGANIC SOLVENTS

Column: 150 mm × 4.6 mm BSA-Polygosil 60-7. Mobile phase: 50 m*M* phosphate buffer (pH 7.0, 6% 1-propanol), 0.8 ml/min. UV detection at 254 nm.

	k'_1	k'_2	α	R_s
a	3.1	4.4	1.4	2.4
b	3.0	4.0	1.3	1.9

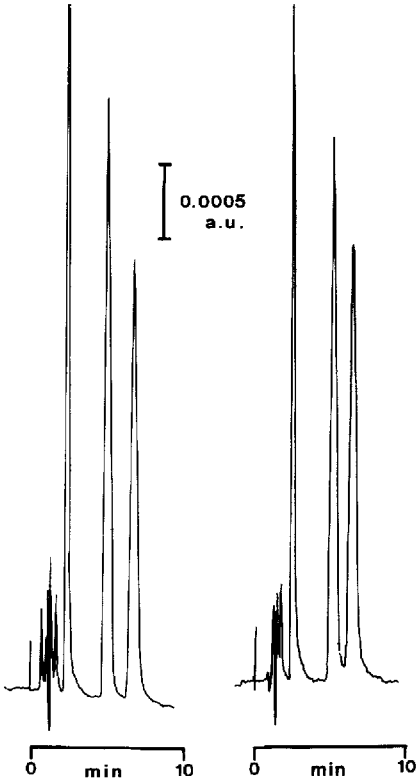

Fig. 3. Optical resolution of (±)-benzoin on a 200 mm × 2.6 mm BSA-Polygosil 60-7 column before and after treatment with organic solvents. Phosphate buffer (50 n*M*, pH 7.0) + 6% 1-propanol. Flow-rate 0.8 ml/min UV detection at 247 nm. A 25 μM solution was injected.

immobilized protein therefore behaves quite differently from its free state in solution. The most likely reason for this is the decreased conformational mobility caused by the multipoint attachment to the solid phase.

TABLE V

CHROMATOGRAPHIC DATA OBTAINED WITH A GLUTARALDEHYDE CROSS-LINKED BSA SORBENT BEFORE (a) AND AFTER (b) EXPOSURE TO ORGANIC SOLVENTS

Column: 150 mm × 4.6 mm BSA-Nucleosil 100-7. Mobile phase: phosphate buffer (pH 6.5, 5% 1-propanol), flow-rate 1.0 ml/min. UV detection 225 nm.

Solute		k'_1	k'_2	η_1	η_2	α	R_s
N-Benzoyl-D,L-serine	a	0.33	0.91	–	–	2.8	2.1
	b	0.12	0.50	–	–	4.2	1.7
N-Benzoyl-D,L-alanine	a	1.8	3.7	2.8	2.1	2.0	3.8
	b	1.6	2.9	1.0	1.0	1.8	2.6
N-Benzoyl-D,L-phenylalanine	a	6.1	21.0	1.0	1.6	3.4	6.8
	b	6.1	23.1	1.0	1.2	3.8	7.1

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