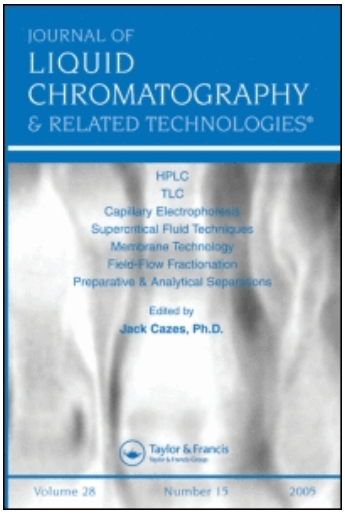

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The Influence of Mobile Phase pH on the Retention and Selectivity of Peptides and Proteins on A C-18 Derivatized Reversed Phase Matrix Based on Nonporous Glycidol-Filled Agarose Beads

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THE INFLUENCE OF MOBILE PHASE pH ON THE RETENTION AND SELECTIVITY OF PEPTIDES AND PROTEINS ON A C-18 DERIVATIZED REVERSED PHASE MATRIX BASED ON NONPOROUS GLYCIDOL-FILLED AGAROSE BEADS

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ABSTRACT

The synthesis of a C-18 derivatized reversed phase matrix based on nonporous agarose is described. The beads, which are stable at alkaline as well as acidic pH, were utilized for a study of the retention and the selectivity of peptides and proteins at different pH values. For most peptides and proteins the retention was strongest at an acidic pH, although basic peptides and proteins were most strongly retained at alkaline pH. The performance of the agarose based reversed phase matrix is demonstrated by isocratic separations of small peptides.

INTRODUCTION

The most commonly used stationary phases for reversed phase chromatography today are silica supports derivatized with aliphatic hydrocarbons (1). Such matrices give highly efficient separations, but the silica has the inherent drawback that it is not stable above a pH of 7-8.0. Recently, several stationary phases that are stable also at alkaline pH values have been introduced.

Among these are matrices made of crosslinked polystyrene, coated alumina, a coated vinyl alchohol copolymer, graphitized carbon (2) and a fluorocarbon resin (3).

Agarose beads have traditionally been considered as soft or semirigid, and thus not suited for HPLC- applications. However, recent reports show that agarose can be used also as an HPLC matrix (4-8). A drawback of porous agarose are its shrinking/swelling properties when organic solvents are used. Nonporous glycidol-filled agarose beads (9, 10) behave better and can be used in organic solvents, even when gradients are applied. This paper describes a synthesis of C-18 derivatized nonporous glycidol-filled agarose beads and their use for reversed phase chromatography of peptides and proteins.

MATERIALS AND METHODS

Materials

Divinyl sulphone (DVS) was from Fluka (Buchs, Switzerland), borontrifluoride diethyletherate (BF_3) from Merck-Schuchardt (Hohenheim bei München, F.R.G.), 2,3-epoxy-1-propanol (glycidol) and 1,4-butandiol diglycidyl ether (BDD) from Aldrich-Chemie (Steinheim, F.R.G.), dextran T 10, T 40 and T 500 from Pharmacia-LKB AB (Uppsala, Sweden) and Vikolox (a C-18 epoxide) from Viking Chemical Company (Minneapolis, MA, U.S.A.). All peptides and proteins were purchased from Sigma (St. Louis, MO, U.S.A.) (listed in Table 1). Chromatography-grade acetonitrile from Merck (Darmstadt, F.R.G.), was used in all chromatography experiments. All other chemicals were of analytical grade.

Instrumentation

The chromatographic system, including a Model 2150 HPLC pump, a Model 2152 HPLC controller, a 2158 UV-detector and a Model 2210 recorder, was from LKB (Bromma, Sweden). The loop injector was purchased from Rheodyne (Berkeley, CA, U.S.A.). The column tubes

with inner diameters of 3 and 6.5 mm were made of glass and purchased from Omnifit (New York, NY, U.S.A.). Pressure-stable variable-length endpieces were made at the workshop at the Uppsala Biomedical Center.

Methods

All experiments were performed with 12 per cent agarose beads with diameters between 10 and 20 μm prepared as described previously (11). Crosslinking of agarose beads was done with DVS (8). Deactivation of the nonreacted DVS (and simultaneous reduction in pore volume) were performed with dextran according to reference 8. In brief: 4 grams of crosslinked agarose beads were suspended in 20 ml of water (adjusted to pH 13 by addition of KOH) to which 50 mg sodium borohydride and 0.4 grams of dextran T 10, T 40 and T 500, respectively, were added. The reaction was performed in a 100-ml beaker with stirring at room temperature for 6 hours. The beads were washed with water on a Büchner funnel to a neutral pH, and then transferred stepwise to dioxane (10). The filling of the bead pores was completed by polymerization of glycidol (9). In brief: 4 grams of agarose beads were suspended in 30 ml of dioxane to which 4 ml of glycidol and 0.3 ml of BF_3 were added.The reaction was performed for 1 hour as described above. The beads were then washed with dioxane and crosslinked with BDD to stabilize the glycidol structure. 4 gr of gel was suspended in 20 ml of dioxane to which 1 ml of BDD and 0.2 ml of BF_3 were added. The reaction was performed for 45 minutes as described above. The beads were washed with dioxane and transferred to a beaker in which the derivatization with the C-18 epoxide was performed. 20 ml of dioxane and 0.8 gr of Vikolox were added. After the Vikolox had dissolved (ca. 30 minutes), 0.2 ml BF_3 was added and the reaction was run for 18 hours as described above. The beads were washed and transferred stepwise to a mixture of water:methanol (1:1).

The columns were slurry packed in water:methanol (1:1).

The characterization of the matrix was performed at different pH values. For pH 2, 0.1 % TFA was used as buffering agent; for pH

8, 100 mM NH_4HCO_3; for pH 9.8, 50 mM pentylamine/HCl and for pH 11, 100 mM NH_4OH. Linear gradients in acetonitrile, from 7 to 60 % in 60 minutes, were run at 0.25 ml/min. All absorbance measurements were made at 226 nm.

The retentions of the different peptides and proteins were expressed as V_e/V_T values, where V_e is the elution volume of the different peptides/proteins and V_T is the elution volume of tetraglycine which was not retarded at any of the pH values studied. The characterization studies were done with 6.5 x 35 mm beds, whereas 3 x 200 mm beds were used for isocratic separations. The isocratic separations, which were made to illustrate the performance of the synthesized matrix, were done at a flow rate of 0.1 ml/min.

RESULTS AND DISCUSSION

Agarose, like other carbohydrate-based chromatography matrices, has a tendency to shrink (or swell) when the solvent polarity is changed. This, together with the relatively low rigidity of these porous matrices, make them unsuitable as reversed phase supports. Both of these shortcommings can be overcome by using a nonporous matrix of agarose, the pores of which have been filled with dextran and glycidol (9). The glycidol polymer chains are also crosslinked to further increase the rigidity of the beads. These nonporous beads do not shrink or swell noticeably when the solvent polarity is changed. Another attractive characteristic of these beads is their pH stability: they can be used at both acidic and alkaline pH.

After derivatization with a C-18 epoxide, the beads were characterized by running standard peptides and proteins at different pH values: 2, 8, 9.8 and 11. The result is shown in Table 1, where the retention volumes of the different solutes are expressed as V_e/V_T values.

From this table one can see that the retention of the different peptides and proteins are weakest at "zwitterionic" pH

TABLE 1

Retention of peptides and proteins (expressed as V_e/V_T values), on a nonporous agarose based reversed phase matrix, as a function of mobile phase pH. V_e is the elution volume of tetraglycine and V_T is the elution volume of the different peptides/proteins. NE = Not Eluted.

			V_e/V_T			
	mw	pI	pH 2	pH 8	pH 9.8	pH 11
Tetraglycine	300		1.0	1.0	1.0	1.0
Leu-enkephalin	555		3.8	1.5	2.0	1.4
Met-enkephalin	634		3.0	1.7	2.0	1.2
Bradykinin	1,040		1.6	1.4	1.5	4.0
Insulin chain A (oxidized, bovine)	2,300		8.0	1.0	1.3	1.0
ACTH (porcine)	4,500		9.2	6.9	8.0	9.0
Insulin (bovine)	5,700	5.7	9.9	9.4	9.8	8.6
Cytochrom C (horse)	12,400	9.4	11.2	1.0	3.0	NE
Ribonuclease (bovine)	13,700	8.9	7.8	1.0	1.0	1.0
Lysozyme	14,400	11.0	11.1	10.2	NE	NE
Myoglobin (horse)	17,200	6.9, 7.4	14.1	16.0	17.3	20.4
Carbonic anhydrase (bovine)	29,000	5.9	13.4	1.0	1.0	1.0
Ovalbumin	43,000	4.7	16.3	1.0	1.0	22.0
Human serum albumin	66,400	5.8	14.5	12.7	15.3	16.8

values where they have the highest number of positive and negative charges, and therefore are most hydrophilic. The retention of the different components tested was strongest at pH 2 and 11.

Some of the peptides and proteins tested were more strongly retained at pH 9.8 than at pH 8 or 11. This is caused by the relatively hydrophobic ion pair that is formed between the peptide/protein and the pentylamine used as buffering agent at pH 9.8. This can be exemplified by met- and leu-enkephalin, which

contain one tyrosine (tyrosine is ionized at pH values above 9.7). The ammonium ion that is used as buffering agent at pH 11 lacks the hydrophobic character of pentylamine and the enkephalins are thus less retained at pH 11 than at pH 9.8. Various hydrophobic amines used as ion pairing agents have been shown to retard acidic peptides run on a polymeric reversed phase column (12).

The two arginine residues of bradykinin start to lose their charges at pH 11, which can explain its stronger retention at that pH compared to other pH values. At "zwitterionic" and alkaline pH the oxidized chain A of insulin is highly negatively charged, and is thus not retained on the column except at pH 9.8, where pentylamine forms a hydrophobic ion pair with the peptide. The observations discussed above are in agreement with what was reported in reference 13.

The explanation of the retention behavior of proteins, compared to peptides, is not that obvious, although one can see from Table 1 that the basic proteins are more strongly retained at higher pH values. An exception is ribonuclease, which is not retained even at a pH of 11. Proteins with lower isoelectric points seems either to be less effected by buffer pH (ACTH, insulin, myoglobin and human serum albumin) or less retained at "zwitterionic" pH:s (ovalbumin and carbonic anhydrase) and alkaline pH (carbonic anhydrase). The reversed phase material discussed in this paper has a selectivity similar to that of the fluorocarbon resin presented in reference 3, although the agarose based material is somewhat less hydrophobic.

Typical separations of peptides are illustrated in figure 1, which shows two chromatograms run at pH 2 and 9.8, respectively. The separations were done isocratically. The chromatograms obtained are similar at these two pH:s, although met- and leu-enkephalin are not resolved at the higher pH. It should be noted that the bead size is around 15 μm, and that better resolution can be expected if smaller beads are used.

The above results show that derivatized nonporous agarose beads can be used for reversed phase chromatography of peptides

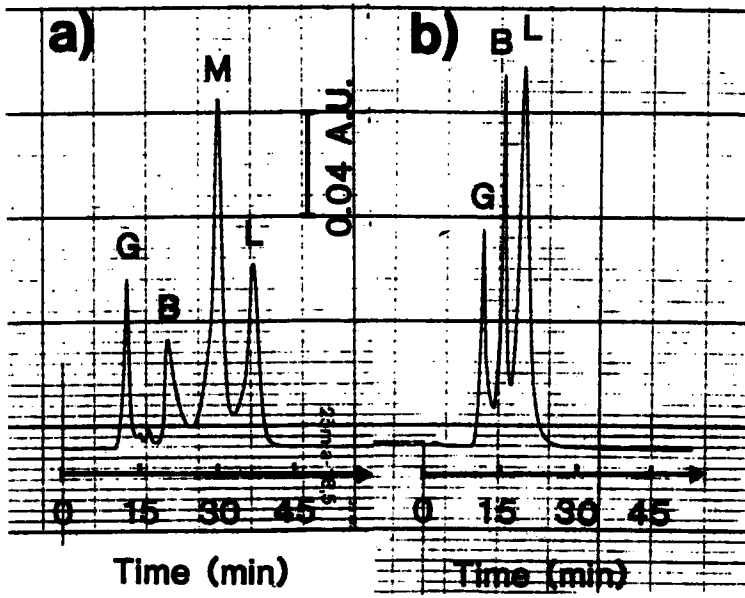

Figure 1: Isocratic separations of peptides. Sample: tetraglycine (T), bradykinin (B), met-enkephalin (M) and leu-enkephalin (L). Column dimensions: 3 x 200 mm, flow rate: 0.1 ml/min. (a) pH 2 (0.1 % TFA), 14 % acetonitrile. (b) pH 9.8 (50 mM pentylamine/HCl) 14 % acetonitrile (met-enkaphalin was not included in the sample run at pH 9.8).

and proteins. With their attractive characteristics, ease of derivatization, no need for end-capping and their pH stability, they could be an alternative to silica-based (or other) materials. But further studies are needed to optimize the preparation of these beads and to refine their use as a reversed phase matrix.

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