Journal of Chromatography, 598 (1992) 181–187
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 007

High-performance hydrophobic interaction chromatography of proteins on reversed-phase supports coated with non-ionic surfactants of polyoxyethylene type

Purification of a fungal aspartic proteinase

Youne Lie Kong Sing, Yolande Kroviarski*, Sylvie Cochet*, Didier Dhermy and Olivier Bertrand*,*

INSERM U 160 and Centre de Recherche sur les Enzymopathies de l'Association Claude Bernard, Hopital Beaujon, 92118 Clichy Cedex (France)

(First received July 19th, 1991; revised manuscript received December 20th, 1991)

ABSTRACT

On coating reversed-phase supports with polyoxyethylene-type non-ionic surfactants, proteins are no longer retained on such supports at moderate or low ionic strength, but they are retained at high ionic strength and can be desorbed by a decreasing ionic strength gradient. These reversibly modified supports were used for hydrophobic interaction chromatography (HIC). The proteins probably interact with the polyoxyethylene tail of the non-ionic surfactant while the hydrophobic part of the surfactant anchors the surfactant to the reversed-phase support by interactions with its alkane coverage. Although the interactions between non-ionic surfactant and reversed-phase support are non-covalent and the HIC mobile phases contained no surfactant, the modified columns were stable and could be used repeatedly. A surfactant-modified reversed-phase column provided a rapid, efficient, one-step purification of a fungal aspartic proteinase from a commercial crude preparation.

INTRODUCTION

Hydrophobic interaction chromatography (HIC) is a powerful method for purifying proteins. It was discovered as an adjunct to affinity chromatography when it was realized that the alkyl spacers used to immobilize affinity ligands could themselves interact with hydrophobic pockets situated on the surface of proteins and so retain them [1]. HIC columns are generally loaded with proteins at moderate to high salt concentration and the proteins are eluted with a decreasing salt concentration gradient [1–3].

HIC was initially performed with soft gels as a matrix for column preparation, but mechanically resistant supports based on a silica or a polymeric backbone can also be used [3–13]. Various ligands have been grafted to the chromatographic supports to confer the hydrophobic character needed for HIC; these ligands range from weakly hydrophobic types such as polyoxyethylene ethers [4,6,8–10,12–14] and poly(vinyl alcohols) (the name mild hydrophobic interaction was coined some years ago for this particular type of column [14]) to highly hydrophobic types such as alkane and phenyl ligands (3–5, 7, 11, 13). The influence of the nature of the ligand has been studied [4], as also have the differences between reversed-phase chromatography and HIC. The former uses strongly hydrophobic, highly

* Present address: INSERM U 76, INTS, 6 Rue Alexandre Cabanel, 75739 Paris Cedex 15, France.

0021-9673/92/$05.00

substituted supports and proteins lose their native structure when bound on such supports, proteins being eluted with a mobile phase containing an organic solvent [8–10]. HIC columns are less hydrophobic because they carry less hydrophobic ligands or smaller amounts of ligands, and proteins remain in their native form on such columns and no organic solvent is needed for elution.

Polyoxyethylene-type non-ionic surfactants in aqueous solution can be retained on reversed-phase supports by their alkane tails, leaving the comparatively polar parts of the surfactant molecule (*i.e.*, the polyoxyethylene part) exposed to an aqueous mobile phase [15–18]. As polyoxyethylene covalent grafting has been used to prepare HIC columns [4,6,8–10,12,13], we attempted to modify reversibly the surface properties of a reversed-phase column by coating it with a polyoxyethylene-type surfactant so as to use it for the HIC of proteins. While this paper was in the review process, we became aware that it had been noticed before that high salt conditions could promote the retention of proteins on columns prepared (for other purposes) by surfactant coating [15,17].

EXPERIMENTAL

Materials

Thermolysin and bovine trypsinogen were obtained from Merck (Darmstadt, Germany). Bovine α-chymotrypsinogen A and a fungal protease (protease from *Rhizopus* species, type XVIII) were obtained from Sigma (St. Louis, MO, USA) and bovine ribonuclease A from Boehringer (Mannheim, Germany). Proteins were used as received.

The non-ionic surfactant Brij 76 (decaethylene glycol *n*-octadecyl ether) was obtained from Sigma. All other chemicals and solvents were from Merck or Carlo Erba (Milan, Italy).

Two reversed-phase materials were purchased from SFCC (Neuilly Plaisance, France): Davisil C_{18} (irregular, mean particle diameter 20 μm, pore size 25 nm) and Ultrabase C_{18} (spherical, particle size 10 μm, pore size 30 nm).

METHODS

Chromatographic apparatus and buffers

The liquid chromatograph was a modular apparatus from Gilson. The mobile phases were 0.5 *M* ammonium acetate buffer (pH 6.0) containing 3 *M* ammonium sulphate (buffer A) and 0.5 *M* ammonium acetate buffer (pH 6.0) (buffer B).

Capacities of reversed-phase supports for non-ionic surfactant

A 150-mg amount of the reversed-phase support was slurried in a small volume of buffer B and settled in a Pharmacia 5/5 FPLC column. The column was then equilibrated with buffer B and 0.05% Brij 76 in buffer B was pumped through the column for 16 h at 1 ml/min. The column was then flushed with five column volumes of buffer B, five column volumes of water and finally twenty column volumes of methanol–isopropanol (1:1, v/v). The water and organic solvent washings were combined and dried, the residue was taken up in water and the surfactant content measured as indicated below.

Preparation of the test HIC column

Davisil C_{18} or Ultrabase C_{18} were packed into 10 cm × 4.6 mm I.D. columns by the method of Kuwata *et al.* [19]. The freshly packed columns were washed with several hundred column volumes of deionized water at 1.0 ml/min. Surfactant was adsorbed on the packing by pumping 0.05% (w/v) Brij 76 in buffer B through the column at 1.0 ml/min for 16 h. The columns were rinsed with several column volumes of buffer B, followed by buffer A.

Chromatography of model proteins

The performances of the columns were evaluated with a mixture of globular proteins dissolved in buffer A at concentrations given in the legends to Figs. 2 and 3. Sample mixture (50 μl) was injected and the columns were developed with a 20-min linear gradient from 0 to 100% B at a flow-rate of 1.0 ml/min at room temperature. The behaviour of the columns was examined by repeated chromatography of the protein mixture.

Determination of non-ionic surfactant concentration

Surfactant was assayed spectrophotometrically using Coomassie Brilliant Blue G-250 (CBBG) [20]. Briefly, aliquots of unknown or standard surfactant (40 μl) were mixed with 960 μl of CBBG reagent (BioRad Labs, Richmond, CA, USA) and the absorbance at 620 nm was read. The calibration graph

is not linear; the slope is steeper when the surfactant concentration in the assay mixture exceeds the critical micelle concentration.

Aspartic protease activity assays

Rhizopus aspartic protease was assayed by monitoring the hydrolysis of Leu–Ser–Phe(NO_2)–Nle–Ala–Leu–OCH_3 (Bachem, Bubendorf, Switzerland). The concentration of the stock substrate solution was determined spectrophotometrically [21]. The final peptide concentration in the assay mixture was 0.73 m*M* and the buffer was 25 m*M* ammonium formate (pH 3.1) containing 0.1 *M* NaCl. The peptide assay mixture also contained 20 μM 6,9-diamino-2-ethoxyacridine lactate as internal standard. Hydrolysis was allowed to proceed at 37°C for 20 min and stopped by adding one tenth of the reaction volume of 1 mg/ml Pepstatin solution in dimethyl sulphoxide. The reaction product was separated from undigested substrate by reversed-phase chromatography on a Nucleosil C_{18} (250 mm × 4.6 mm I.D.) column or on a laboratory-prepared (as indicated above) Ultrabase C_{18} column. Both columns were operated in the isocratic mode with 0.1% trifluoroacetic acid in acetonitrile–water (35:65) as the mobile phase. The absorbance of the column effluent was monitored at 280 nm. The apparatus was calibrated by injecting defined amounts of product. One enzyme unit is defined as the enzyme activity which releases 1 μmol of product per minute under the above assay conditions.

Protein assay

Proteins were determined either by dye-binding assay [22] or by measuring the absorbance at 280 nm.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli [23] using 12.5% polyacrylamide gels under reducing conditions.

RESULTS AND DISCUSSION

Properties of the surfactant-modified reversed-phase column

A reversed-phase column loaded with non-ionic surfactant and equilibrated with a low-salt aqueous mobile phase (*e.g.*, buffer B) did not retain measurable amounts of any of the test proteins. An unloaded column retained all the proteins, and they were eluted only by adding organic solvent to the mobile phase.

This result, together with the known structure of the non-ionic surfactant, indicates that the non-ion-

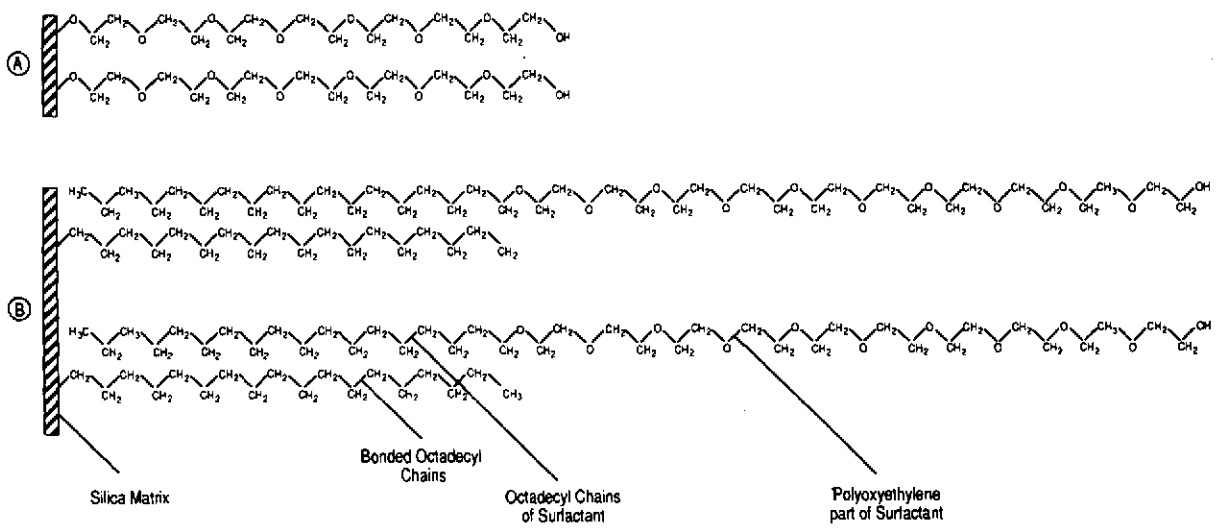

Fig. 1. Conceptual view of (A) an HIC column prepared by grafting polyoxyethylene glycol on silica and (B) the interaction between a non-ionic surfactant and a reversed-phase C_{18} support.

ic surfactant molecules are anchored to the reversed-phase support through strong hydrophobic interactions between alkane bristles of the reversed-phase support and their hydrophobic tails. The polar parts of the non-ionic surfactant molecules probably extend outwards, preventing the interaction of proteins with the buried hydrophobic layer composed of hydrophobic surfactant tails and the hydrocarbon layer grafted on to the support. This changes the surface properties of the reversed-phase support to those of the supports grafted with polyoxyethylene (as illustrated in Fig. 1).

Davisil C_{18} retained 154 μmol of surfactant per millilitre of packing and Ultrabase C_{18} retained 146 μmol/ml.

Surfactant is retained by the support only by non-covalent bonding, because of a dynamic equilibrium. It follows that some surfactant must be desorbed from the support when a surfactant-loaded column is used with a mobile phase that does not contain dissolved surfactant [18]. The extent to which this could affect the column properties was checked by measuring the surfactant load of an Ultrabase column that had been rinsed with 1000 column volumes of buffer B before eluting the surfactant with methanol–isopropanol. The value was the same as that given above, showing that surfactant leaching was indeed very low. Perhaps even more significant is the reproducibility of the chromatograms (see below), demonstrating that the column properties remained unaltered with use.

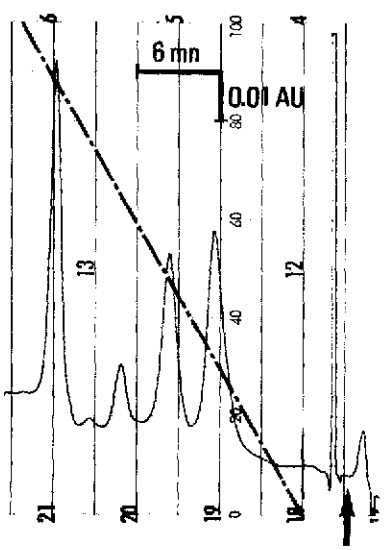

Fig. 2. Separation of a model protein mixture on a Davisil C_{18} column (10 cm × 0.46 cm I.D.) modified with Brij 76. The arrow indicates injection of the protein mixture (0.21 mg of cytochrome *c*, 0.30 mg of myoglobin, 0.15 mg of ribonuclease A and 0.18 mg of chymotrypsinogen). The peak eluting immediately after injection is a system peak; proteins are eluted through the gradient (dashed line) in the order given above. Absorbance was monitored at 280 nm. The numbers shown are from the chart paper and have no significance. mn = Minutes.

Hydrophobic interaction chromatography of a test mixture

All the test proteins were retained if dissolved in buffer A and deposited on to a surfactant-loaded column equilibrated with buffer A. Satisfactory resolution of all proteins was obtained by running a linear gradient between buffers A and B (Figs. 2 and 3). The back-pressures generated by the Davisil column were 14–24 bar and those for the Ultrabase column were 20–30 bar. A transient pressure surge to about 50 bar occasionally occurred at the time of sample injection.

Fig. 3 demonstrates that this good separation is reproducible; virtually identical chromatograms were obtained with the first and fortieth injections of the same test mixture.

Purification of an aspartyl protease from Rhizopus

Rhizopus species fungi produce several different aspartyl proteases collectively named rhizopuspepsin. Rhizopuspepsin is known to exist as several isozymic forms [24]. The commercial enzyme preparation used as the starting material for the purification showed several protein bands on SDS-PAGE. Fig. 4 shows the chromatogram obtained by running this material through a surfactant-loaded reversed-phase column and Table I gives quantitative data on the purification. About 65% of protease activity was found in pool B, and the SDS gel (Fig. 5) demonstrates that it is associated with a single band, whose molecular weight is consistent with published data [24]. Some protease activity was also found in other pools (probably corresponding to rhizopuspepsin isozymes), but the specific activity was lower than that in pool B.

Reutilization of a column in reversed-phase mode after use for HIC

The same column was used in the reversed-phase

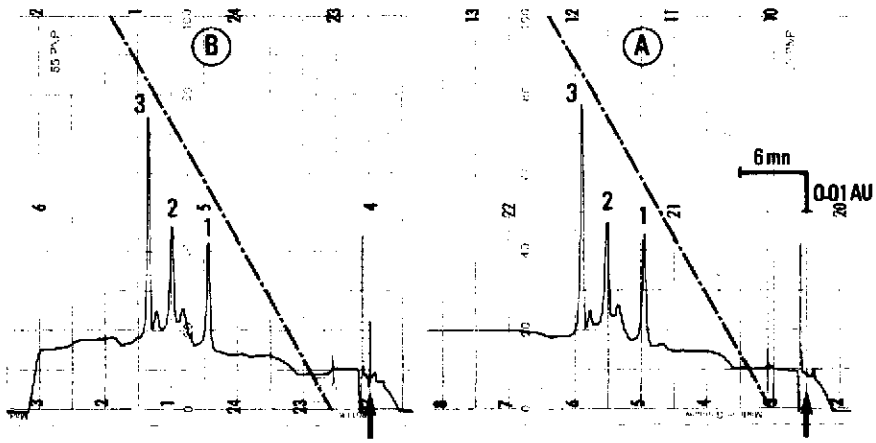

Fig. 3. Separation of a model protein mixture on an Ultrabase C_{18} column (10 cm × 0.46 cm I.D.) modified with Brij 76. The arrows indicate injections of the protein mixture (98 μg of ribonuclease A, 83 μg of trypsinogen and 53 μg of chymotrypsinogen). Peaks 1, 2 and 3 correspond to ribonuclease, trypsinogen en chymotrypsinogen, respectively, and other peaks are unidentified impurities. The dashed lines indicate the gradient profile. Absorbance was monitored at 280 nm. A and B are the recorder tracings for the first and the fourtieth injection of the same mixture, respectively.

mode for measuring protease activity (see Experimental) and then, after surfactant coating, for HIC of rhizopuspepsin (fifteen injections made as indicated above). The surfactant coverage was then removed with organic solvent and the column used again for proteolytic acitivity measurements. The chromatograms (Fig. 6) recorded before and after use in the HIC mode demonstrate that the column can be used successively in both modes. The column seemed to be more hydrophobic when used in the reversed-phase mode after the HIC mode (the k' values were higher, Fig. 6). The reason for this changed behaviour is unknown but has no significant effect on column usage.

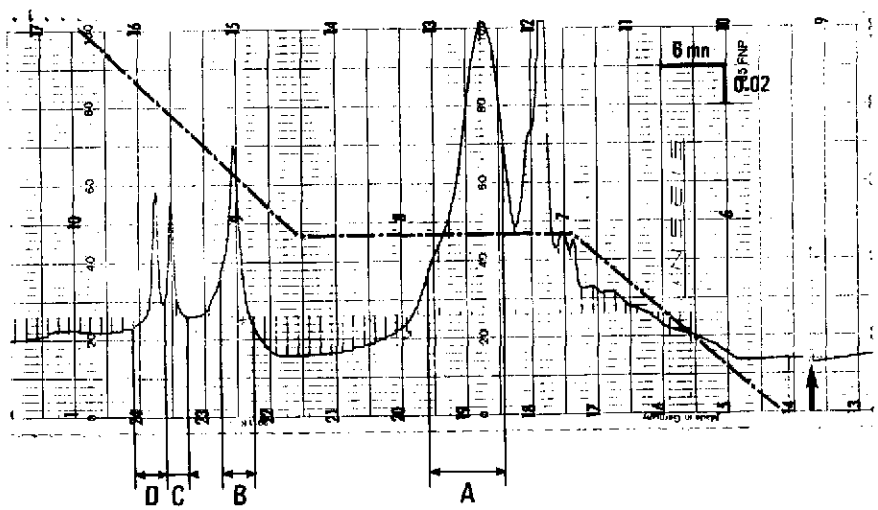

Fig. 4. Chromatogram of crude rhizopuspepsin on an Ultrabase C_{18} column (100 cm × 0.46 cm I.D.) modified with Brij 76. Crude rhizopuspepsin (1.36 mg) was injected at the arrow; the gradient profile is shown by the dashed line. Double arrows A to D indicate the pools of proteolytic activity.

TABLE 1

QUANTITATIVE RESULTS FOR THE PURIFICATION OF *RHIZOPUS* ASPARTYL PROTEASE

Pool B was collected as shown in Fig. 4.

Material	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Starting material	1.36	14.7	10.8	100
Pool B	0.25	9.5	38.1	64.6

CONCLUSION

This study has demonstrated that a reversed-phase column is readily modified by loading it with a non-ionic surfactant, and that such a column can be used for HIC. The use of a reversed-phase support for column preparation implies that the HIC support has the mechanical resistance of the silica backbone, and is thus superior to the classical agarose-based HIC supports in terms of flow-rate. Commercial mechanically resistant HIC supports (with more or less hydrophobic ligands covalently linked to the supporting matrix) are adequate substitutes for agarose-based HIC supports and can be used for the efficient purification of a range of proteins. However, most if not all are much more expensive than reversed-phase chromatographic supports. Our approach of modifying the surface properties of reversed-phase supports provides efficient HIC columns at a reasonable cost.

Deliberate modification of the properties of a stationary phase by interaction with some mobile phase component is a long established procedure. For instance, in ion-pair chromatography, the origin of which can be traced to the early 1970s [25], one important contribution to separation seems to be the coating of the support wiht a layer of the ion-pairing agent. This even led to the extreme view that ion-pair chromatography is a form of disguised ion-exchange chromatography because of adsorp-

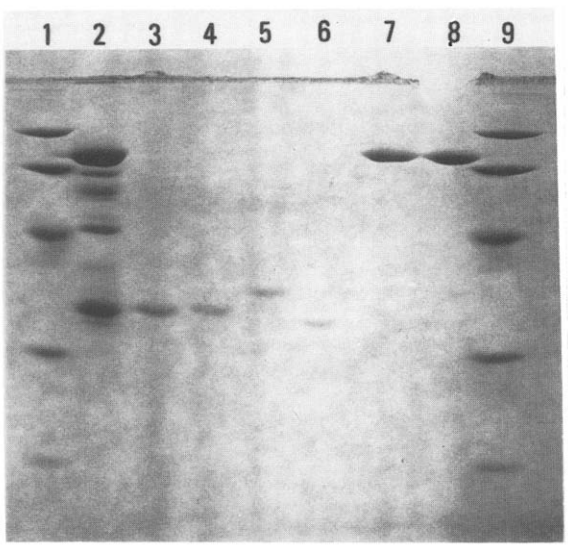

Fig. 5. SDS-PAGE of the pools obtained by chromatography of crude rhizopuspepsin on an Ultrabase C_{18} column modified with Brij 76. Starting material, lane 2; pool B, lanes 3 and 4; pool C, lane 5; pool D, lane 6; pool A, lanes 7 and 8. Molecular weight standards are on lanes 1 and 9 (from top to bottom, 94 000, 67 000, 43 000, 30 000, 20 100 and 14 400).

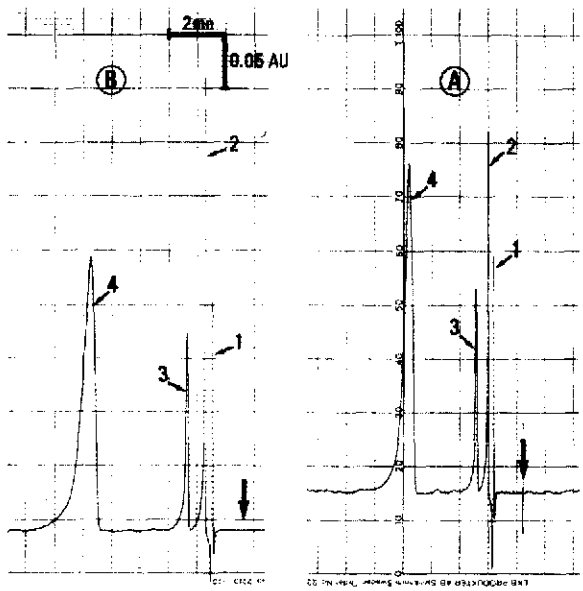

Fig. 6. Peptidolytic assay of *Rhizopus* protease activity on an Ultrabase C_{18} column (10 cm × 0.46 cm I.D.). Assay mixtures were injected at the arrows. Peak 1, 2, 3 and 4 are a system peak, reaction product [Leu–Ser–Phe(NO_2)], ethoxyacridine (internal standard) and undigested substrate, respectively. Chromatogram A was obtained with a freshly packed column and chromatogram B on the same column after use in the HIC mode and removal of the non-ionic surfactant coating.

tion of the ion-pairing agent on the stationary phase [26]. In fact, the secondary equilibria that occur in a mobile phase with an intentionally maintained concentration of ion-pairing agent are important [27]. In micellar liquid chromatography also multiple equilibria exist between the eluite, (modified) stationary phase and surfactant molecules present both as monomeric or associated (micellar) species [28]. Modification of reversed-phase support properties was also obtained by addition to the mobile phase of peculiar alcohols such as pentanol [29,30] or methoxyethanol [31] Lastly, it has been shown that the stationary phase adsorbs a certain amount of the organic component of the mobile phase in reversed-phase chromatography [32].

In all the instances cited above, the stationary phase modifier is present in the mobile phase also during chromatography. In contrast, we have used a non-ionic surfactant to modify the reversed phase before using it with pure aqueous mobile phases with no surfactant added.

The results showing that a reversed-phase column can be readily converted into a column usable for HIC provide further evidence of the versatility of reversed-phase supports used in conjunction with surfactants. Reversed-phase supports coated with non-ionic surfactants can be used for the size-exclusion chromatography of proteins [15], and reversed-phase supports can operate as ion exchangers if modified with ionic surfactants [33]. It is possible to use derivatives of non-ionic surfactants (with some suitable ligand grafted to their polar head) to convert reversed-phase columns into affinity [16] or immobilized dye chromatographic supports [18], whereas reversed-phase columns coated with non-ionic surfactants can be used as restricted access media for the chromatography of small molecules (these columns allow the analysis of small molecules by direct on-column injection of samples containing protein [17]).

All these modifications are readily performed, and might even be possible using the same reversed-phase silica backbone. Finally, the data presented above indicate that the surfactant can be stripped from the modified reversed-phase columns and the ordinary reversed phase columns thus obtained can be used thereafter in a conventional fashion.

REFERENCES

1 S. Shaltiel, *Methods Enzymol.*, 104 (1984) 69.
2 S. Hjertén, *J. Chromatogr.*, 87 (1973) 325.
3 Y. Kato, T. K. Itamura and T. Hashimoto, *J. Chromatogr.*, 298 (1984) 407.
4 S. H. Chang, K. M. Gooding and F. E. Regnier, *J. Chromatogr.*, 120 (1976) 321.
5 D. L. Gooding, M. P. Schmuck and K. M. Gooding, *J. Chromatogr.*, 296 (1984) 107.
6 N. T. Miller, B. Feibush and B. L. Karger, *J. Chromatogr.*, 316 (1984) 519.
7 J. L. Fausnaugh, E. Pfannkoch, S. Gupta and F. Regnier, *Anal. Biochem.*, 137 (1984) 464.
8 N. T. Miller and B. L. Karger, *J. Chromatogr.*, 326 (1985) 45.
9 N. T. Miller, B. Feibush, K. Corina, S. Powers Lee and B. L. Karger, *Anal. Biochem.*, 148 (1985) 510.
10 J. P. Chang, Z. E. El Rassi and Cs. Horváth, *J. Chromatogr.*, 319 (1985) 396.
11 A. J. Alpert, *J. Chromatogr.*, 359 (1986) 85.
12 Y. Shibusawa, U. Matsumoto and M. Takatori, *J. Chromatogr.*, 398 (1987) 153.
13 N. Cooke, P. Shieh and N. Miller, *LC · GC Int.*, 3 (1990) 8.
14 T. G. I. Ling and B. Mattiasson, *J. Chromatogr.*, 254 (193) 83.
15 J. P. Chang, *J. Chromatogr.*, 317 (1984) 157.
16 J. L. Torres, R. Guzman, R. G. Carbonell and P. K. Kilpatrick, *Anal. Biochem.*, 171 (1988) 411.
17 C. Desilets, M. A. Bourns and F. E. Regnier, *J. Chromatogr.*, 544 (1991) 25.
18 Y. L. Kong Sing, E. Algiman, Y. Kroviarski, D. Dhermy and O. Bertrand, *J. Chromatogr.*, 558 (1991) 43.
19 K. Kuwata, M. Uebori and Y. Yamazaki, *J. Chromatogr.*, 211 (1981) 378.
20 K. S. Rosenthal and F. Koussale, *Anal. Chem.*, 55 (1983) 1115.
21 M. N. Raymond, E. Bricas, R. Salesse, J. Garnier, P. Garnot and R. Dumas, *J. Dairy Sci.*, 56 (1973) 419.
22 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
23 U. K. Laemmli, *Nature (London)*, 277 (1970) 680.
24 K. Takahashi, *J. Biochem.*, 103 (1988) 162.
25 S. Eksborg and G. Schill, *Anal. Chem.*, 45 (1973) 2092.
26 P. T. Kissinger, *Anal. Chem.* 49 (1977) 883.
27 Cs. Horváth, W. Melander, I. Molnar and P. Molnar, *Anal. Chem.*, 49 (1977) 2295.
28 M. F. Borgerding, R. L. Williams, Jr., W. L. Hinze and F. H. Quina, *J. Liq. Chromatogr.*, 12 (1989) 1367.
29 K. G. Wahlund, *J. Chromatogr.*, 115 (1975) 411.
30 K. G. Wahlund and U. Lund, *J. Chromatogr.*, 122 (1976) 269.
31 W. Mönch and W. Dehnen, *J. Chromatogr.*, 147 (1978) 415.
32 R. M. McCormick and B. L. Karger, *J. Chromatogr.*, 199 (1980) 259.
33 D. E. Keller, J. L. Torres, R. G. Carbonell and P. K. Kilpatrick, *Anal. Biochem.*, 176 (1989) 191.