Journal of Chromatography, 359 (1986) 111–119
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 782

REVERSED-PHASE CHROMATOGRAPHY OF PROTEINS ON RESIN-BASED WIDE-PORE PACKINGS

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SUMMARY

PLRP-S, a macroporous poly(styrene–divinylbenzene) HPLC packing material, was evaluated for analysis of proteins. Materials with a particle size of 8 μm and pore sizes of 100 Å, 300 Å, and 1000 Å were tested under similar chromatographic conditions to determine the effect of pore size and surface area on retention, selectivity, and efficiency. This resin-based material has a highly homogeneous hydrophobic surface, based on phenyl moieties contributed by the copolymers of the packing. Thus, unlike silica, PLRP-S has no bonded alkyl groups or residual silanol sites. In addition, the PLRP-S material is stable over a wide range of solvents, pH, and ionic strengths. The elution order of a series of proteins on PLRP-S 300 Å was similar to that observed on a variety of alkyl-bonded silica-based reversed-phase columns. The percentage of acetonitrile in the mobile phase necessary to elute three of these proteins (ribonuclease A, cytochrome *c*, and ovalbumin) was determined. Protein desorption occurred over a very narrow range of 2–4% increase in concentration of the organic modifier. No apparent change in protein selectivity or peak area occurred with repetitive injections of a mixture of proteins, indicating little relative loss of protein on the column. Pore size appeared to have little effect on the selectivity or retentivity of the test proteins. It appeared that the loss of total surface area due to increased pore size was offset by the increased availability of pore surface area for protein-packing interaction. The resin-based PLRP-S 300 Å was used for analysis of wheat proteins, bovine pancreatic enzymes, high-molecular-weight proteins, and whey proteins. Over the broad range of molecular weights and hydrophobicities in these applications, proteins were eluted as sharp, symmetrical peaks. Thus, PLRP-S offers an effective alternative to reversed-phase silica for the analysis of proteins.

INTRODUCTION

In recent years reversed-phase liquid chromatography has been adopted for the analysis of peptides and proteins. The utility of a variety of macroporous silica-based packings for reversed-phase separation of proteins has been widely recognized[1–5]. Because proteins generally carry a net charge under the mobile phase con-

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ditions used for reversed-phase chromatography, of major concern in the use of silica-based packings is the presence of residual and accessible surface silanols. Interaction of proteins with the silanol groups leads to mixed-mode separation with resultant poor chromatographic resolution due to peak broadening and tailing[6].

A new high-resolution resin-based packing (PLRP-S 300 Å) has been introduced for reversed-phase analysis of proteins. While offering the advantage of compatibility with a wide range of solvents, pH, and ionic strengths, the highly cross-linked poly(styrene–divinylbenzene) PLRP-S can also be operated at normal flow-rates and column pressures without loss of column performance.

The resin-based material has a highly homogeneous, non-polar surface due to phenyl moieties contributed by the copolymers of the packing material. Thus, unlike silica-based material, PLRP-S has no bonded alkyl groups or residual silanol groups. In the present study, PLRP-S material of different pore sizes was evaluated for its utility in the chromatography of proteins. The efficiency and selectivity of the material for the chromatography of proteins having a wide range of hydrophobicities and molecular weights was determined.

EXPERIMENTAL

Apparatus

High-performance liquid chromatography (HPLC) was performed on PLRP-S 100 Å, 300 Å, and 1000 Å columns (Polymer Labs., Church Stretton, U.K.) using two Knauer Type 64 pumps (Polymer Labs.), a Knauer Type 50B HPLC gradient programmer (Polymer Labs.), a Knauer dynamic mixing chamber (Sonntek, Woodcliff Lake, NJ, U.S.A.) and a Rheodyne Model 7125 loop injector (Rheodyne, Cotati, CA, U.S.A.). All columns were 150 × 4.6 mm I.D. and contained 8-μm packing. A Knauer Type 87 variable-wavelength detector (Polymer Labs.) and a Trilab 2000 chromatography data system (Trivector, West Chester, PA, U.S.A.) were used for detection, data collection, and analysis.

Reagents

All proteins were obtained from Sigma (St. Louis, MO, U.S.A.). Acetonitrile and water were of HPLC quality and were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.) as was the trifluoroacetic acid (TFA).

Chromatographic conditions

All chromatographic separations were carried out at ambient temperature. Mobile phases were vacuum-degassed and sonicated. Flow-rates generally were 1.0 ml/min, and proteins were injected in 10-μl volumes. Unless otherwise stated for gradient elution, solvent A was 0.1% TFA in acetonitrile–water (1:99) and solvent B was 0.1% TFA in acetonitrile–water (95:5). Proteins were detected at 220 nm.

RESULTS AND DISCUSSION

Effect of organic modifier concentration on protein retentivity

The percentage of acetonitrile in the mobile phase necessary to elute ribonuclease A, cytochrome *c*, and ovalbumin was determined. PLRP-S 300 Å columns

were equilibrated with a mobile phase containing increasing (1% increments) acetonitrile concentrations, individual proteins were injected, and retention times were determined.

The elution characteristics of the proteins on PLRP-S were similar to those reported for reversed-phase silica[7–11]. Following injection of the proteins into the column, there appeared to be little or no desorption until a critical concentration of acetonitrile was reached (Fig. 1). At this point, the retention of the various proteins greatly decreased. With a further 2–4% increase in organic modifier concentration, the proteins were no longer retained. Preliminary results (data not shown) suggested that when the organic modifier concentration was increased to high levels (greater than 70% acetonitrile for ribonuclease A and cytochrome *c*) proteins were once again adsorbed on the PLRP-S packing. Increased protein retention at high modifier concentrations has been reported for reversed-phase bonded silica[9,12].

The percentages of acetonitrile required for initial desorption of ribonuclease A and cytochrome *c* were 26% and 31%, respectively. The desorption of ovalbumin was observed to be more complex. Initial desorption was detected at 48% acetonitrile but multiple, distorted peaks eluted. This may be due to the presence of ovalbumin species in varying states of unfolding[13], column deglycosylation[14], or the partial separation of the heterogeneous glycosylated forms of ovalbumin[15–17]. Upon increasing the organic modifier concentration to 50%, the k' for ovalbumin decreased five-fold, and a single species of protein was eluted.

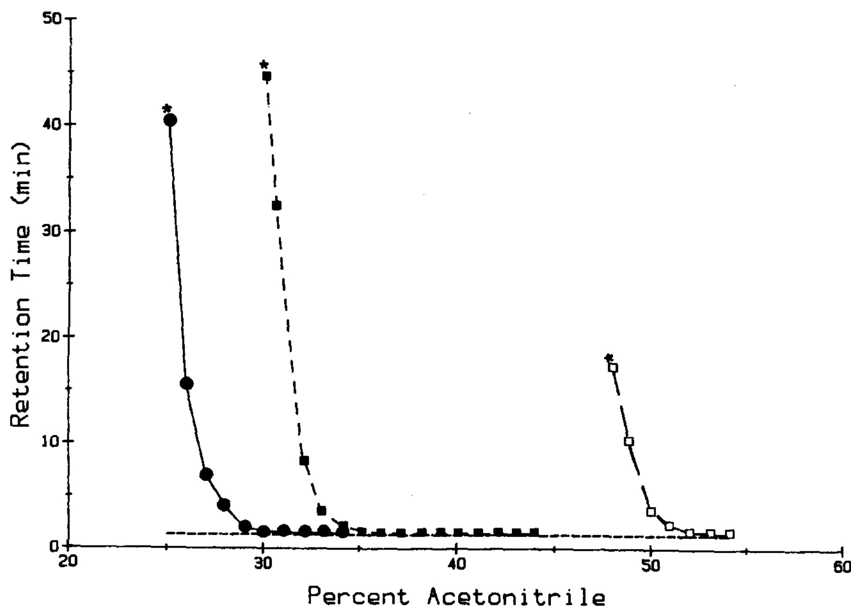

Fig. 1. Effect of organic modifier concentration on protein retentivity. Proteins were injected into a PLRP-S 300 Å column equilibrated with increasing (1% increments) of acetonitrile. Retention times were determined for ribonuclease A (●—●), cytochrome *c* (■---■), and ovalbumin (□—□). The asterisks indicate the modifier concentrations below which no protein desorption was detected.

Protein recovery

To determine protein recovery from PLRP-S 300 Å, increasing amounts of ribonuclease A, cytochrome *c*, and ovalbumin were individually injected in a series (2-fold increments) from 1.5 μg to 100 μg back to 1.5 μg. Mobile phases containing acetonitrile concentrations giving k' values of about 4 were used (0.1% TFA in acetonitrile–water (27:73), acetonitrile–water (33:67), and acetonitrile–water (51:49) for ribonuclease A, cytochrome *c*, and ovalbumin, respectively). After each injection, protein retention time, peak area, and peak height were determined at 220 nm.

For all three proteins, there was a very good linear response between the amount of protein injected and both detector peak area and peak height response up to 100 μg. The peak area resulting from the initial injection of 1.5 μg of each protein was compared to that of the final injection of 1.5 μg at completion of the series. An increase of about 8% in peak area for the final injection relative to the initial injection was detected for the various proteins and may indicate some protein loss on the packing during the initial injection. A certain amount of column "conditioning" may be required, especially when small quantities of protein are injected into a new column.

Selectivity and column performance of PLRP-S in protein chromatography

To further investigate protein recovery on PLRP-S 300 Å and to examine column selectivity and efficiency, repetitive injections of a mixture of ribonuclease A and ovalbumin were made over a two-day period. Proteins were eluted with a 20–60% gradient of solvent B over 15 min. Retention times, peak areas, and peak heights were determined, and the results are summarized in Table I. Throughout the series of injections, the column demonstrated good selectivity, as peak areas and retention times for both proteins remained relatively constant. Whereas ribonuclease A showed no decrease in peak height, some reduction in peak height for the more hydrophobic ovalbumin was observed with time. These results indicate that PLRP-S has reproducible selectivity over a broad range of protein hydrophobicities. Because of the homogeneous surface characteristics of the PLRP-S, it is expected that selectivity variations between batches of the packing would be minimized.

Effect of pore size on protein retentivity, pore permeation, and column performance

PLRP-S packings of two different pores sizes, 300 Å and 1000 Å, were compared for chromatography of proteins over a molecular weight range of 12 000–

TABLE I

EFFECT OF REPETITIVE INJECTIONS OF PROTEIN ON PLRP-S 300 Å SELECTIVITY AND COLUMN PERFORMANCE

$\bar{k}$ = the gradient elution capacity factor, $\bar{x}$ = the mean value of 32 injections and s = the standard deviation.

Protein	$\bar{k}$	*Retention time (s)*		*Peak height*		*Peak area*	
		$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
Ribonuclease A	2.9	405	4.5	381	15	4320	91
Ovalbumin	7.7	899	8.6	156	35	3918	117

335 000. Equal amounts of protein were injected into each column and were eluted using a 20–60% gradient of solvent B over 15 min (ribonuclease A, cytochrome *c*, ovalbumin) or 30–60% solvent B over 12 min (β-amylase, apoferritin, thyroglobulin). Peak heights, retention times, and areas were determined.

Elution times for each of the proteins were similar on both columns. An increase in pore size from 300 Å to 1000 Å appeared to have no significant effect on peak heights over the molecular weight range investigated (Table II). The loss of surface area which accompanied the increase in pore size from 300 Å to 1000 Å appeared to be offset by the increase in the region of the pore surface available for interaction of the proteins with the packing material. Preliminary results (data not shown) indicated a significant increase in peak height when high-molecular-weight fibrous proteins (collagen and fibrinogen) were chromatographed on PLRP-S 1000 Å compared to PLRP-S 300 Å.

Ribonuclease A, cytochrome *c*, apoferritin, ovalbumin, β-amylase, and thyroglobulin were chromatographed on PLRP-S with pore sizes of 100 Å, 300 Å, and 1000 Å. The proteins were chromatographed under conditions where they were not retained (70% acetonitrile). The elution times of the proteins were compared to that of thiourea, which was used as a marker for the total permeation volume of the columns. The elution times for thiourea on the 100-Å, 300-Å, and 1000-Å columns were 87, 98 and 110 s, respectively. The proteins all eluted prior to the thiourea and their order of elution suggests some selective permeation of pores in all three column types (Fig. 2). The time required for the proteins to be eluted from the columns was observed to decrease with increasing molecular weight. On the 300-Å and 1000-Å columns all the proteins appeared to be able to enter the packing pores. On the 100-Å column, selective permeation was restricted to proteins with a molecular weight of less than 200 000. The 300-Å pore type, based on these results and those of the column performance studies would appear to offer the most adequate ratio between surface area and accessible pore volume for highest-efficiency separations of globular proteins. The 1000-Å column, on the other hand, demonstrated potential for more efficient separations of fibrous proteins.

Applications of PLRP-S 300 Å

A variety of protein samples were chromatographed on PLRP-S 300 Å. Analysis of a mixture of standard proteins demonstrated that the order of elution of these

TABLE II

EFFECT OF PLRP-S PORE SIZE ON PROTEIN PEAK HEIGHT

Protein	*Molecular weight (daltons)*	*Peak area*		*Peak height*	
		300 Å	*1000 Å*	*300 Å*	*1000 Å*
Cytochrome *c*	12 400	3680	3665	305	300
Ribonuclease A	13 600	4066	4045	376	395
Apoferritin	22 150*	10 649	10 996	353	355
Ovalbumin	45 000	4873	4215	277	276
β-Amylase	200 000	5846	5872	242	253
Thyroglobulin	335 000*	3216	3184	49	50

* Subunit molecular weight.

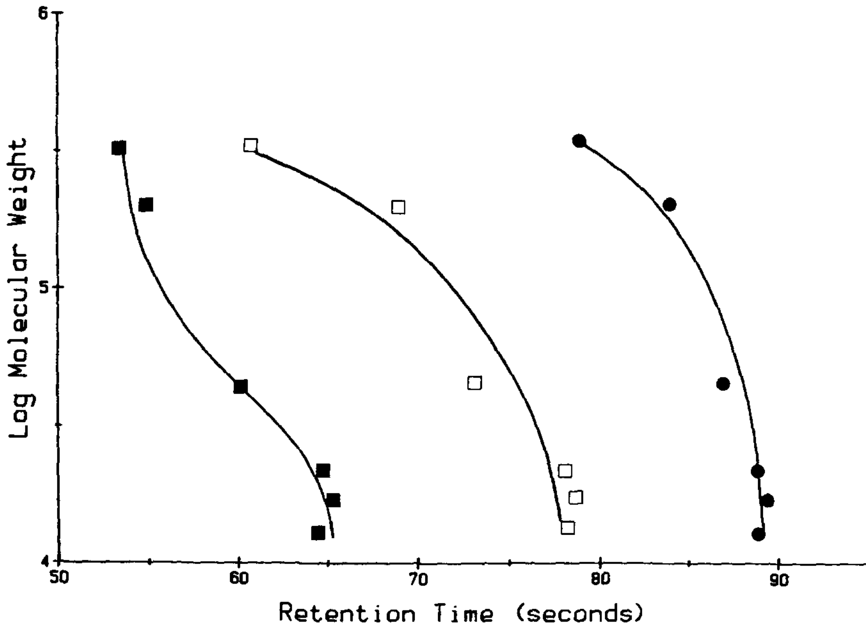

Fig. 2. Effect of pore size on selective permeation of packing pores by proteins. Ribonuclease A, cytochrome *c*, ovalbumin, apoferritin, *β*-amylase, and thyroglobulin were chromatographed on PLRP-S 100 Å (■—■), 300 Å (□—□), and 1000 Å (●—●) with 0.1% TFA in acetonitrile–water (70:30) and retention times were determined.

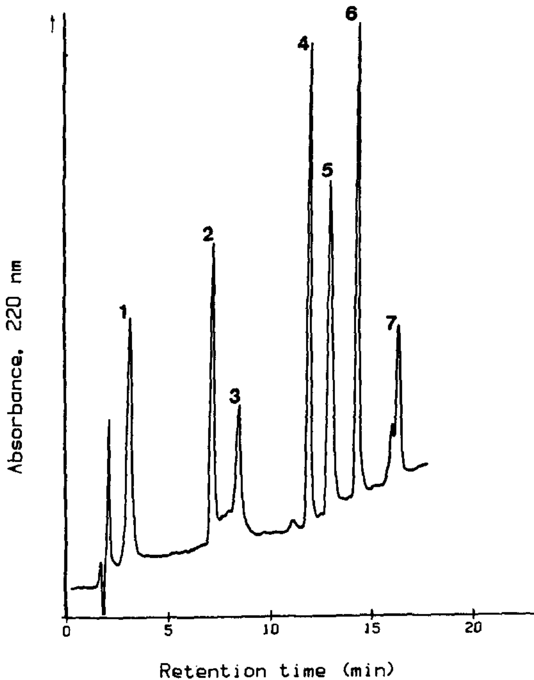

Fig. 3. Analysis of a mixture of standard proteins on PLRP-S 300 Å. Proteins were chromatographed on a 250 × 4.6 mm I.D. column with a linear gradient of 20–60% solvent B over 22 min at a flow-rate of 1.5 ml/min. Peaks: 1 = ribonuclease A, 2 = insulin, 3 = cytochrome *c*, 4 = lysozyme, 5 = bovine serum albumin, 6 = myoglobin and 7 = ovalbumin. (Courtesy of L. L. Lloyd, Polymer Labs.).

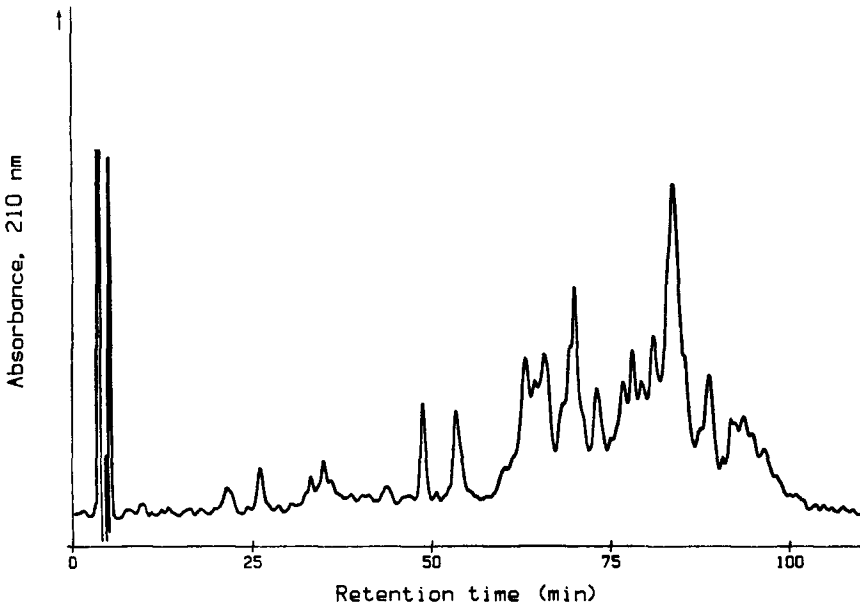

Fig. 4. Analysis of gliadin proteins from Scout wheat flour on PLRP-S 300 Å. The ethanol-extracted wheat proteins were chromatographed on a 150 × 4.6 mm I.D. column. Solvent A was 0.1% TFA in acetonitrile–water (15:85) and solvent B was 0.1% TFA in acetonitrile–water (80:20). A gradient of 20–50% B over 120 min at 0.5 ml/min was used. (Courtesy of D. L. Wetzel, Kansas State University).

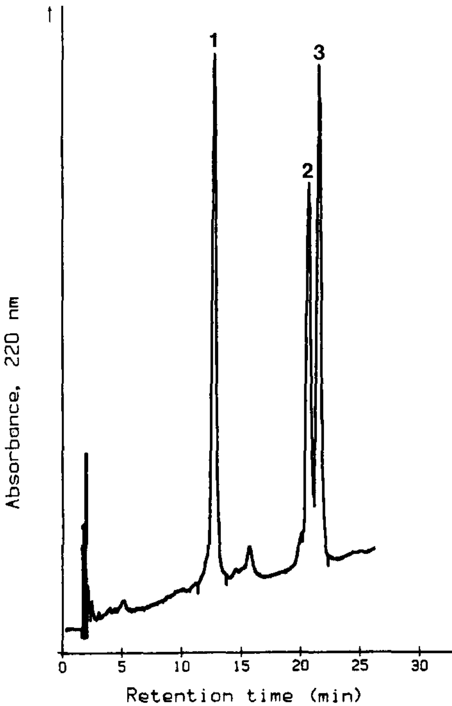

Fig. 5. Analysis of representative whey proteins on PLRP-S 300 Å. The proteins were chromatographed on a 150 × 4.6 mm I.D. column with a gradient of 36–48% solvent B over 24 min. Peaks: 1 = α-lactalbumin, 2 = β-lactoglobulin (B chain), and 3 = β-lactoglobulin (A chain).

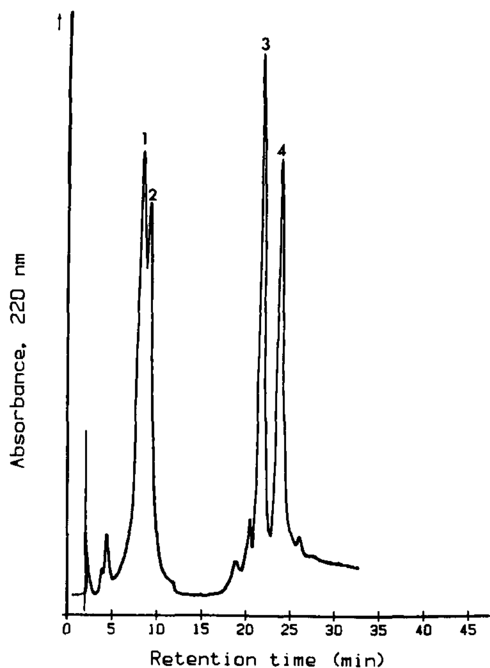

Fig. 6. Analysis of bovine pancreatic enzymes on PLRP-S 300 Å. A gradient of 37–40% solvent B over 15 min, followed by 40–45% solvent B over 10 min, was used for chromatography of the enzymes on a 150 × 4.6 mm I.D. column. Peaks: 1 = trypsinogen, 2 = trypsin, 3 = chymotrypsin, and 4 = chymotrypsinogen.

proteins from PLRP-S (Fig. 3) is similar to that seen on a variety of alkyl-bonded silica columns. A separation of gliadin proteins from Scout wheat flour is shown in Fig. 4. Reversed-phase chromatography has been shown to be an effective method for finger-printing of wheat varieties[18]. PLRP-S provided high resolution of the proteins in the flour extract. Also of commercial importance is the ability to compare proteins in various whey preparations[19]. A separation of the major proteins present in whey is shown in Fig. 5. Near-baseline resolution was obtained for the A and B chains of β-lactoglobulin. The PLRP-S was able to provide excellent separation of chymotrypsin and its proenzyme, chymotrypsinogen (Fig. 6). Both of these proteins were readily separated from the bovine pancreatic enzyme trypsin and its precursor form, trypsinogen. Over the broad range of molecular weights and hydrophobicities illustrated by these applications, proteins were eluted as sharp peaks with no observable tailing. Thus, the PLRP-S packing with its homogeneous, non-polar surface provided for high resolution separations of proteins.

ACKNOWLEDGEMENTS

We thank L. L. Lloyd and F. P. Warner (Polymer Labs.) for advice and M. F. Barnett and Z. Dryzek (Polymer Labs.) for supplying experimental PLRP-S columns.

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