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SEPARATION OF PROTEINS BY MICROCOLUMN LIQUID CHROMATOGRAPHY BASED ON THE REVERSED-PHASE AND SIZE-EXCLUSION PRINCIPLES

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SUMMARY

Slurry-packed fused-silica microcolumns of 250 μm I.D., are characterized for use in high-performance liquid chromatographic studies of proteins. The present work utilizes the reversed-phase and size-exclusion chromatographic modes for the separation of standard protein mixtures. A 5-μm, 300-Å octyl material is utilized for the reversed-phase studies, and the size-exclusion studies are accomplished with 5-μm diol material of 60-, 100- and 300-Å pore sizes. Column efficiency and packing reproducibility, as well as sample capacity in a micropreparative mode, are discussed. In addition, the inherent mass sensitivity of a microcolumn liquid chromatography system as applied to protein detection is demonstrated.

INTRODUCTION

Modern liquid chromatography (LC), using rigid particle materials, has been firmly established as a vital method for rapid and effective separation of proteins. Various retention mechanisms (size exclusion, reversed phase, hydrophobic interaction, ion exchange, bioaffinity, etc.) have been under extensive development to satisfy the needs of modern biochemical research and biotechnology. Particle technologies leading to the preparation of reversed-phase and size-exclusion columns appear less involved than those of the remaining systems.

An overwhelming majority of modern analytical protein separations involves 4.6 mm I.D., columns which permit microgram to milligram amounts of injected samples, leading to concentrations of the recovered protein solutes on the order of μg/ml. These methods are generally compatible with subsequent amino acid determination and sequencing methods. However, proteins in some biological samples are present only in nanogram quantities.

Several studies[1–5] have demonstrated the trace enrichment and micropreparative capabilities of smaller diameter columns (either 2.1 mm or 1 mm I.D.). These column types were chosen to minimize analysis time and contact time between the stationary phase and a protein, thereby decreasing bandbroadening and increasing protein recovery. Due to the strict dependence of protein capacity factors on the

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organic modifier concentration[6,7], submicrogram amounts of peptide or protein, present in a large sample volume, can be concentrated onto the reversed-phase support at less-than-critical organic modifier concentrations. Trace enrichment has been demonstrated by Nice *et al.*[1], who concentrated protein contained in 2 ml of sample down to an eluent volume as small as 25 μl. The recovered concentrations are on the order of mg/ml, which allows the use of fractions in gas-phase sequencers without further manipulations and sample loss. Nice *et al.*[1] also demonstrated low nanogram levels of detectability of epidermal growth factor (EGF_{α_1}) by using a 7.5 × 0.21 cm column.

Short columns have also been shown to be advantageous in similar directions[1,3,4,8]. Since column length does not appear to significantly affect resolution of protein peaks[9], other inherent advantages of shorter columns can be exploited. There are indications of improved recovery and fewer problems with underloading while using such columns[10]. Although the maximum loading capacity is only a few milligrams of protein, this is usually adequate for isolation and purification in a micropreparative mode.

During the last several years, microcolumn LC has been increasingly utilized for the separation of biologically important molecules[1–5,8,11]. Slurry-packed, fused-silica capillary columns[12–24] impart analytical advantages to relatively small biological solutes, such as high column efficiencies, increased mass sensitivity of concentration-sensitive detectors, and the ability to work with very small specimens. Our laboratory has recently become interested in extending certain of these capabilities to biopolymers.

The isolation of small quantities of proteins appears to be among the most desirable features of microcolumn (capillary) LC. Encouraged by the successful scale-down results with 2.1 and 1.0 mm I.D., columns[1–5], this direction is further justified by the recent developments in ultrahigh-sensitivity determinations of protein degradation products[2,4,8,25] for the sake of structural characterization.

We feel that fused-silica, slurry-packed microcolumns have the following major advantages to offer to the field of protein chromatography: (a) significantly less adsorptive behavior, due to the column materials inherently associated with this column technology, as well as the drastically reduced quantities of the sorption materials contacting the protein molecules; (b) enhanced mass sensitivity of the concentration-sensitive detectors (based on UV-absorption or native fluorescence) that are typically employed in protein detection; and (c) compatibility with small-scale sample manipulations. We have already demonstrated in a previous report[26] that subpicomolar amounts of model proteins can be detected and isolated using these microcolumns. The purpose of the investigations described here has been to characterize several different types of microcolumns with respect to the separation of common protein mixtures. The current scope of such studies has been limited to the reversed-phase and size-exclusion systems. Basic parameters of the protein analytical separations addressed here include column efficiency, sample capacity, and relative retention. Finally, the findings of these studies are correlated with various literature data obtained on larger-diameter columns.

TABLE I

PROTEINS USED IN THIS STUDY

Protein	*Abbreviation*	*Source*	*Molecular weight in kilodalton*
Thyroglobulin	Thy	Porcine	165
Transferrin	Trans	Bovine	77
Albumin	BA	Bovine	66
Ovalbumin	Ova	Chicken egg	45
β-Lactoglobulin A	LA	Bovine milk	35
Carbonic Anhydrase	CA	Bovine erythrocytes	29
α-Lactalbumin	Lalb	Bovine milk	14.2
Ribonuclease A	Rnase	Bovine pancreas	13.5
Cytochrome *c*	Cyt	Horse heart	11.7
Insulin	Ins	Bovine pancreas	11.5

MATERIALS AND METHODS

Reagents

Chromegabond MC-8 (ES Industries, Marlton, NJ, U.S.A.), and SynChropak GPC 60, 100, and 300 (SynChrom, Lafayette, IN, U.S.A.), having nominal particle sizes of 5 μm, were chosen as the stationary phases. HPLC grade acetonitrile and 85% phosphoric acid, and sodium phosphate dibasic were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.). ChromAR HPLC grade methanol and sodium phosphate monobasic were purchased from Mallinckrodt (Paris, KY, U.S.A.). Distilled and deionized water was prepared in our laboratory. Mobile phase solutions were filtered with either 0.45-μm Millex-HV units (Millipore, Bedford, MA, U.S.A.), or a 0.5-μm in-line filter unit (Upchurch Scientific, Oak Harbor, WA, U.S.A.).

The protein standards, listed in Table I, were purchased from Sigma (St. Louis, MO, U.S.A.). Blue dextran (BD) and L-serine (Ser) (Sigma), as well as tetraglycine [$(Gly)_4$], a gift from Dr. Frank R. N. Gurd (Indiana University), were also utilized in this study. Samples were dissolved in 0.1 *M* phosphate buffer solutions of pH 2 (for reversed phase) or pH 7 (for size exclusion), and were filtered with Millex-HV units.

Column packing

After a porous frit was fixed at the column end[27], fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, U.S.A.) (50 cm × 250 μm I.D.) were slurry-packed using a Model LC-5A pump (Shimadzu, Kyoto, Japan). The volume of the slurry reservoir, 1.6 mm I.D., varied according to the packing material.

Chromegabond (reversed-phase) MC-8 material required a fast packing technique[28]. An amount of 55 mg of the material was slurried with 200 μl of 1.5% Nonidet P-40 (Sigma) in acetonitrile, and the column was packed using acetonitrile as the solvent.

With the size-exclusion packings, the best results were obtained when methanol was used as the solvent. A slurry ratio of 40 mg packing to 120 μl methanol, and a slow packing technique[28] produced columns with the best efficiencies. The column

was slurry-packed at 5 μl/min constant flow-rate until the pressure reached 210 kg/cm². The pump was turned off, and the pressure allowed to decrease to zero. The column was then equilibrated with the mobile phase used for column evaluation.

Chromatographic systems

Reversed-phase chromatography (RPC). Separations were carried out on columns of 30-, 20-, and 10-cm in length. Analyses were done using a Model μLC-500 syringe pump (ISCO, Lincoln, NE, U.S.A.) operated in the constant-flow mode, and a μLC-10 UV–VIS detector (ISCO) fitted with a 1-mm pathlength (30 nl volume) flow cell. Proteins were detected at 215 nm. Injections were made using a four-port internal loop (air-actuated) injection valve (Model ACI4W, Valco Instruments, Houston, TX, U.S.A.) with a 0.2-μl rotor, and the moving-injection method[29]. Proteins were eluted using a stepwise gradient[19] from 20% to 55% acetonitrile in 0.1 *M* H_3PO_4–NaH_2PO_4 buffer at pH 2.

The step gradient utilizes PTFE reservoir tubes between 3 and 15 μl in volume. The tubes are filled and stacked from weakest eluent to next weakest, etc. The syringe pump is filled with the strongest eluent, in this case, the highest acetonitrile concentration. As the chromatographic run begins, eluent is displaced from segment to segment, through the column, thereby generating the step gradient.

Size-exclusion chromatography (SEC). Separations were carried out on columns between 40 and 50 cm in length. Analyses were done using the ISCO syringe pump in the constant-pressure mode, and a UVIDEC-100-V detector (Jasco, Tokyo, Japan) fitted with an in-house modified 150-nl cell, at 215 nm. Sample injections were made with an electrically-activated Valco valve, Model ECI4W, fitted with a 0.2-μl rotor. The samples were eluted isocratically using a 0.1 *M* NaH_2PO_4–Na_2HPO_4 buffer at pH 7, with 10% methanol added to the mobile phase for the GPC 100 and 300 materials.

The injection time and, consequently, the injected sample amount were controlled by an IBM personal computer, connected to the electrical actuator. The same computer, employing programs developed in this lab, acquired and calculated the peak moment data. Column efficiency data were compared using the totally included species, which were Ser for GPC 60, and $(Gly)_4$ for GPC 100 and 300. The mobile phase linear velocity was calculated using the totally excluded species, BA for GPC 60 and BD for GPC 100 and 300.

RESULTS AND DISCUSSION

The RPC and SEC principles for protein separation have been useful in different directions. RP-HPLC systems are very adaptable due to the wide variety of stationary phase–mobile phase combinations. Examples of their utilization include the study of hormonal polypeptides and proteins[30,31], histones[32], immunoglobulins[33], and pancreatic proteins[34], purification of hydrophobic virus membrane proteins[35], as well as highly efficient separations of cyanogen bromide[36,37] and tryptic[38,39] fragments of proteins. SEC has also been utilized extensively due to the ease with which separation of proteins based on size, or hydrodynamic volume, can be accomplished. Different size-exclusion materials allow the preliminary characterization of unknown mixtures as for their molecular weight ranges. The wide variety of

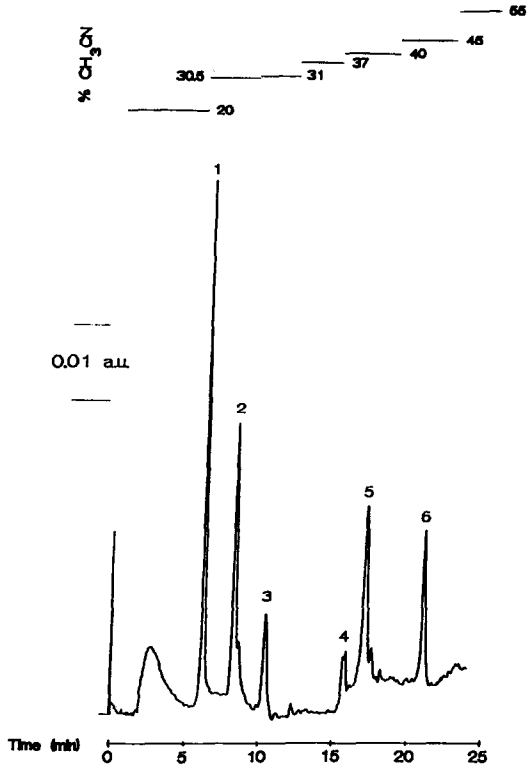

Fig. 1. Chromatographic separation of standard proteins on 10 cm × 250 μm I.D., octyl column. Proteins in order of elution: 1 = 3 ng Rnase, 2 = 3 ng Ins, 3 = 2 ng Cyt, 4 = 3 ng BA, 5 = 2 ng Lalb, 6 = 2 ng LA. Acetonitrile gradient in 0.1 *M* sodium phosphate buffer, pH 2, as shown above the chromatogram. Flow-rate = 2.1 μl/min.

buffers and mobile phase additives often makes the retention of native structure and activity possible, although, if desirable, denaturants[40,41] or surfactants[42,43] can also be added. The general utility of RP-HPLC and SEC have prompted our initial efforts toward column miniaturization in these areas.

Since the earlier investigations[9,37,44–47] had shown the superiority of large-pore materials in RP-HPLC, an octyl substituted, 300-Å pore size material was chosen in our studies. Among various alternatives available for efficient SEC, we chose silica-based materials derivatized with a glycerylpropylsilane to form a diol surface moiety[48,49]. Packing procedures for these materials were based on our experience[28] with somewhat similar packings for the separations of smaller molecules.

The first example of protein separation, shown in Fig. 1, was obtained on a 10-cm microcolumn packed with a 300-Å octyl material, with the gradient profile and sample amounts indicated. The advantages of high mass sensitivity with the miniaturized UV detector are indicated. Clearly, subpicomolar amounts of the model proteins form well-shaped peaks. As shown in a recent communication[26] concerned with the micro-isolation capabilities of such microcolumns, there are only very small losses of proteins involved; a small peak of insulin was still detectable at the 58-pg

TABLE II

PROTEIN CAPACITY FACTORS AS A FUNCTION OF COLUMN LENGTH FOR THE 300-Å OCTYL PACKING

Proteins	*Capacity factors**		
	10 cm	*20 cm*	*30 cm*
Rnase	2.4	1.3	0.8
Ins	4.4	2.1	1.3
Cyt	5.6	2.4	1.4
BA	7.9	3.8	2.2
Lalb	8.5	4.3	2.6
LA	10.0	5.1	3.2

* t_0 measured by dead volume peak that occurred due to slight differences in buffer concentrations of solute and mobile phase.

level. For the proteins with native fluorescence, even smaller quantities could be detectable.

Table II lists capacity factors for the model proteins on the C_8 microcolumns of different lengths. When continuous gradients in conventional systems are utilized, the values are not expected to vary with length. We attribute the changes observed in Table II to the difficulty in decreasing the volumes of the gradient segments proportionally to the column length. These volume differences effectively change the gradient slope, which has been shown to affect both peak shape and recovery in protein separations[33,50,51]. Despite the differences in values indicated by the table, we observed that the proteins do elute at the same acetonitrile concentration on different columns, indicating that the column length does not influence the relative retention of proteins[9].

Perhaps it is pertinent to address the problems associated with the use of microcolumns at this point. As with any column, clogs may develop due to sample precipitation or the presence of "foreign matter". Because fused silica is easy to cut, this problem can be addressed by simply removing the first few millimeters of column, without seriously affecting retention times or separation efficiency.

A more serious drawback of the microcolumn technique is the utilization of a step gradient. The step gradient itself is reproducible, because one has control over the volumes and concentrations of the eluents used. However, it would be prohibitively difficult for use in the analysis of complex protein samples, due to the large number of segments that would be required. The use of a continuous gradient, either linear or exponential, would aid in the ease and speed of analysis, particularly by decreasing the amount of time devoted to choosing proper gradient concentrations and volumes for optimum component separation. However, we are severely limited by the mixing volumes allowable. Total gradient volumes are typically on the order of 40–50 μl for a 30-cm column, and even less for shorter columns. Unfortunately, these volumes are much lower than the mixing-delay volumes available in most commercial and homemade gradient devices[52–55]. Until other options are made available, we appear to be limited to the utilization of the step gradient for microcolumn LC.

As can be seen in Fig. 2, the resolution for the Ins/Cyt pair decreases as the

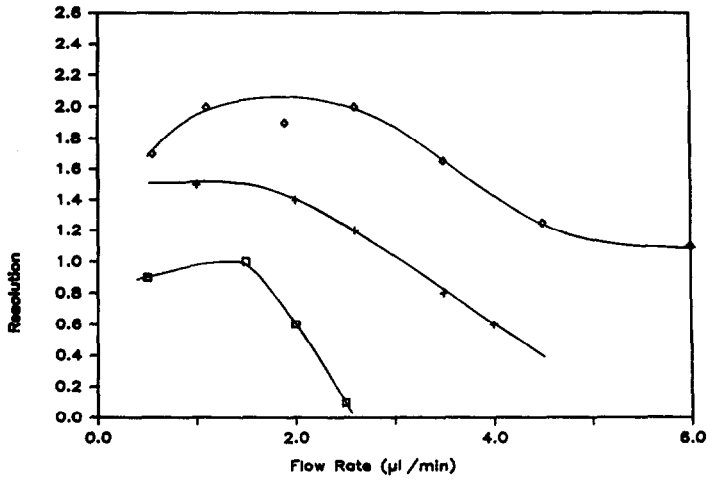

Fig. 2. Ins/Cyt resolution as a function of flow-rate for (□) 30-cm, (+) 20-cm, and (◇) 10-cm columns. Gradient conditions similar to those of Fig. 1. Resolution is $2(t_{R_B} - t_{R_A})/(W_A + W_B)$.

flow-rate increases for all column lengths tested, as has been observed in other systems[2,56]. As indicated by Schlabach and Wilson[2] and Jones *et al.*[56], lower flow-rates are better for protein resolution in gradient elution. Similar results were obtained for the BA/Lalb pair. Fig. 2 also seems to indicate that resolution increases as column length decreases, as was noted by Blanquet *et al.*[50]. Obviously, similar behavior is

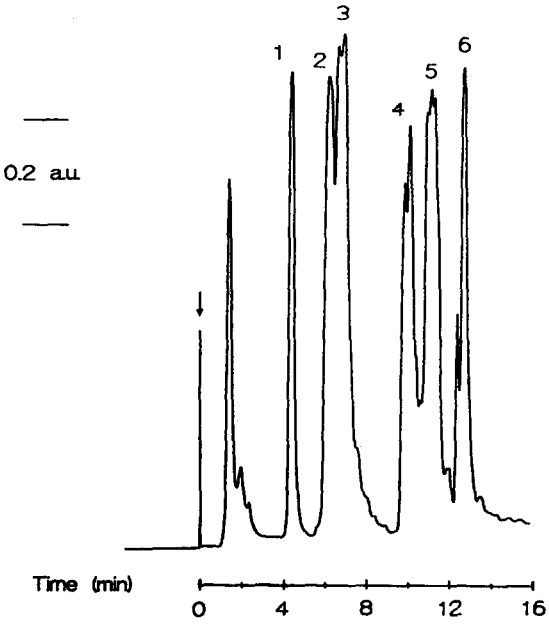

Fig. 3. Loadability of a 10 cm × 250 µm I.D., octyl column. Proteins in order of elution: 1 = 1.6 µg Rnase, 2 = 1.7 µg Ins, 3 = 1.8 µg Cyt, 4 = 1.5 µg BA, 5 = 1.7 µg Lalb, 6 = 1.8 µg LA. Gradient conditions as in Fig. 1. Flow-rate = 2.5 µl/min.

noticed here with our microcolumns. In terms of both relative retention and component resolution, microcolumns prepared successively from the same sorption material were found to be reproducible.

Column loading capacity is an important consideration if one wishes to take full advantage of the micropreparative capabilities of a microcolumn system with samples containing varying amounts of proteins. As demonstrated in Fig. 3, adequate component resolution can be achieved at microgram quantities. A blank gradient then was initiated to estimate the extent of "ghosting", that is, the appearance of solute peaks in subsequent runs. At the maximum loading capacity of the column, a peak corresponding to bovine albumin reappeared, with a peak area of approximately 2% of that from the previous solute run. No peaks corresponding to the other proteins were seen, and another blank run failed to produce another bovine albumin "ghost" peak. This phenomenon is not a product of the use of a step gradient, but a problem that exists in protein chromatography when the solubility limits of hydrophobic proteins, in particular, are exceeded. In fact, the use of microcolumns seems to reduce the number of subsequent blank gradients needed to clean the column, even at maximum loading. Our microcolumns thus exhibit an adequate "dynamic range" for the protein samples, from the microgram quantities down to the minimum detectable amounts, as demonstrated in a related publication[26], in the high picogram range.

In the area of SEC, we have evaluated three packing materials that are applicable to different molecular weight ranges. The plate height values as a function of the mobile phase velocity were determined, as demonstrated in Fig. 4. The results for the GPC 60 and 100 materials reflect a general increase in the slope of the linear portion as the molecular weight increases; this trend is seen more clearly in the data obtained on the GPC 300 column. It has been observed[48] that proteins eluting immediately after the dead volume tend to have broader peaks than the others. This is due to limited diffusion as the molecular dimensions approach those of the pore, and could account for the non-linearity observed in the thyroglobulin data. It is also possible that thyroglobulin aggregation or denaturation could be occurring; at this point, only speculations are possible.

Similar to the observations of Yamamoto *et al.*[57] with the HW55F gel, the height equivalent to a theoretical plate (HETP) value extrapolated to $u=0$ for the GPC 300 material did not change appreciably with the molecular weight. The GPC materials also demonstrate that HETP depends very little on flow-rate for Ser and $(Gly)_4$, because their movement is not limited by their diffusion coefficients[58]. Table III summarizes the efficiencies in plates per meter and the reduced plate height values, averaged from three consecutively prepared microcolumns for each packing material. The results obtained for efficiencies are comparable to those obtained by Rokushika *et al.*[58] with the TSK-GEL 2000 and 3000 SW columns, and appear to be better than those obtained by SynChrom[59] on conventional columns.

Protein standards were separated on the GPC 60 and 100 columns as shown in Figs. 5 and 6, respectively, with good resolution. It is apparent that the mass detection sensitivity inherent to microcolumn LC makes the detection of nanogram amounts of proteins routine.

The GPC 300 material was studied in more detail, because of the larger molecular weight range. Fig. 7 demonstrates the typical sigmoidal calibration curve

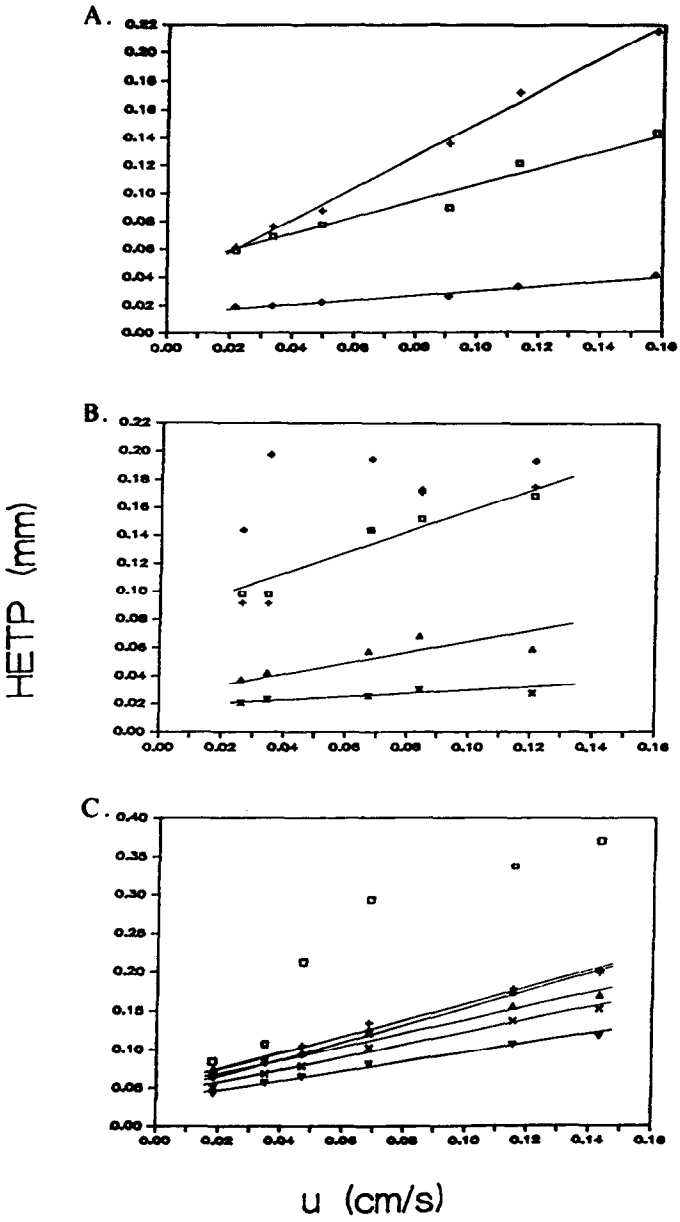

Fig. 4. HETP as a function of mobile phase linear velocity (u) for (A) GPC 60, (B) GPC 100, and (C) GPC 300. Mobile phases: (A) 0.1 M sodium phosphate buffer, pH 7; (B) and (C) 10% methanol in 0.1 M sodium phosphate buffer, pH 7. Key: A, (□) BA; (+) Rnase; (◇) Serine; B, (□) Thy; (+) Trans; (◇) LA; (△) Lalb; (×) $(Gly)_4$; C, (□) Thy; (+) Trans; (◇) BA; (△) LA; (×) CA; (▽) Rnase.

TABLE III

AVERAGE COLUMN PERFORMANCE FOR SYNCHROPAK GPC MATERIALS

Mobile phase flow-rate was 0.4 μl/min for all determinations.

	N/m	*h*
GPC 60		
BA	11300	19
Rnase	10200	20
Ser	42000	4.9
GPC 100		
Thy	9700	20
Trans	11000	19
LA	6000	34
Lalb	22000	9.2
$(Gly)_4$	43700	4.6
GPC 300		
Thy	9000	22
Trans	11300	18
BA	12300	16
LA	10000	21
CA	15400	13
Rnase	17300	12
$(Gly)_4$	42100	4.8

obtained with the model proteins, plus the excluded and included solutes. All the proteins behaved linearly, with a correlation coefficient of 0.98.

Fig. 8 demonstrates the separation of a standard mixture of proteins and $(Gly)_4$. Problems were encountered with the coelution of Trans and BA, and, as the

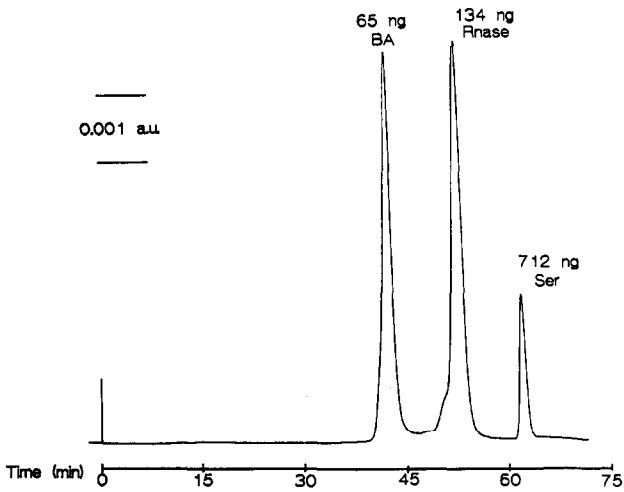

Fig. 5. Chromatography of standard proteins on a 53 cm × 250 μm I.D., GPC 60 column. Mobile phase: 0.1 *M* sodium phosphate buffer, pH 7, at a flow-rate of 0.27 μl/min.

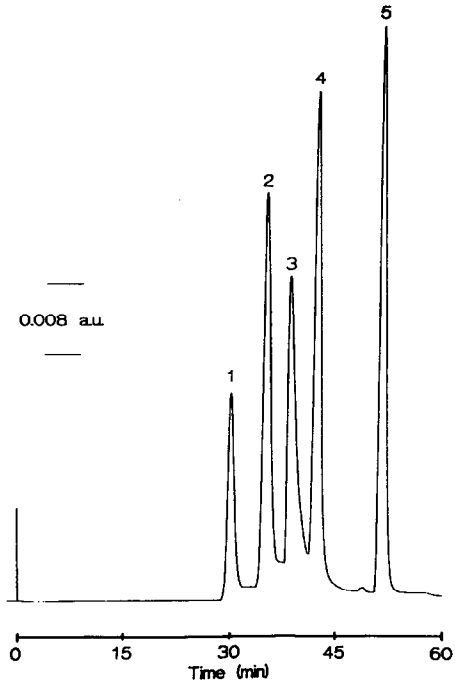

Fig. 6. Chromatography of standard proteins on a 51 cm × 250 μm I.D., GPC 100 column. Proteins in elution order: 1 = 86 ng Thy, 2 = 124 ng Trans, 3 = 94 ng LA, 4 = 124 ng Lalb, 5 = 166 ng $(Gly)_4$. Mobile phase: 10% methanol in 0.1 *M* sodium phosphate buffer, pH 7, at a flow-rate of 0.33 μl/min.

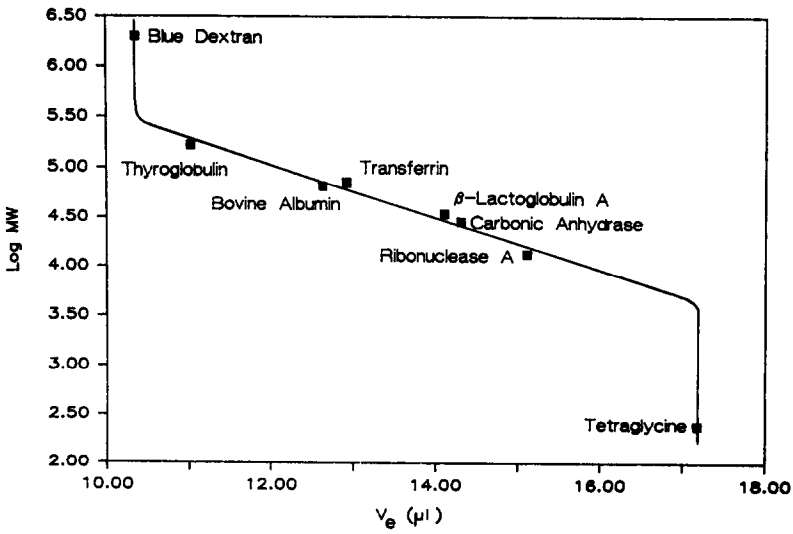

Fig. 7. Relationship of log MW to elution volume on a 48 cm × 250 μm I.D., GPC 300 column. Mobile phase: 10% methanol in 0.1 *M* sodium phosphate buffer, pH 7.

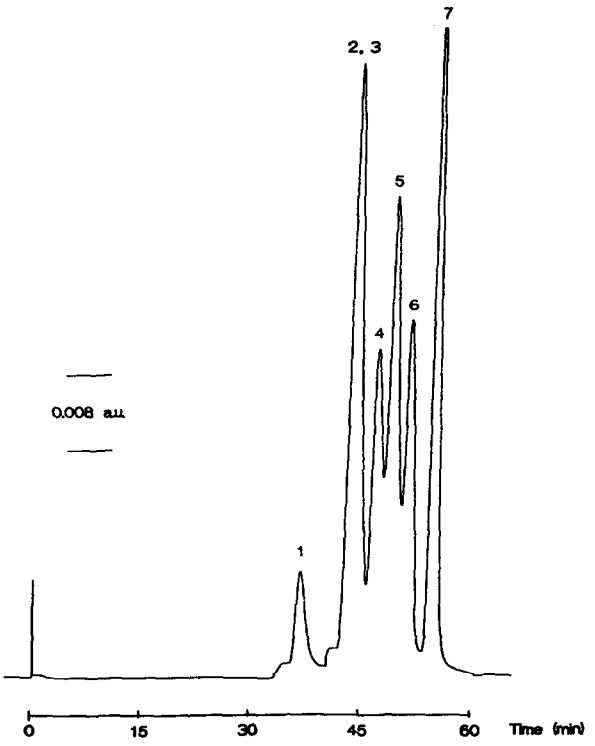

Fig. 8. Chromatography of standard proteins on a GPC 300 column. Proteins in order of elution: 1 = 90 ng Thy, 2 = 101 ng Trans, 3 = 89 ng BA, 4 = 86 ng LA, 5 = 95 ng CA, 6 = 92 ng Rnase, 7 = 178 ng $(Gly)_4$. Other conditions same as in Fig. 7, at a flow-rate of 0.33 μl/min.

sample was diluted further, LA and CA also coeluted. It has been observed that there must be a two-fold difference in molecular weight before two solutes may be resolved, even with a good SEC column[48]. This fact seems to be supported by the observed coelution problem. Although Rokushika *et al.*[58] demonstrated the separation of low nanogram amounts of protein at 205 nm on a TSK-GEL 3000 SW column, our results reported elsewhere[26] demonstrate separation and detection of subpicomolar amounts.

As shown in this communication, microcolumn LC technologies have considerable potential in the investigations of very small protein samples. The initial work shown with the reversed-phase and size-exclusion systems must now be expanded to the other separation modes that include ion-exchange, hydrophobic interaction and affinity principles for the retention of biological activity. Microcolumns can be used to isolate proteins in quantities that are compatible with structure elucidation procedures that are currently available. However, we have also demonstrated the ability to isolate low nanogram quantities of proteins, which are clearly too low for use with gas-phase sequencers. Consequently, highly sensitive protein characterization techniques must be developed to match the protein microisolation capabilities. Work is underway in this laboratory to achieve these goals, and preliminary

results on the ultrahigh-sensitivity determination of amino acids[26] through laser-based fluorescence detection have already been reported.

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