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Selectivity optimization of reversed-phase high-performance liquid chromatographic peptide and protein separations by varying bonded-phase functionality

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Abstract

Several chemical bonded-phase modified silicas were prepared using sterically protected monofunctional silane reagents which varied widely in structure and polarity. Since some of these bonded-phase packing materials are highly polar (hydrophilic), resistance to acid-catalyzed bonded-phase loss by hydrolysis was examined, and observed to remain high even for the highly polar Diol bonded-phase functionality. Modification of the surface of 300 Å pore size, fully hydroxylated and base-deactivated silica microspheres with these sterically protected silanes yielded HPLC column packing materials for examination of separation selectivities in reversed-phase separations of peptide and protein mixtures. Distinct separation selectivities were apparent for each bonded-phase functionality. Selectivity differences ranged from limited band spacing changes for steric-protected C_{18} and C_8 bonded-phases, to reversal of elution order for the more polar C_3 and CN bonded phases. The use of column-based selectivity differences between sequential reversed-phase separation steps is used for the two-step HPLC isolation of a recombinant human amyloid precursor polypeptide fragment from a crude bacterial extract.

1. Introduction

Separation of peptide and polypeptide mixtures by reversed-phase high-performance liquid chromatography (RP-HPLC) is a widely used protein chemical separation technique, due to the very-high-resolution separations that can be obtained. Such high-resolution separations can be performed because of the ready availability of small-particle, high-performance RP-HPLC column packings. Additionally, high-resolution separations result from using elution conditions which reduce peak tailing, band broadening, peak ghosting, etc. Specifically, many RP-HPLC separations of peptides and polypeptides use a low-pH mobile phase with an elevated column temperature, using gradient elution with an organic modifier, usually acetonitrile. Trifluoroacetic acid (TFA) is widely favored as the acidic mobile phase modifier due to its high available purity, excellent solubilizing properties, and low UV absorbance. Octadecyl silane-modified silicas (ODS or C_{18}) are commonly used for peptide HPLC, and butyl- (C_4) or propyl- (C_3) silane-modified silicas for protein separations.

Optimization of selectivity, or band spacing, in the RP-HPLC separation of small molecules is generally achieved by systematic changes in

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mobile phase composition [1,2]. In gradient elution this includes optimizing gradient steepness of the organic solvent modifier [2]. Significant changes in separation selectivity can also be obtained by manipulating column temperature, although relatively few workers take advantage of this useful procedure. A further source of changing separation selectivity results from varying the stationary phase interactive surface [3,4]. In this approach, practitioners often optimize separation selectivity by varying the interactive surface of C_{18} columns, by using columns from various manufacturers. Unfortunately, this approach to separation optimization often produces a method with limited robustness [3].

Reversed-phase separations of peptides and polypeptides permit fewer options for mobile phase optimization. Problems associated with sample solubility, aggregation, unfavorable silanol interactions, eluent transparency at low wavelengths, and eluent viscosity, limit the ability to change the mobile phase. Therefore, in some circumstances, it is desirable to use a favorable mobile phase, such as TFA–acetonitrile–water, then manipulate separation selectivity by varying the stationary phase functionality. While there has been limited systematic investigation of the effects of bonded-phase functionality on selectivity manipulation of peptide and polypeptide separations, the advantages for separations of small organic molecule mixtures have been amply demonstrated [3,4].

It is generally acknowledged that peptide separations exhibit selectivity differences with different bonded phases. However, this effect is infrequently employed for method development. This probably is a result of experience with the poor stability of short-chain, polar, bonded-phase silicas in reversed-phase operation at low pH. Literature reports on the selectivity effects of bonded-phase chemistry for protein separations are more sparse. Comparisons of bonded-phase mediated selectivity effects should be approached with caution, using the same base silica support to minimize the potentially confounding effects of varying silanol contributions to retention processes. Early studies by Cooke et al. [5] demonstrated significant selectivity differences for protein separations on C_8 and C_3 bonded-phase porous silica columns. In contrast, significant selectivity differences were not observed for protein mixtures separated on nonporous silica particles modified with C_{18}, C_8, C_4, C_2 and phenyl bonded-phases [6]. Several studies have noted selectivity differences for proteins separated on *n*-alkyl bonded-phase porous silica columns, when compared to the cyanopropyl and/or phenyl bonded phases [7–12]. Recently, Hanson et al. [13] observed significant protein separation selectivity differences when comparing several polymethacrylate-based polymer- and copolymer-coated silica packings.

A variety of wide-pore, small particle silica column packings have been successfully prepared using the so-called steric-protected silane reagents [14,15]. These monofunctional silane reagents use bulky hydrophobic side chains (i.e. diisopropyl, diisobutyl) to protect the hydrolytically sensitive silica–siloxane bond. The loss of bonded-phase from the surface of conventional silica RP-HPLC column packings at low pH leads to progressive deterioration of polypeptide separations [16]. The marked hydrolytic stability of columns of steric-protected C_{18} and C_8 bonded-phase packing materials towards the aggressive mobile phase conditions used for peptide and protein separations (low pH, elevated temperature), has previously been described [15,17,18]. The bulky side groups on the bonded phase also affords steric protection of short-chain, more polar, bonded phases [15], resulting in much higher column stability. When such column packings are free of the complications associated with instability in low pH applications, then bonded-phase functionality can be widely varied to find surface properties to produce useful and stable selectivity characteristics for peptide and polypeptide RP-HPLC.

The purpose of the present study was to examine the potential for selectivity changes in peptide and polypeptide separations by using various steric-protected silica-based stationary phases, including short-chain bonded phases. These column packings differ only in the nature of the bonded-phase functionality, being uniformly produced on fully hydroxylated and base-

deactivated silica microspheres. Mixtures of small synthetic peptides and commercially available proteins were used as sample mixtures to study column selectivity.

There are few reports of the successful expression of the amyloid precursor polypeptides (APPs) in bacteria [19–21], and very limited information available on methods for the purification of recombinant APPs. Attempts to isolate an 400-amino acid C-terminal fragment of APP were complicated by low yield, and contamination of the ca. $50 \cdot 10^3$ molecular mass polypeptide by low-molecular-mass impurities [19]. Expression of sequences in the interior portion of the molecule, spanning regions as large as 572 residues (amino acids 20–591 of the APP695 sequence), have been successful [20]. At the time that the current work was in progress, Gardella et al. [21] reported on the successful bacterial expression of an 109-amino acid C-terminal fragment of APP fusion polypeptide. This recombinant polypeptide was partially purified by a combination of immobilized metal–chelate affinity chromatography, followed by RP-HPLC, both under denaturing conditions. In the current report, we describe the expression and purification of a 132-amino acid C-terminal fragment of APP, the so-called APP-C132 polypeptide. We examine the utility of column-based selectivity manipulation for the RP-HPLC purification of APP-C132, and demonstrate the use of sequential reversed-phase HPLC steps, differing in selectivity, for isolation of APP-C132.

2. Experimental

2.1. Apparatus

Analytical separations and column-stability studies were performed using an HP 1090 LC pump (Hewlett-Packard, Avondale, PA, USA). Preparative separations used an HP 1050 LC or DuPont Model 8800 pump fitted with 4 × flow pump heads. Data were acquired with PE-Nelson A/D converters using ChromPerfect software (Justice Innovations, Mountain View, CA, USA) with a personal computer.

2.2. Columns

Stationary phases were prepared in the laboratory by reacting steric-protected silanes with 300 Å pore size, 5 μm particle size, fully hydroxylated high-purity porous silica microspheres [22]. Silanes were obtained from Hüls America (Bristol, PA, USA), or prepared in the laboratory. Bound ligand concentration was determined by elemental analysis for C, H, N and F by Microanalysis (Wilmington, DE, USA). The monofunctional steric-protected silanes all yielded silica surface substitutions of 2.0 ± 0.2 $\mu mol/m^2$, which is the maximum theoretically available for these bulky silane reagents. Stainless-steel column blanks slurry-packed at high pressure by conventional techniques yielded >10 000 plates for analytical columns (150 mm × 4.6 mm I.D.) or 18 500 plates for semi-preparative columns (250 mm × 9.4 mm I.D.). Similar columns designated as Zorbax 300 SB-C_{18}, 300 SB-C_8, 300 SB-CN and 300 SB-C_3 are commercially available in the USA from MacMod Analytical (Chads Ford, PA, USA) and in Canada from Chromatographic Specialties (Brockville, Canada). The Vydac C_4 column (part 214TP5415; The Separations Group, Hesperia, CA, USA) was obtained from the manufacturer, and had 5 μm particles with 300 Å pores in a 150 mm × 4.6 mm I.D. column format.

2.3. Samples

Commercially available standard proteins included; bovine ribonuclease A (RNase; Sigma Chemical, St. Louis, MO, USA), chicken egg white lysozyme (Lyso, Sigma), bovine cytochrome *c* (Cyt c, Sigma), bovine insulin (Ins, Sigma), bovine brain S-100A (S-100α/S-100β subunit heterodimer, Sigma), bovine brain S-100B (S-100β subunit homodimer, Sigma), equine myoglobin (Myo, Sigma), bovine erythrocyte carbonic anhydrase (CA, Sigma), rabbit muscle parvalbumin (Parv, Sigma) and bovine brain calmodulin (CDR; Calbiochem, San Diego, CA, USA). Solutions of standard proteins were prepared in 6 *M* guanidine–HCl/0.1 *M* phosphate buffer, pH 7.8, and kept at room

temperature (23°C) for at least 4 h before use. Synthetic peptides were obtained from Bachem (Philadelphia, PA, USA). The abbreviations used are as follows ($-NH_2$ refers to the modification of the carboxy-terminal to an amide): L1 = Leu–Gly–Leu; L2 = Leu–His–Leu; L3 = Leu–Arg–Leu; L4 = Leu–Leu–Leu–NH_2; L5 = Leu–Leu–Val–Tyr; L6 = Leu–Leu–Leu; L7 = Leu–Leu–Phe–NH_2; L8 = Leu–Leu–Phe; L9 = Leu–Leu–Val–Phe.

The recombinant amyloid precursor protein (APP) fusion polypeptide (APP-C132) is composed of 132 amino acids of the carboxy-terminal sequence of the human amyloid precursor protein sequence (amino acids 563–695, see ref. [23]), with an additional 38 amino acids at the amino-terminus. Twenty-nine residues of the N-terminal sequence can be released from APP-C132 by cleavage with enterokinase. The APP-C132 recombinant protein was prepared as follows: complementary DNA (cDNA) was prepared by reverse transcription of a sample of human brain mRNA, as described [24]. The APP-C132 coding sequence was isolated by polymerase chain reaction (PCR) amplification of human brain cDNA using a synthetic oligonucleotide with the sequence 5′-GCAAGCTTCAC - GATGGAAGTCGACCC-TGTTGATGCCCGCC-3′ as the sense primer and the sequence 5′-GACTCGAGTCCGCTG-TCCAACTTCAGAGGTGC-3′ as the antisense primer. PCR amplification was carried out as described previously [24]. The single amplified band of 468 base pairs (bp) was cleaved with the restriction enzymes Sal I and Xho I (Gibco-BRL, Gaithersburg, MD, USA) and directionally ligated to the Sal I/Xho I sites of the linearized plasmid pFLAG (International Biotechnologies, New Haven, CT, USA). Ligated plasmid was used to transform *Escherichia coli* (strain DH5α, Gibco-BRL). The DNA sequence of the cloned APP-derived insert was determined to confirm its identity and the absence of PCR generated mutations in the sequence. When following the supplier's recommended procedures, we were unable to isolate secreted, soluble APP-C132 from bacterial extracts, before or after induction of the bacteria by isopropylthiogalactoside (IPTG). Analysis of this expression system suggests that APP-C132 is recoverable only from dilute buffer-insoluble materials in the cells. It appears likely that the polypeptide is present in inclusion bodies, although this has yet to be directly examined.

Recombinant plasmid containing bacteria were grown in Luria Broth (LB) [5% tryptone (Difco; Fisher Scientific, Pittsburg, PA, USA), 2.5% yeast extract (Difco) and 5% sodium chloride] at 37°C, with vigorous shaking, until cultures had an optical density at 600 nm of 0.3. IPTG was added to a final concentration of 1.7 m*M* and cells were incubated for a further 4 h. The cells from 1 l of culture were lysed by treatment with lysozyme (1 mg/ml) in lysozyme buffer [50 m*M* Tris, 50 m*M* EDTA (pH 8.0), 8% sucrose, 100 mg/ml phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml aprotinin, 1 mg/ml leupeptin] and sonication. The extract was centrifuged at 10 000 *g* for 10 min, the supernatant discarded and the pellet resuspended in 10 m*M* Tris–HCl/1 m*M* EDTA (pH 8.0) containing DNAase (100 mg/ml, Sigma) and RNAase (100 mg/ml, Sigma) for 30 min at room temperature. The insoluble material was collected by centrifugation (10 000 *g*, 15 min), resuspended in 100 m*M* NaCl/10 m*M* Tris–HCl/1 m*M* EDTA, pH 7.5, and again pelleted by centrifugation. The chromatographic feed stock was prepared by homogenization of the pellet with 6 *M* guanidine–HCl/0.02 *M* Tris–HCl, pH 8.0. The resulting extract was cleared by centrifugation (15 000 *g*, 60 min); the supernatant fraction typically contained 1.5–2 mg/ml of protein. The cleared extract was subjected to RP-HPLC on the 250 mm × 9.4 mm I.D. Zorbax 300 SB-C_8 column. Fractions of interest from the separation were lyophilized to dryness, then resolubilized using 5% acetic acid/6 *M* urea, at a protein concentration of 2–3 mg/ml. This protein solution was either used for further purification, or sampled for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Miscellaneous procedures

Protein determinations were carried out using the bicinchoninic acid (BCA) reaction, using reagents supplied by Pierce (Rockford IL,

USA). Discontinuous buffer SDS polyacrylamide gel electrophoresis (SDS-PAGE) was conducted on 12% or 10–20% polyacrylamide gradient minigels. Following electrophoresis, the gels were negatively stained with a copper stain kit (Bio-Rad, Hercules, CA, USA). A digital image was obtained of the gel using a UMAX 1260 scanner in the reflected light mode, and then the proteins were electrophoretically transferred to nitrocellulose membranes. The recombinant APP fragments were detected immunochemically on the membranes using a rabbit polyclonal antibody specific to the 14 C-terminal amino acids of APP (681–695) (antibody R37, Ref. [25]), and an alkaline phosphatase labeled anti-rabbit immunoglobulin secondary antibody. Immunoreactive bands were localized by reaction with 5-bromo-4-chloro-3-indoyl phosphate/Nitro-blue tetrazolium (BCIP/NBT) alkaline phosphatase substrate. Alternatively, gels were directly stained after electrophoresis using the Silver Stain Plus procedure and reagents from Bio-Rad. Electrophoretic analysis of protein samples containing guanidine–HCl required precipitation of proteins by cold 10% trichloroacetic acid. The resulting protein precipitate was collected by centrifugation, washed twice with cold ethanol–diethyl ether (50:50, v/v), lyophilized, and then resolubilized using 2% SDS/20 mM Tris–HCl, pH 7.5.

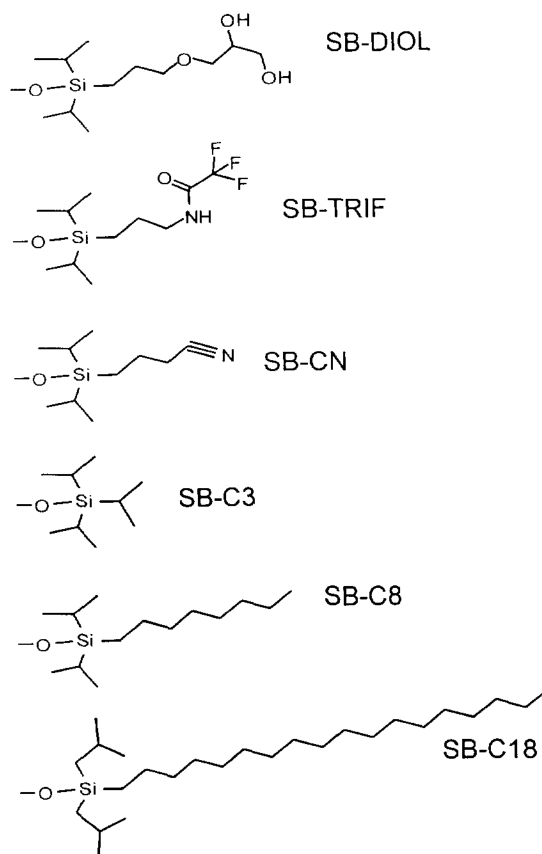

Fig. 1. Structures of the steric-protected silanes used in this study. The reagents are listed in the top-to-bottom order of increasing retention for small organic test molecules. SB-TRIF = [3-(trifluoroacetamido)propyl]-diisopropylsilane.

3. Results and discussion

A variety of organosilane reagents have been successfully prepared with bulky hydrophobic side chains that provide steric protection of the silica surface-bound silane [15,18]. Several examples of such reagents are illustrated in Fig. 1. These silica-based bonded phases have either the diisopropyl steric-protecting groups, or, in the case of the SB-C_{18} reagent, diisobutyl steric-protecting groups. As seen in this figure, a wide variety of bonded-phase surface chemistries are potentially available for changing selectivity in RP-HPLC separations. The corresponding stationary phases vary widely in polarity, as judged by retention of non-ionogenic hydrophobic organic compounds. A previous report [3] demonstrates the small molecule separation selectivity differences available with some of the bonded–phases shown in Fig. 1.

Hydrolysis of covalently attached bonded phases from the surface of silica occurs more rapidly at low pH and elevated temperatures [15,17]. At low pH, dimethyl-substituted bonded phases degrade more rapidly as the ligand chain is shortened, or the effective polarity of the bonded phase increases [15,18]. Highly stable peptide separations resulting from the use of steric-protected SB-C_8 and SB-C_{18} n-alkyl bonded phases have previously been reported [18]. Since some of the bonded phases listed in Fig. 1 are highly hydrophilic, there was a question that the protection afforded by bulky side groups may be insufficient to yield reasonable

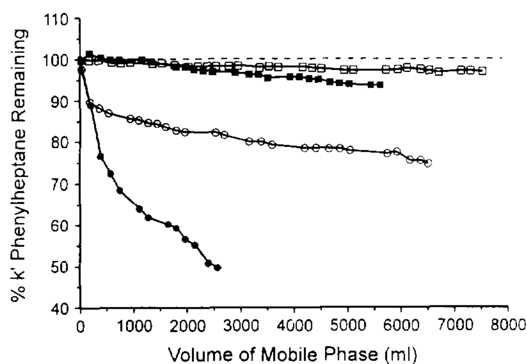

Fig. 2. Test of the stability of bonded-phase silica column packing materials. Loss of retention of phenylheptane as a function of volume of mobile phase passed through the column. Retention of phenylheptane was determined isocratically following 0–100% gradients of water to acetonitrile, both containing 0.5% TFA. The 150 mm × 4.6 mm I.D. columns were maintained at 60°C for the gradients, and tested isocratically at room temperature (ca. 22°C). □ = SB-C_3; ■ = SB-TRIF; ○ = SB-Diol; ■ = C_4 phase.

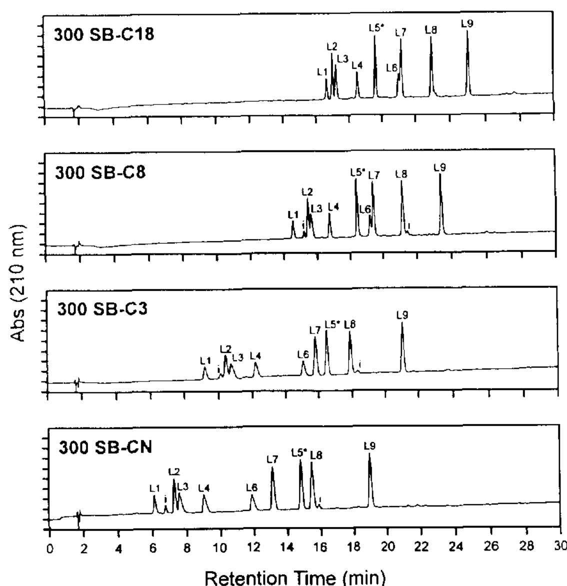

Fig. 3. Gradient RP-HPLC separations of a mixture of nine peptides on various bonded-phase column packings: columns, 150 mm × 4.6 mm I.D.; mobile phase gradient 0–36% water to acetonitrile, both with 0.1% TFA, over 30 min; flow-rate, 1 ml/min; temperature, 40°C; 2 μg of each peptide was injected in a volume of 20 μl. For L1–L9 see text; i = unidentified impurities.

stability for use under the aggressive conditions typical for peptide and polypeptide separations (pH 1–2, 20–60°C). Fig. 2 presents a comparison of bonded-phase stabilities for very polar steric-protected bonded phases versus a widely used, less polar, C_4 bonded-phase packing material. These plots show the retention of a neutral hydrophobic analyte (phenylheptane) as a function of the number of column volumes of the challenging mobile phase passing through the column. Loss of retention for this analyte has previously been shown to correlate with bonded-phase loss [15].

To investigate the potential for selectivity shifts with different bonded-phase functionalities, we compared peptide and polypeptide gradient RP-HPLC separations using columns packed with the SB-C_{18}, -C_8, -C_3 (triisopropyl) and -CN (cyanopropyl) bonded phases. Fig. 3 compares the elution profiles obtained for identical 30-min gradient separations of a nine-component mixture of 3- and 4-amino acid-residue peptides. Based on analyses of small organic molecules, retention decreased significantly, as expected, for all peptides when shifting from the longer-chain, more hydrophobic SB-C_8 and SB-C_{18} *n*-alkyl bonded phases, to the short-chain SB-C_3 and the nitrile-containing SB-CN bonded phase. For peptide L5, elution order shifts with bonded-phase type, with this peptide eluting between L4 and L6 on the *n*-alkyl bonded phases, and between L7 and L8 for the more polar SB-C_3 and SB-CN bonded phases. Less radical selectivity differences are noted for several band pairs when comparing the separations on each column, for example, band spacing for L6/L7, or L8/L9. The elution patterns were not completely reordered, however, as apparent by the similar selectivities of these packings for separating peptides L1, L2, L3 and L4.

Changes in gradient elution conditions lead to band spacing changes for separations such as those in Fig. 3 [26]. To define the dependence of gradient elution conditions on the selectivity differences between the bonded-phase packings, separations of the nine-component peptide mixture were conducted on each column at 20, 40 and 60 min. These retention data sets were used with DryLab software (LC Resources, Walnut

Creek, CA, USA) to predict optimum separations of this mixture both in bonded-phase functionality and gradient time. Fig. 4 presents the experimental verification of the predicted best separations of the nine-component peptide mixture on SB-CN and SB-C_{18} columns. The predicted and observed retention times were within 0.15 min. For this peptide mixture, the SB-CN bonded-phase column produced the best result, yielding baseline resolution in about 23 min. The SB-C_{18} bonded phase gave an acceptable separation, but required a longer gradient time with less resolution than obtained with the SB-CN or SB-C_3 bonded phases. The SB-C_3 column was also able to separate these components with near-baseline resolution ($R_s = 1.4$), but required a 35 min gradient time. Optimum separation with the SB-C_8 column required the least time (L9 elutes at 19.5 min), but gave poor resolution of bands L6/L7. The data in Fig. 4 show that judicious choice of bonded phase, combined with gradient optimization, yielded complete and rapid separation of all components of this peptide mixture.

Comparison of the separations for several polypeptides using the SB-C_{18}, -C_8, -C_3 and -CN bonded-phase columns demonstrated significant selectivity differences. Fig. 5 shows the elution profiles for a ten-component mixture of polypeptides using identical gradient conditions for each column. In this case nine standard proteins were denatured in 6 *M* guanidine–HCl before injection. As was observed previously for the small peptides, a general reduction in retention is apparent in shifting from the most hydrophobic SB-C_{18} bonded phase to the short-chain, more polar bonded phases. Significant selectivity differences are apparent with each of these bonded-phase packings for separating the ten polypeptides, as readily seen by comparing the

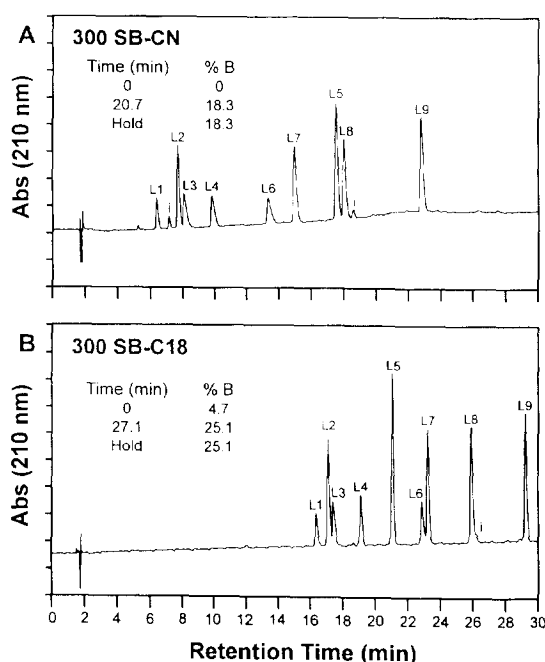

Fig. 4. Gradient RP-HPLC separations of a peptide mixture using gradient optimized elution conditions for two bonded-phase columns, packed with (A) SB-CN or (B) SB-C_{18} modified silicas: mobile phase gradients as indicated on the figure (solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in acetonitrile); columns, 150 mm × 4.6 mm I.D.; flow-rate, 1 ml/min; temperature, 40°C; 2 μg of each peptide was injected in a volume of 20 μl.

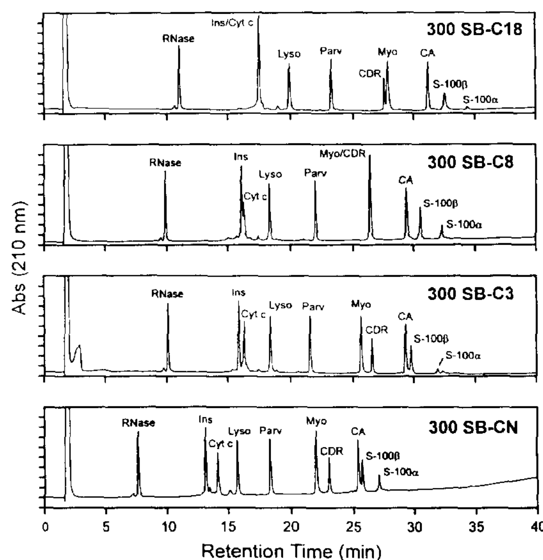

Fig. 5. Gradient RP-HPLC separations of a polypeptide mixture on various bonded-phase columns: columns, 150 mm × 4.6 mm I.D.; flow-rate, 1 ml/min; temperature, 60°C; gradient conditions, 25–70% B in 40 min, solvent A = 0.1% TFA in water, solvent B = 0.09% TFA in acetonitrile–water (80:20, v/v); 3 μg of each protein in 20 μl 6.0 *M* guanidine–HCl/0.1 *M* sodium phosphate, pH 7.8. For abbreviations, see text.

retentions of Ins and Cyt c, or Myo and CDR. Comparing the separations obtained on each column using gradients of 20, 40 and 60 min, as was described above for the peptide separations, confirmed that selectivity differences between bonded-phase types were not unique to the specific gradient conditions chosen for Fig. 5. The apparent differences in peak recovery between columns for the S-100 α-subunit is an artifact of the sample preparation buffer conditions. This polypeptide appears to slowly precipitate under the conditions used. No differences were noted in sample recovery between the columns for the ten polypeptides of this sample.

RP-HPLC separations of complex polypeptide samples can yield highly purified components. In some cases, insufficient selectivity differences exist to permit isolation of pure components in a single separation step. It becomes increasingly difficult, however, to isolate pure components from a complex sample as sample load increases, as is usually the case for preparative separations. Fig. 6 shows a semi-preparative-scale gradient RP-HPLC separation of proteins from a crude extract of recombinant protein expressing *E. coli* cells, using a 250 mm × 9.4 mm I.D. column packed with SB-C_8 bonded-phase silica (3.8 mg protein injected). The recombinant bacteria were engineered to express the fusion polypeptide APP-C132, which contains the carboxy-terminal sequence of the human amyloid precursor polypeptide. In this example of a complex sample separation, the target APP-C132 polypeptide (identified by western blot analysis in Fig. 7) is observed by gel electrophoresis to coelute with a variety of higher-molecular-mass polypeptides. Attempts to obtain pure material by gradient or temperature optimization of this separation provided insufficient resolution. The purity of the material produced by a single RP-HPLC step was, at best, 60–70% using the semi-preparative SB-C_8 column.

Fig. 8 compares separations of the partially purified APP-C132 preparation using SB-C_8 and SB-CN bonded-phase packings. Gradient conditions for these separations used the same rate changes in acetonitrile (0.36% acetonitrile/ml), while yielding similar retention times of the peak of interest. This comparison shows that there are significant selectivity differences between these two bonded-phase functionalities for this sample. Most of the impurity peaks apparent for the SB-C_8 separation elute close to the APP-C132 peak, as expected, considering the source of the crude material (SB-C_8 semi-preparative separation). A major contaminant peak (labeled I in Fig. 8) is only partly resolved from the APP-C132 peak. In contrast, contaminants eluting from the SB-CN column are shifted considerably further from the target peak, for example, comparing the resolution of APP-C132 to the major contaminant peak I.

The purity of APP-C132 preparations isolated using the SB-CN and SB-C_8 columns, as de-

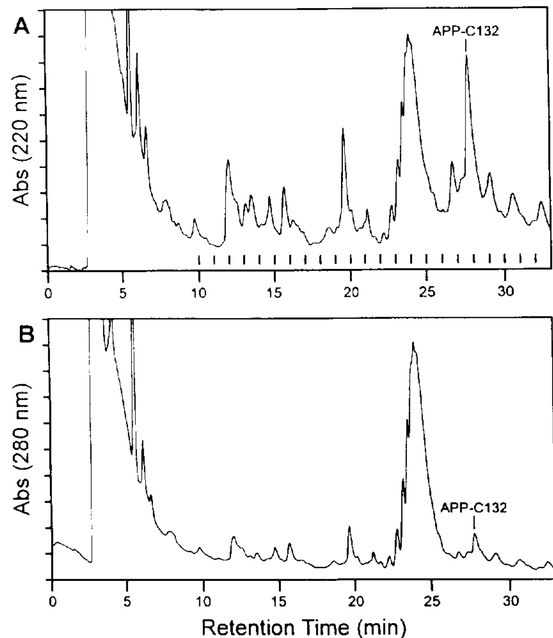

Fig. 6. Separation of APP-C132 from bacterial proteins: column, 250 mm × 9.4 mm I.D. Zorbax 300 SB-C_8; flow-rate, 4 ml/min; temperature, 40°C; gradient conditions, 35–47% B in 30 min, solvent A = 0.1% TFA in water, solvent B = 0.09% TFA in acetonitrile; sample, 2.5 ml of 6.0 *M* guanidine–HCl extract of recombinant protein expressing *E. coli* cells (3.8 mg protein); detection, absorbance at (A) 220 nm and (B) 280 nm. Fractions were collected at 1-min intervals starting at 10 min, as indicated in (A). The peak containing the $22 \cdot 10^3$ molecular mass immunoreactive APP-C132 polypeptide elutes at 28 min (see Fig. 7).

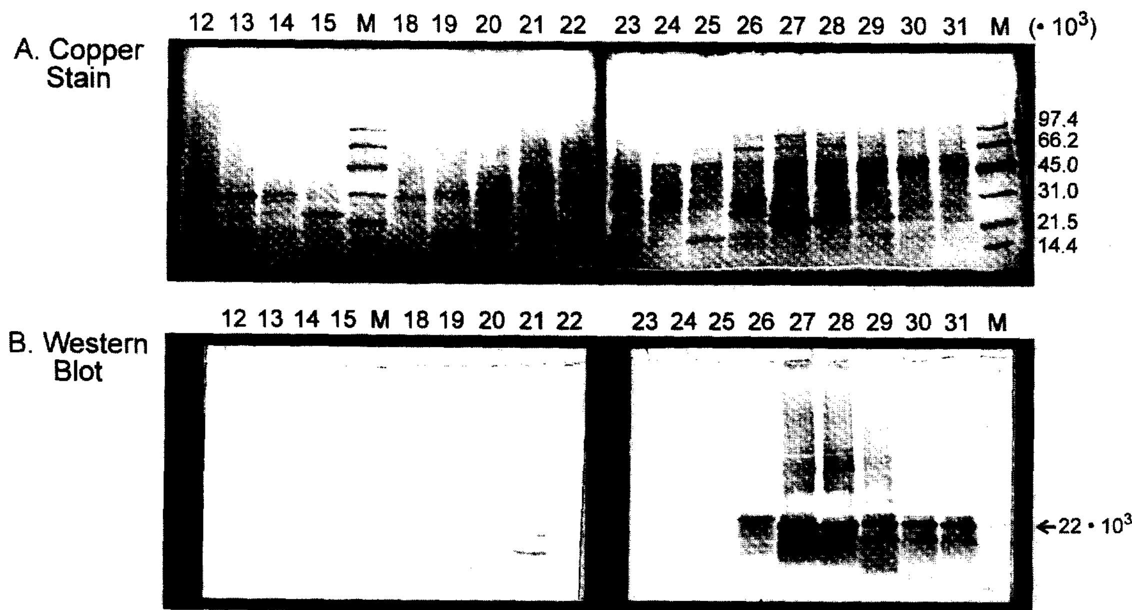

Fig. 7. SDS-PAGE analysis of fractions collected from the RP-HPLC separation of the bacterial extract (Fig. 6). Lyophilized samples obtained from RP-HPLC were submitted to SDS-PAGE separation on 10–20% gradient gels. Proteins were identified in the gels by the reversible copper stain (A), then transferred to nitrocellulose sheets for immunochemical staining (B). The immunoreactive APP-C132 band, eluting from the RP-HPLC column at 27–29 min, is identified on the immunoblot to migrate as an apparent $22 \cdot 10^3$ molecular mass band. M = Standard molecular mass marker proteins.

scribed above, were analyzed by reinjecting collected fractions on the opposite column. Fig. 9 shows the analysis of APP-C132 peaks from the SB-CN column, using SB-C_8 (A), and from the SB-C_8 column, using SB-CN (B). The separation shown in Fig. 9A reveals a single contaminant eluting at 11.8 min, representing about 2.5% of the area counts, compared to the APP-C132 peak. Fig. 9B shows that the APP-C132 isolate, obtained using the SB-C_8 column, is more contaminated, with several impurities eluting on the front of the target peak. Isolation of APP-C132 from the semi-purified sample by the use of the SB-CN column results in a material with higher purity than can be obtained using the SB-C_8 column.

Thus, the use of sequential reversed-phase steps, with a selectivity switch from the SB-C_8 to the SB-CN bonded-phase, yields highly purified APP-C132. Confirmation of the purity of APP-C132 produced by this scheme, and comparison with the intermediate purification steps, was carried out by SDS-PAGE analysis, as shown in Fig. 10. The final protein preparation appeared as a single band on the electropherogram.

4. Conclusions

Bonded-phase silica column packing materials were successfully prepared on 300 Å pore size fully hydroxylated, high-purity, silica microspheres with a variety of steric-protected silane reagents. In agreement with previous results [15,17,18], the sterically protected silane bonded phases exhibited excellent resistance to acid-catalyzed bonded-phase hydrolysis, even in cases where the bonded-phase moiety was highly hydrophilic (e.g., SB-Diol). Bonded-phase loss was minimal, even with highly aggressive mobile phase conditions ($pH < 1$, elevated temperature). Since the molar surface coverages of these monofunctionally reacted surfaces are highly

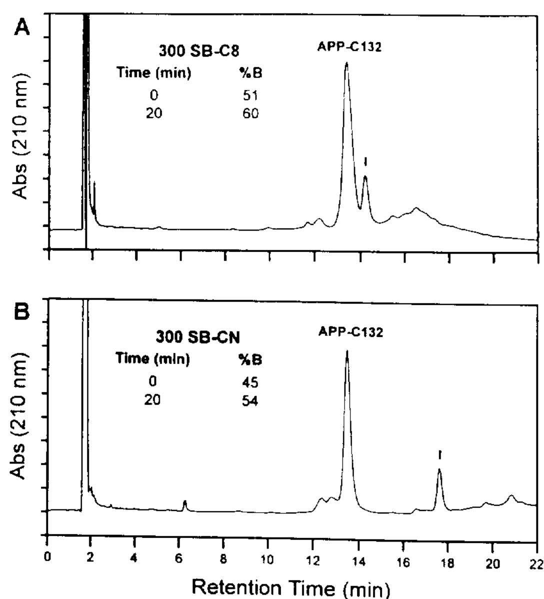

Fig. 8. Separation of partially purified APP-C132 from contaminants using the SB-C_8 (A) and SB-CN (B) bonded-phase columns: columns, 150 mm × 4.6 mm I.D.; flow-rate, 1 ml/min; temperature, 40°C; gradient conditions as described on the figure, solvent A = 0.1% TFA in water, solvent B = 0.09% TFA in acetonitrile–water (80:20, v/v); sample, 9 μg protein samples from the semi-preparative SB-C_8 separation were injected. I represents a major impurity peak.

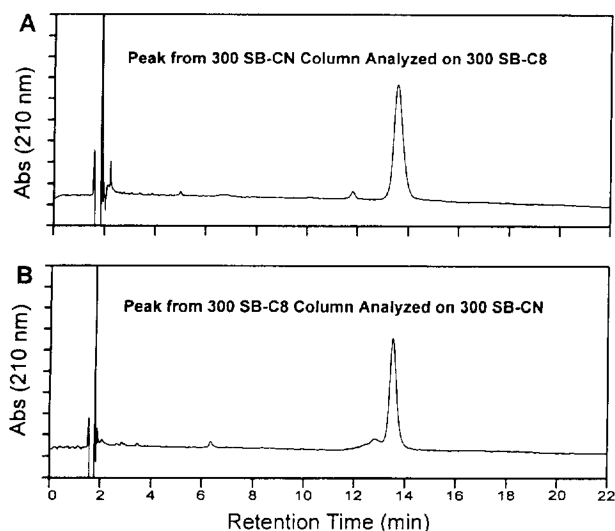

Fig. 9. Analysis of purified APP-C132 preparations. (A) Analysis of APP-C132 protein, obtained using the SB-CN column, as shown in Fig. 8B. This analysis used the SB-C_8 column, and conditions for the SB-C_8 column described in Fig. 8A. (B) Analysis of APP-C132 protein, obtained using the SB-C_8 column, as described in Fig. 8A. This analysis used the SB-CN column, and conditions for the SB-CN column, as described in Fig. 8B. For both analyses 4 μg of purified protein were injected.

similar (ca. 2.0 $\mu mol/m^2$), it is safe to assume that any selectivity changes observed between bonded-phase packings are not the result of surface changes resulting from use of the column, or due to gross differences in accessibility of the underlying silica silanol groups.

The present study has demonstrated significant differences in peptide and protein separation selectivities with bonded-phase functionality. The marked selectivity differences for separations on the sterically protected C_8 and CN bonded phases are in agreement with other recent studies [11,12], and extend these observations to a wider variety of stationary phases. Thus, columns of these short-chain bonded phases can be used to obtain favorable band-spacing changes because of their unique surface interactive properties, which result from varying the steric-protected silane functionality.

The utility of bonded-phase selectivity manipulation has been demonstrated by the two-step RP-HPLC purification of recombinant APP-C132 fusion polypeptide from a crude bacterial lysate. Although definitive proof of the purity of the APP-C132 preparation will require further independent methods of analysis, the use of column-based selectivity shifting between consecutive RP-HPLC steps yields a material of higher purity than could be obtained by using a single bonded-phase functionality. The recombinant construct used for expression of APP-C132 gave rather poor yields of the polypeptide, which resulted in a challenging sample for purification. Because the sequence of interest contains the amyloidogenic Aβ sequence [23], it was anticipated that poor expression efficiency might result, and that the resulting polypeptide would display inconvenient aggregation and solubility properties. Using the RP-HPLC approach described, APP-C132 fusion polypeptide could be prepared in a highly purified form, in a single working day. The successful isolation of APP-C132 will permit further analysis of the solubility

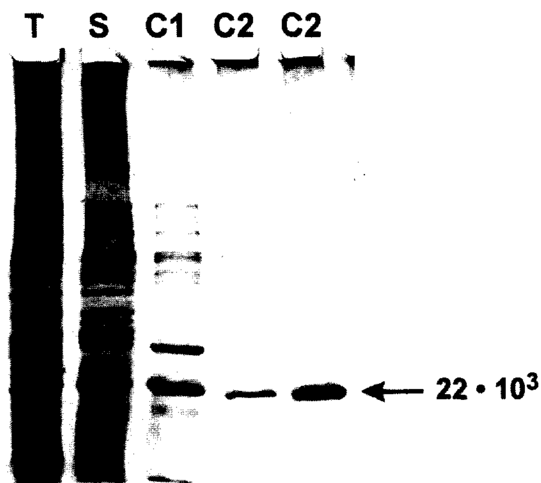

Fig. 10. Electrophoretic analysis following the purification of APP-C132. Samples were separated using a 12% SDS-PAGE gel, then visualized by silver staining. Sample identity: T = total protein in the lysed bacterial pellet (4 μg loaded); S = 6.0 *M* guanidine-HCl solubilized sample (4 μg loaded); C1 = pooled column eluate from the separation on Zorbax 300 SB-C_8 column (2 μg loaded); C2 = pooled column eluate from the separation of C1 on the Zorbax 300 SB-CN column, using conditions described in Fig. 8B (0.4- and 2.5-μg samples loaded).

and aggregation properties of the polypeptide, as well as supply material to characterize the biological role of APP and its fragments.

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