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High temperature fast chromatography of proteins using a silica-based stationary phase with greatly enhanced low pH stability

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Abstract

Reversed-phase liquid chromatography (RPLC) is very widely used for the separation and characterization of proteins and peptides. A novel type of highly stable silica-based stationary phase has been developed for protein separations. A dense monolayer of dimethyl-(chloromethyl)phenylethyl)-*chlorosilane* (DM-CMPES) on the surface of silica is "hyper-crosslinked" with a polyfunctional aromatic crosslinker through Friedel-Crafts chemistry resulting in stationary phases with extraordinary stability in acidic media. Elemental analysis data confirm the high degree of cross-linking among the surface groups. The hyper-crosslinked phases are extremely stable under highly acidic mobile phase conditions even at a temperature as high as 150 °C. A wide-pore (300 Å) material made in this way is used here to separate proteins by a reversed-phase mechanism and compared to a commercially available "sterically protected" C_{18} phase. For small molecules, including neutral and basic compounds, these crosslinked phases give comparable peak shape and efficiency to the commercial phase. Our results show that no pore blockage takes place as commonly afflicts polymer coated phases. In consequence, protein separations on the new phases are acceptable. Using strong ion-pairing reagents, such as HPF_6, improves the separation efficiency. Compared to the commercial phases, these new phases can be used at lower pHs and much higher temperatures thereby enabling much faster separations which is the primary focus of this work. Better efficiency for proteins was obtained at high temperature. However, at conventional linear velocities the instability of proteins at high temperature becomes a problem which establishes an upper temperature limit. Uses of a narrowbore column and high flow rates both solves this problem by reducing the time that proteins spend on the hot column and, of course, speeds up the separation of the protein mixture. Finally, an ultrafast gradient (<1 min) protein separation was obtained by utilizing the high temperature and thus high linear velocities afforded by the extreme stability of these new phases. The phases are stable even after 50 h of exposure to 0.1% TFA at 120 °C. This paper is dedicated to the memory of Csaba Horvath whose work in high temperature HPLC inspired the development of the stationary phases described here.

Keywords: High temperature; Proteins

1. Introduction

Silica-based bonded phases dominate the reversed-phase separations of proteins and peptides [1–4]. Low pH mobile phases are preferred for such mixtures to suppress undesirable interactions between basic amino acid side chains in the proteins and the stationary phase surface by protonating the surface silanol groups [5–7]. However, the narrow range of pH stability of most silica-based packing materials limits their use to pHs between about 3 and 7. Acidic eluents hydrolyze the siloxane bond between the bonded silane and the silica surface resulting in a continuous change of chromatographic retention [8]; in contrast at higher pHs the silica substrate dissolves more readily and extensively [6,8–10].

The significance of temperature as a major operational variable in liquid chromatography is becoming well recognized [11–20]. Over the past several years we have investigated various aspects of increasing separation speed by the use of much higher temperatures. As Antia and Horvath [21] have pointed out high temperature decreases viscosity and increases diffusion rates both of which work in concert to allow the use of higher linear velocities with conventional pumps and hardware [22,23]. Theoretical work indicates that in iso-

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cratic chromatography faster analyses are promoted by the use of high temperature and water rich eluents [22]. Finally, we [24] showed that as predicted by Antia and Horvath [21] analyte instability due to the elevated temperature is not as inhibiting as had been thought provided that residence times at high temperature are decreased. In fact, 10 years ago Chen and Horvath were able to separate a mixture of four proteins in less than 10 s by using a temperature of 120 °C [15]. Such work has not become widespread because traditional silica-based stationary phases are not sufficiently stable at such high temperatures (>80 °C), especially in acidic eluents, to be useful [25]. The major focus of this work is the demonstration that reversed phases capable of doing acceptable protein chromatography in acid at high temperature have been designed.

There are many advantages to speeding-up HPLC. The cost of a separation would be reduced if the lifetime of the chromatographic column could be prolonged to allow it to be used at higher temperature. Furthermore the throughput in proteomic studies could be enhanced if analysis speed could be increased. Therefore, the need for a low pH and high temperature stable material motivated this study of hyper-crosslinked silica-based phase for protein separations. Gradient elution RPLC is now quite fast enough to be used for monitoring the progress of preparative scale separations by displacement development.

A novel type of highly stable silica-based *wide-pore* stationary phase, developed in this laboratory for protein separations [26–29], is the topic of the current work. As in prior work on a narrow pore (80 Å) material [26–29], a dense monolayer of dimethyl-(chloromethyl)phenylethyl)-*chlorosilane* (DM-CMPES) on the silica surface is hyper-crosslinked with multivalent aromatic crosslinkers through Friedel-Crafts chemistry to significantly enhance the acid stability of the resulting stationary phase. The first step in the modification is to covalently bond a dense monolayer of DM-CMPES to the silica. This monolayer provides nearly 3 μmol/m^2 of reactive chlorine on the silica surface and is the basis for further treatment by Friedel-Crafts catalyzed self-condensation and crosslinking with additional reagents. In the second step, a crosslinkable aromatic compound (styrene heptamer) and aluminum trichloride are used to extensively crosslink the phenyl rings which were previously attached to the silica. The third reaction used methoxychloromethane to further crosslink the stationary phase and provide additional chloromethyl groups for the fourth reaction. In the final step, 1-phenyloctane groups are added to the residual chloromethyl groups to produce the hyper-crosslinked C_8 (HC-C_8) material. Elemental analysis data confirm the high degree of cross-linking among the surface groups [29]. The loss in amount of this stationary phase under highly acidic mobile phase conditions (5% trifluoroacetic acid) even at a temperature as high as 150 °C [27–29] is quite small and is less than 40 μg of carbon/m^2 out of a total initial coverage of 800 μg of carbon/m^2, i.e. less than 5%.

The resulting wide-pore HC-C_8 stationary phase was used here to separate proteins by a reversed-phase mechanism and compared to a commercially available "sterically protected" C_{18} phase. In our study, a mixture of standard proteins was chosen to study the applications at high temperature, high flow rate, and low pH conditions on our newly developed phase. For small neutral compounds, the hyper-crosslinked phase gave comparable peak shape and efficiency as the commercial phase. Inverse size exclusion chromatography showed that no pore blockage [27–29], which commonly afflicts polymer coated phases, occurs. As a result, protein separations on the new phases are acceptable. Compared to the commercial phases, these new phases can be used at lower pHs and much higher temperatures. Therefore, they offer alternatives for fast protein separations. The effects of different ion-pairing agents and temperatures are evaluated.

2. Experimental

2.1. Chemicals

HPLC-grade acetonitrile (ACN) was from Burdick and Jackson (Muskegon, MI). HPLC water was obtained from a Barnsted Nanopure deionizing system (Dubuque, IA) and run through an "organic-free" cartridge followed by a 0.2 μm particle filter. The water was boiled to remove carbon dioxide. All solvents were filtered through a 0.45 μm filter (Lida Manufacturing Corp., Kenosha, WI) before use. Trifluoroacetic acid (TFA), heptafluorobutyric acid (HFBA), and hexafluorophosphonic acid (HPF_6) were obtained from Aldrich (Milwaukee, WI, USA). Tris(carboxyethyl)phosphine (TCEP) chloride and proteins were from Sigma (St. Louis, MO).

2.2. Chromatographic columns

The Zorbax StableBond C_{18} (SB-C_{18}) column was a gift of Agilent Technologies Inc. (Wilmington, DE). The column dimension is 5.0 cm × 0.46 cm. The particle size is 5 μm with a pore size of 300 Å.

The HC-C_8 was synthesized using the Friedel-Crafts crosslinking method [29]. The particle size is 5 μm with a pore size of 300 Å. The stationary phase was packed in 5.0 cm × 0.46 cm and 3.3 cm × 0.21 cm columns. Stainless steel column hardware and 0.5 μm stainless steel frits were obtained from Isolation Technologies (Hopedale, MA). The particles were slurried in 2-propanol (1.0 g particle in 23.5 mL solvent) and sonicated for 20 min prior to packing. Columns were packed by the downward slurry technique at a packing pressure of 350 bar (He gas) using pure 2-propanol as the driving solvent. About 60 mL solvent were collected before depressurizing. The column was then left on the packer for 10 more minutes before it was disconnected and closed with an end-fitting.

Before the column was used to do any separations, it was washed with 47.5/47.5/5.0 ACN/water/TFA (v/v/v) (pH = 0.5) mobile phase at a flow rate of 1.0 mL/min and a

column temperature of 150 °C for 2 h. The purpose of this washing is to remove the residual aluminum(III) on the stationary phase left from the synthesis and to hydrolyze remaining alkyl chloride and open the labile Si–O–Si bonds.

2.3. Chromatographic experiments

Chromatographic experiments were performed on an HP 1100 chromatograph. For high temperature experiments (≥80 °C), a heating apparatus from Systec Inc. (New Brighton, MN) was used to control the column temperature. This apparatus consists of a mobile phase preheater assembly and feedback controlled insulated jacket, which allows the column to be heated to temperatures as high as 200 °C. A cold-water bath was also used to cool the eluent coming out of the column before reaching the detector. Unless otherwise noted, all data were obtained using 1.0 μL injection volumes of solute with an absorbance detector set to a wavelength of 220 nm. Protein samples were typically prepared as 5 mg/mL solutions in 0.1% aqueous trifluoroacetic acid.

3. Results and discussion

The purpose of this work was to demonstrate the separation of proteins on the new material but our primary interest is in their use and stability at high temperature. The sterically protected SB-C_{18} column was chosen as our benchmark for protein separations because it is a very acid stable material. The SB-C_{18} phase was also chosen because it was synthesized on the same Zorbax type B silica particles, thus eliminating any differences due to acidity of silanol groups and pore size of the underlying silica.

3.1. Separations of small, neutral compounds

Table 1 compares the chromatographic data on the SB-C_{18} and HC-C_8 phases. The goal was to quantify any differences in retention for hydrophobic compounds and differences in chromatographic efficiency due to packing effects. It is very clear that both phases give comparable performance towards neutral compounds (the plate counts on both columns are in excess of 70,000 m^{-1}). Very similar plate counts and asymmetry factors were obtained. This result agrees with the previous finding in Trammell's work [29] on small pore material.

Table 1
Comparison of neutral compound separations on StableBond-C_{18} and hypercrosslinked C_8 phases[a]

	k'		N[d]		USP T_f [d]	
	SB[b]	HC[c]	SB	HC	SB	HC
Uracil	0.00	0.00	3500	3900	1.32	1.32
Phenol	0.27	0.22	3900	4200	1.34	1.34
Benzene	0.89	0.60	4500	4700	1.2	1.27
Naphthalene	1.99	1.67	4600	4600	1.14	1.27

[a] Chromatographic conditions: 50/50 ACN/H_2O, 35 °C, 1 mL/min, 254 nm.
[b] SB-C_{18} phase.
[c] HC-C_8 phase.
[d] Plate count (N) and USP tailing factor (USP T_f) were given by the ChemStation software (ChemStation for LC 3D, Rev. A.08.03 [847], Agilent Technologies 1990–2000, Hewlett Packard S.A., Wilmington, DE).

Table 2
Characteristics of proteins studied

Protein	MW[a]	D[b]	pI[c]	N[d]
Insulin	6030	1.6	5.7	51
Lysozyme	14310	1.2	11.0	129
Myoglobin	17200	1.1	7.1	153

[a] Molecular weight.
[b] Diffusion coefficient ($\times 10^{-6}$ cm^2/s) at 20 °C.
[c] Isoelectric point.
[d] Total number of amino acid residues in the protein.

3.2. Protein separations

Insulin, lysozyme, and myoglobin were chosen as probes because they are commonly studied [6,15,30] and have quite different isoelectric points and molecular weights (see Table 2) [31,32]. A mixture of these three proteins was separated on both SB-C_{18} and HC-C_8 columns under the same chromatographic conditions. The chromatograms are shown in Fig. 1. All three proteins eluted under the gradient condition on both phases, but have relatively shorter retention times on the HC-C_8 phase. This is mainly because, as shown in the previous work [29], the HC-C_8 phase is less hydrophobic as indicated by a smaller free energy of retention per methylene group than for the SB-C_{18} phase. Acceptable peak shapes were obtained in both situations. However, the peak widths on the HC-C_8 phase were somewhat larger than on the SB-C_{18} phase (see Table 3). The ratios of the peak widths on the HC-C_8 to those on the SB-C_{18} were between 1.6 and 1.8.

3.3. Zero retention test

In order to make sure there is no pore blockage on our HC-C_8 phase, we deliberately chose conditions to force the retention of all the solutes to zero. If some of the pores in the particles were blocked, the mass transfer rate would be slower; therefore the peak width for the unretained solutes should be wider. Table 4 shows the comparison between HC-C_8 and SB-C_{18}. It is very clear that on both columns, the peak widths for all solutes are comparable. For some of the solutes, the HC-C_8 gives the narrower peaks. This means that there is no or very little pore blockage on the HC-C_8 phase. We do have some concern that the k' values are slightly different and the proteins are somewhat more excluded on the highly crosslinked phase than on the commercial phase.

3.4. Effect of ion-pairing reagent

Trifluoroacetic acid is the most commonly used ion-pairing reagent in reversed-phase chromatography of proteins

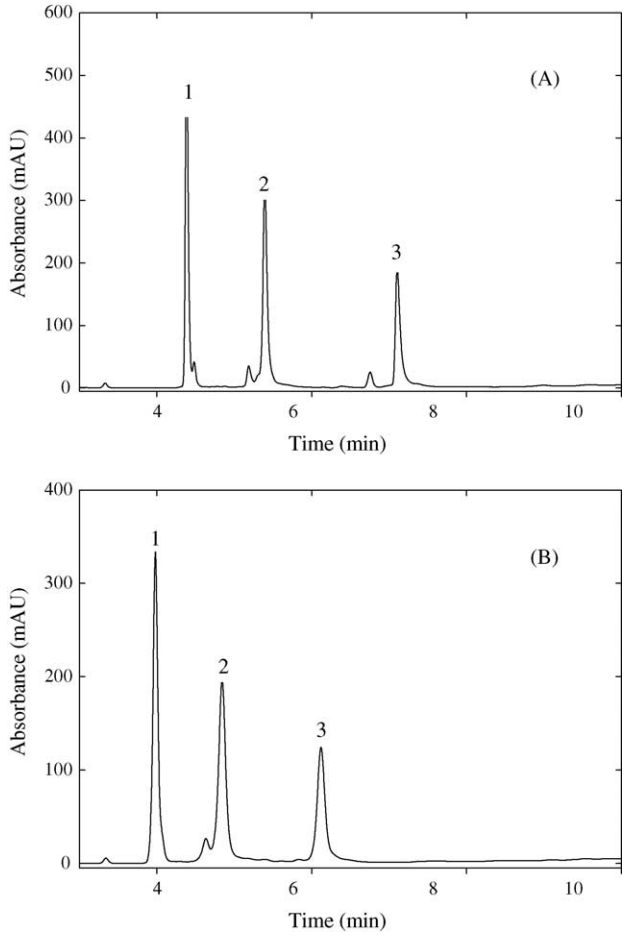

Fig. 1. Chromatograms of protein separations on SB-C_{18} (A) and HC-C_8 phases (B). Chromatographic conditions: 35 °C, 1 mL/min, 1 μL, 220 nm. Solvent (A) 0.1% TFA in water; solvent (B) 0.067% TFA in ACN. Linear gradient from 20 to 60% B in 10 min. Solutes: (1) insulin; (2) lysozyme; (3) myoglobin (5 mg/mL). Detection at 220 nm.

and peptides [6,33]. The main purpose of using an ion-pairing reagent is thought to be the shielding of amino groups in proteins to prevent their interaction with surface silanol groups [6,33]. To find the appropriate ion-pairing reagent so as to shield the positive charges on proteins, we studied three potential ion-pairing reagents.

Table 3
Comparison of protein and peptide separations on StableBond-C_{18} and hyper-crosslinked C_8 phases[a]

	t_R (min)			$w_{1/2}$[c] (min)		
	SB	HC	Ratio[b]	SB	HC	Ratio
Insulin	4.37	3.98	0.91	0.041	0.065	1.60
Lysozyme	5.36	4.82	0.90	0.057	0.104	1.83
Myoglobin	7.10	6.10	0.86	0.066	0.108	1.65

[a] Chromatographic conditions: 35 °C, 1 mL/min, 1 μL, 220 nm. Solvent (A) 0.1% TFA in water; solvent (B) 0.067% TFA in ACN. Linear gradient from 20 to 60% B in 10 min.
[b] Ratio was calculated by t_R-HC/t_R-SB or $w_{1/2}$-HC/$w_{1/2}$-SB.
[c] $w_{1/2}$ is the peak width at half peak height given by ChemStation.

Table 4
Comparison of the peak widths of unretained solutes on StableBond-C_{18} and hyper-crosslinked C_8 phases[a]

	k'		$w_{1/2}$[b] (min)	
	SB	HC	SB	HC
Insulin	−0.05	−0.18	0.031	0.027
Lysozyme	−0.12	−0.24	0.029	0.026
Myoglobin	−0.14	−0.28	0.041	0.028
Uracil	0.00	0.00	0.021	0.022
Benzene	0.91	0.63	0.035	0.031
Toluene	1.43	0.96	0.045	0.036
Naphthalene	2.06	1.68	0.062	0.063

[a] Chromatographic conditions: 35 °C, 1 mL/min, 1 μL, 220 nm, 50/50/0.1 ACN/H_2O/TFA.
[b] $w_{1/2}$ is the peak width at half peak height given by ChemStation.

These are trifluoroacetic acid (TFA), heptafluorobutyric acid (HFBA), and hexafluorophosphonic acid (HPF_6). HFBA is also a commonly used ion-pairing reagent for protein separations. It is well known that HFBA is a much stronger ion-pairing reagent than is TFA [6]. HPF_6 is also a very strong ion-pairing reagent [34]. Table 5 shows that these ion-pairing reagents have a significant effect on the retention and peak width of all three proteins. In one model of the effect of ion interaction reagent, each anion (ion-paring reagent) [34] sorbs on the stationary phase to a different extent. When a positively charged protein molecule encounters an increased concentration of anions near the stationary phase surface, an ion pair forms which then provides longer retention. This model also accounts for the improved peak width values shown in Table 5. The formation of ion pairs between the protein and the sorbed additive anion reduces the relative importance of protein retention by ion-exchange with ionized surface silanol groups. As a result, the retention time *became* longer and the peak widths *became* narrower when a stronger ion-pairing reagent was used. It is interesting that PF_6^- has as much effect as heptafluorobutyric acid on retention and has an even greater effect on the peak width. We are not aware of its prior use in protein RPLC.

Table 5
Effect of different ion pairing agent on the retention time and peak width of proteins on the HC-C_8 column

	t_R (min)			$w_{1/2}$ (min)		
	TFA[a]	HFBA[b]	HPF_6[c]	TFA	HFBA	HPF_6
Insulin	3.98	5.47	5.45	0.065	0.060	0.057
Lysozyme	4.82	6.67	6.52	0.104	0.075	0.063
Myoglobin	6.10	8.11	7.94	0.108	0.086	0.073

Chromatographic conditions: 35 °C, 1 mL/min, 1 μL, 220 nm. Solvent (A) 13 mM acid in water; solvent (B) 8.7 mM acid in ACN. Linear gradient from 20 to 60% B in 10 min.
[a] Trifluoroacetic acid.
[b] Heptafluorobutyric acid.
[c] Hexafluorophosphonic acid.

3.5. Effect of temperature

The use of elevated temperatures for the RPLC separation of peptides and proteins has been studied. It has been shown that increasing temperature improves column efficiency and shortens the run time [11,35–37]. However, the combination of low pH and higher temperature generally results in a very short life for most conventional silica columns [25], which in turn limits the use of temperature for optimizing separations. This problem was addressed by the development of hypercrosslinked silica stationary phases which are extremely stable at low pH (as low as pH 0.5) and high temperature (up to 150 °C) [27–29].

Fig. 2 shows the effect of temperature on the retention time and peak width of all three proteins. As predicted, increasing temperature shortens the retention time and decreases the peak width.

The separations of a mixture of seven proteins at different temperatures are shown in Fig. 3. As the temperature is raised from 35 to 80 °C, all peaks become narrower. However, when the temperature was increased to 100 °C and again to 120 °C, the peaks became wider and most significantly peak areas are very obviously decreased. Some new, small peaks start to appear. An important point is that the increases in peak width were not observed during the separation of small neutral compounds (control experiment data are not shown here). This eliminates the possibility that the increase in width is due to a deleterious effect of high temperature on the column packing. The most likely cause of these problems is the instability of the proteins at such high temperature for long periods of time (∼5 min) in the acidic eluent. In order to understand the cause of the peak deterioration, we studied the stability of two proteins, namely lysozyme and myoglobin, at 80 °C.

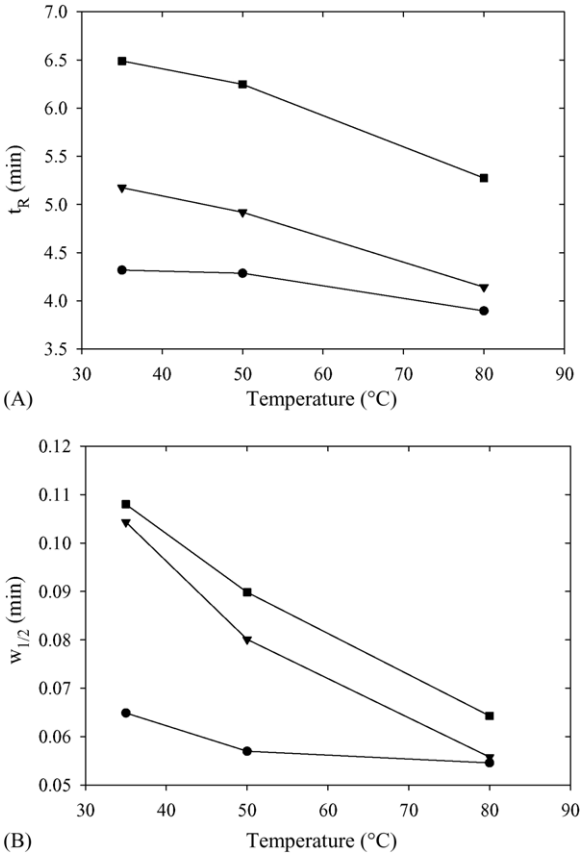

Fig. 2. Effect of column temperature on retention time and peak width of proteins on a HC-C_8 column. Chromatographic conditions: 1 mL/min, 1 μL, 220 nm. Solvent (A) 0.1% TFA in water; solvent (B) 0.067% TFA in ACN. Linear gradient from 20 to 60% B in 10 min. Solutes: (●), insulin; (▼), lysozyme; (■), myoglobin.

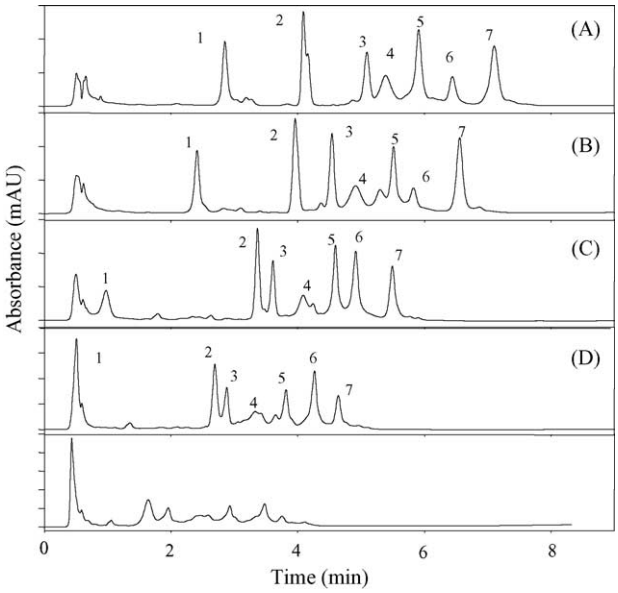

Fig. 3. The effect of temperature on protein separations on the HC-C_8 column. Gradient conditions are the same as in Fig. 2: (A) 35 °C; (B) 60 °C; (C) 80 °C; (D) 100 °C; (E) 120 °C. Proteins: (1) ribonuclease A; (2) insulin; (3) lysozyme; (4) apo-transferrin; (5) α-lactalbumin; (6) α-chymotrypsin; (7) concanavalin A. The concentrations of all the proteins in the mixture were about 5 mg/mL. All the chromatograms are on the same absorbance scale.

3.6. Stability of proteins at 80 °C

Lysozyme has a total of four intramolecular disulfide bonds, which are prone to be broken at high temperature [31]. Thus, we pre-reduced the disulfide bonds as described below to test the temperature stability of the proteins. For the purpose of comparison, we also tested the thermal stability of myoglobin, a protein without any disulfide bonds.

About 10 mg of protein was dissolved in 40 mL of 50/50 ACN/0.1% TFA in HPLC water. For lysozyme, 4 mL 100 mM TCEP (tris(carboxyethyl)phosphine) was added and kept at room temperature for 15 min to reduce the disulfide bonds. An empty 50 mL two-neck, round bottom flask equipped with a condenser, a septum and argon inlet was heated in an oil bath to about 80 °C. Before any solution was added, the system was under total-reflux and under an argon atmosphere. The protein solution was then injected into the flask (time $t = 0$). 0.5 mL aliquots were taken out periodically and were immediately put in an ice bath to terminate any possible fur-

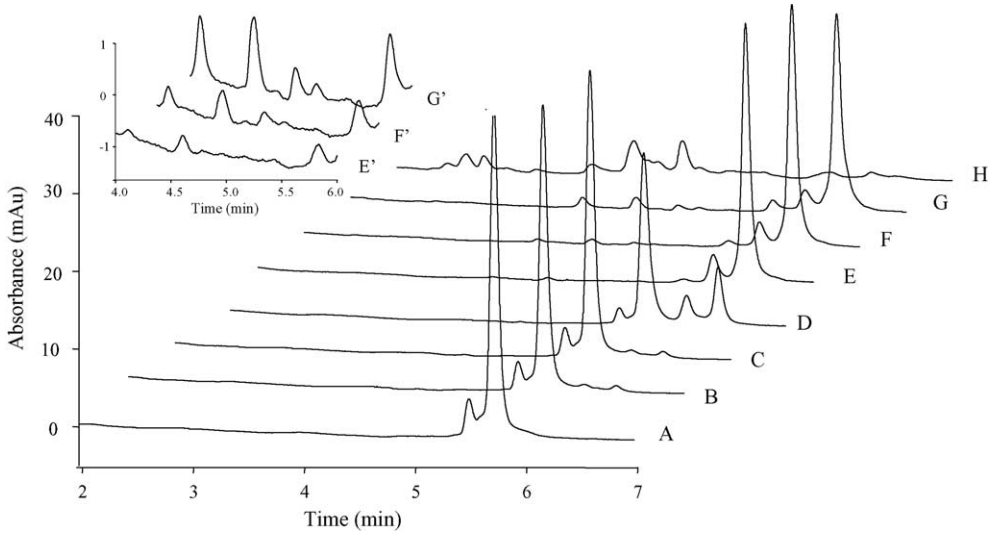

Fig. 4. Stability of lysozyme at 80 °C. Stability test condition see details in text. Chromatographic conditions are the same as in Fig. 1. Column: SB-C_{18} (A) before TCEP solution added; (B) 0 min (no high temperature treatment); (C) 1 min; (D) 2 min; (E) 8 min; (F) 22 min; (G) 60 min; (H) 700 min. E′, F′, and G′ are enlarged partial chromatograms of E, F, and G.

ther reactions. A protein sample before treatment with TCEP solution and a time-zero sample (with TCEP, but not heated at 80 °C) were also taken from the protein solution for the purposes of comparison. All the samples were then separated on the SB-C_{18} column.

Figs. 4 and 5 show the chromatograms of all the lysozyme and myoglobin samples. Clearly, the disulfide bonds in lysozyme were not completely reduced until 8 min after the protein solution was injected into the hot flask had elapsed. More importantly, even after the disulfide bonds were reduced, the protein was still not stable. New, small peaks could be seen in the chromatograms for the samples taken at 22, 35, and 60 min. The peak areas of the reduced forms of lysozyme are shown in Table 6. After the protein solution was kept in the hot environment for about 700 min, the original protein peak completely disappeared. Similar results were obtained for myoglobin, which has no disulfide bond. This indicates that at high temperatures (>80 °C) in the eluents used for protein chromatography the proteins are not stable on the time scale of the typical chromatographic run (10 min), even though our stationary phases are extremely stable at such high temperatures. Since the rupture of the disulfide bonds is clearly not the cause of the instability and it is evident that the proteins are being made into much smaller entities; we assume that the amide bond backbone of the proteins is being hydrolyzed under these aggressive conditions.

Horvath and co-workers [38–40] found that temperature could be adjusted to decrease the peak broadening due to on-column reactions. Both the analyte residence time on the column and the rate of the reaction under the column conditions determined the extent of the on-column reaction. As the column temperature is increased, the reaction rate increases. However, at the same time the residence time also decreases. Horvath and co-workers [21,39,41] showed that the on-column reaction will be unimportant if the decrease in

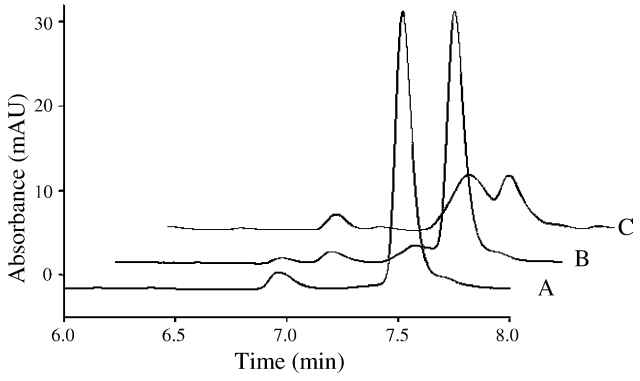

Fig. 5. Stability of myoglobin at 80 °C. Stability test condition see details in text. Chromatographic conditions are the same as in Fig. 1. Column: SB-C_{18} (A) 0 min (no high temperature treatment); (B) 45 min; (C) 660 min.

Table 6
Stability of lysozyme and myoglobin at 80 °C[a]

Lysozyme[b]		Myoglobin	
Time (min)	Peak area	Time (min)	Peak area
8	185	0	180
12	184	1	167
22	180	3	174
35	175	6	169
60	158	8	172
700	0	14	172
		19	162
		26	160
		45	150
		680	43

[a] See Figs. 4 and 5 for stability and chromatographic conditions.
[b] Only the peak areas for the reduced protein are shown.

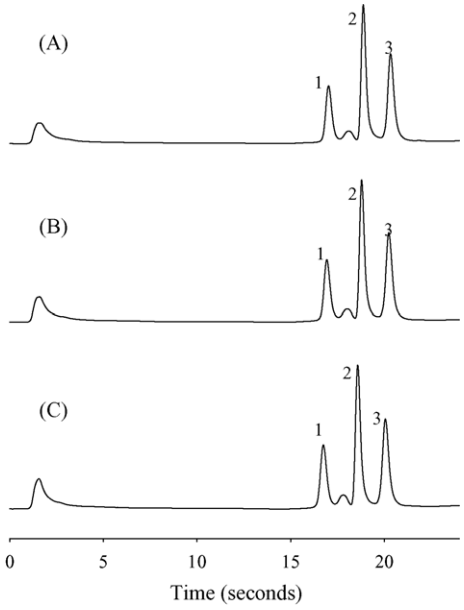

Fig. 6. Chromatograms of a protein mixture separated on the HC-C_8 phase at 120 °C. The column was kept at 120 °C throughout the experiment (about 50 h). (A) Injection was made right after the column was equilibrate at 120 °C; (B) after 24 h; (C) after 50 h. Conditions: 120 °C, 5 mL/min, 1 μL, 220 nm. Solvent (A) 0.1% TFA in water; solvent (B) 0.067% TFA in ACN. Linear gradient from 10 to 90% B in 0.3 min (dwell time is 0.2 min). Solutes: (1) ribonuclease A; (2) insulin; (3) α-chymotrypsin.

the residence time on the column is more important than the increase in the reaction rate. Based on the work of Thompson and Carr [24] who considered the issue of analyte instability (that is, on-column reaction) in detail, the problem of analyte stability can be solved by using a narrowbore columns and high flow rates to reduce the time the proteins spend on the hot column.

3.7. Ultrafast protein separation

In order to perform protein separations at high temperature and high flow rate, a narrowbore column is needed to reduce the thermal mismatch problem encountered at very high flow velocity and high column temperature [42]. A 3.3 cm × 0.21 cm column was prepared for this purpose. The separation of a mixture of three proteins at 120 °C and 5 mL/min is shown in Fig. 6. One complete separation can be done in less than 25 s. From the above stability test, it is clear that the proteins are stable at high temperature *if the separation can be done quickly enough*. Very narrow peaks were obtained. The column was kept at 120 °C in the initial eluent, which is at low pH, throughout the 50 h of the experiment. Clearly the protein chromatograms are reproducible for over 50 h. This offers a large advantage over the work done by Chen and Horvath [15]. Although they were able to do fast high temperature gradient elution protein chromatography, the instability of the columns used in their work greatly shortened the column life and limited the practical application of high temperature separations. This result once again shows that the HC-C_8 phase is stable under low pH and high temperature conditions. We believe that no *silica-based* phase can be used at such high temperatures for such a long time and still provide good performance.

4. Conclusions

The hyper-crosslinked C_8 stationary phase is substantially more acid stable than any silica-based stationary phase available. The extensive crosslinking makes the phase less susceptible to acid catalyzed hydrolysis. For small, neutral molecules, the HC-C_8 phase gives comparable peak shape and efficiency as the SB-C_{18} phase. Our results show that no pore blockage *takes place as* commonly afflicts polymer coated phases. In consequence, protein separations on the new phases are acceptable. Using stronger ion-pairing reagents, such as HPF_6, improves the separation efficiency. Compared to the commercial phases, these new phases can be used at lower pHs and much higher temperatures. Better efficiency for proteins was obtained at high temperature. However, the instability of the proteins at high temperature becomes a problem which limits the use of temperature as a variable to improve separation efficiency and selectivity. Use of a narrowbore column and high flow rates solved this problem by reducing the time that proteins spend on the hot column. Finally, ultrafast (25 s) gradient protein separations were obtained by utilizing the extreme stability of these new phases.

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