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Temperature-Responsive Chromatographic Separation of Amino Acid Phenylthiohydantoins Using Aqueous Media as the Mobile Phase

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Recently, green chemistry has become one of the most important subjects of science for environmental pollution prevention. Here, we report development of a novel chromatographic technology for phenylthiohydantoin (PTH)−amino acid analyses in which only aqueous solution is used as the mobile phase. We have devised HPLC adsorbents (stationary phase) by modifying the surfaces of microparticulate silica gel using functional polymers. The thermoresponsive copolymer, poly(*N*-isopropylacrylamide-*co*-*n*-butyl methacrylate) (IBc) was used to modify the silica stationary phase surfaces. This polymer-grafted surface exhibits temperature-regulated hydrophilic/hydrophobic property changes in water. PTH−amino acid interactions with this surface are readily modulated by changing the column temperature using an isocratic aqueous mobile phase. Increasing hydrophobic interactions between more hydrophobic PTH−amino acids with hydrophobized polymer-grafted surfaces at elevated mobile phase temperatures is used for the effective separation of PTH−amino acids in aqueous solution. This study is aimed at the development of novel separation processes, which are also environmentally benign, for use with biochemical substances in order to meet the growing needs of the life sciences and biotechnology. The method is useful for various separations in life science so that proteins can maintain their biological activity and enzymes, their enzymatic activity.

Polymers that respond to various applied stimuli are widely utilized for drug delivery systems,[1,2,3] cell culture substrates,[4,5] and bioconjugates.[6,7] The stimuli studied to date include changes in concentrations of chemical species and changes in temperature, pH, and electric field. Poly(*N*-isopropylacrylamide) (PIPAAm) is a well-investigated thermosensitive polymer. PIPAAm exhibits thermally reversible soluble−insoluble changes in aqueous solution in response to temperature changes across a prominent lower critical solution temperature (LCST) at 32 °C.[8] Polymer chains of IPAAm hydrate to expand in water below the LCST, while dehydrating to form compact, insoluble conformations above the LCST. We have previously reported dramatic, reversible surface hydrophilic−hydrophobic property alterations for PIPAAm terminally grafted surfaces due to rapid changes in grafted polymer hydration near the polymer's transition temperature.[9] Observed temperature-responsive surface property changes for these terminally grafted surfaces were rapid and significant, which suggests that PIPAAm graft-chain conformational freedom[10] facilitates polymer dehydration and hydrogen bonding with water molecules. We have recently developed a new chromatography system based on grafted PIPAAm.[11−15] Partitioning properties of the stationary-phase surface change in response to external temperature changes (hydrophilic−hydrophobic property).[11] Solute interactions with the PIPAAm-modified stationary phase are regulated by changing the temperature of the chromatography system.

Amino acid sequence analysis of proteins has been widely used for the accumulation of protein primary sequence and structural data by applying protein samples to automated systems for Edman-based phenylisothiocyanate degradation.[16] Conventional analysis uses the reversed-phase columns in which mixtures of organic solvent and buffers are required as a mobile phase to analyze degraded amino acids. Such systems exhibit difficulties with reproducibility when using these organic solvent mixtures and gradient elutions.

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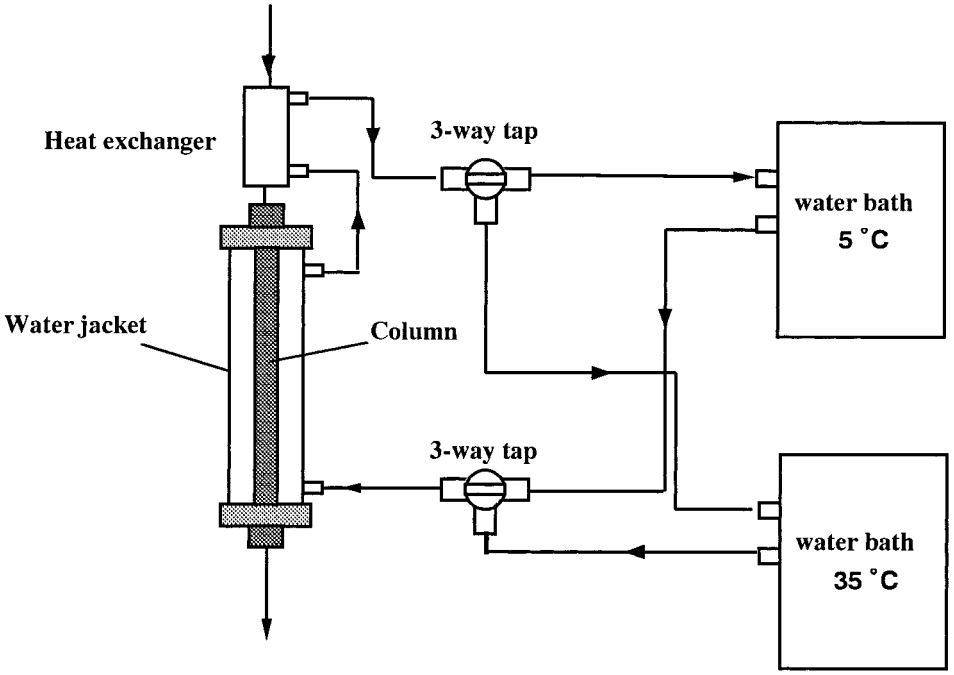

Figure 1. Diagram of a system for stepwise temperature-controlled elution.

Using temperature-responsive chromatography, we have now achieved the successful separation of amino acid phenylthiohydantoins (PTH−amino acids) using only aqueous solution as a mobile phase without organic solvent.

EXPERIMENTAL SECTION

Materials. *N*-Isopropylacrylamide (IPAAm) was kindly provided by KOHJIN (Tokyo, Japan) and was purified by recrystallization from a toluene−hexane mixture and dried at room temperature in vacuo. 3-Mercaptopropionic acid (MPA, Wako Pure Chemicals, Osaka, Japan) was distilled under reduced pressure; fraction boiling at 95 °C (5 mmHg) was used. PTH−amino acids were obtained from Wako Pure Chemicals.

Water was distilled and passed through a Mill-Q purification system (Millipore, Bedford, MA). All other chemicals and solvents were of analytical−reagent grade.

Preparation of Thermosensitive Packing Materials. Synthesis of poly(*N*-isopropylacrylamide-*co*-*n*-butyl methacrylate) [P(IPAAm-*co*-BMA)] (IBc)[17] was as follows: To 50 mL of *N*,*N*-dimethylformamide (DMF), IPAAm (221 mmol), BMA (11.6 mmol), 2,2′-azobisisobutyronitrile (AIBN, 17.7 mmol), 3-mercaptopropionic acid (MPA, 6.52 mmol) were dissolved. After several freeze−thaw cycles to degas this solution, polymerization proceeded at 70 °C for 5 h. Polymer was recovered from precipitation in diethyl ether. Polymer molecular weight was determined to be 6300 by nonaqueous titration, assuming that each molecule has a single carboxyl terminal. This value was in good agreement with gel permeation chromatography (GPC) measurement in DMF. BMA content in the copolymer was determined by ^{1}H NMR to be 3.2 mol %. The LCST for the copolymer was spectrophotometrically estimated to be 24 °C.[17] Terminal carboxyl groups on IBc−3.2 were esterified by *N*-hydroxysuccinimide and *N*,*N*′-dicyclohexylcarbodiimide (mol ratio, 1:2.5:2.5) in ethyl acetate prior to modification of the silica bead surfaces. Active esterified polymer (1.5 g) was dissolved in 50 mL of dry 1,4-dioxane and reacted with 3.0 g of aminopropylsilica for 1 day. The reaction was repeated 3 times per batch using fresh reagents. IBc−3.2-modified silica beads were thus obtained after extensive washing with methanol, distilled water, and Milli-Q water, successively.

Temperature-Responsive HPLC. The polymer-grafted silica support was packed into a stainless steel column (150 mm × 4.6 mm i. d.). The column was connected to an HPLC system (HITACHI model L-6200 intelligent pump, L-4000 UV monitor, D-2500 data processor). Analysis of the samples was performed on the temperature-responsive polymer-modified column using a mobile phase consisting of Milli-Q water. The elution behaviors were monitored by UV 254 nm with a flow-rate of 1.0 mL/min at various temperatures thermostated with a LAUDA RC20 waterbath within a deviation of ± 0.02 °C.

Figure 1 shows diagram of the apparatus for stepwise temperature-controlled elution. A water jacket was used for changing the temperature gradient, and a heat exchanger was used for preheating the mobile phase before it reached the column. The water jacket was connected to two waterbaths with tubing and three-way taps.

Standard solutions of PTH−amino acids were prepared with (1) Arg (initial concentration, 1.000 mg/mL), (2) Asn (0.404 mg/mL), (3) Asp (1.084 mg/mL), (4) Cys (1.000 mg/mL), (5) Gln

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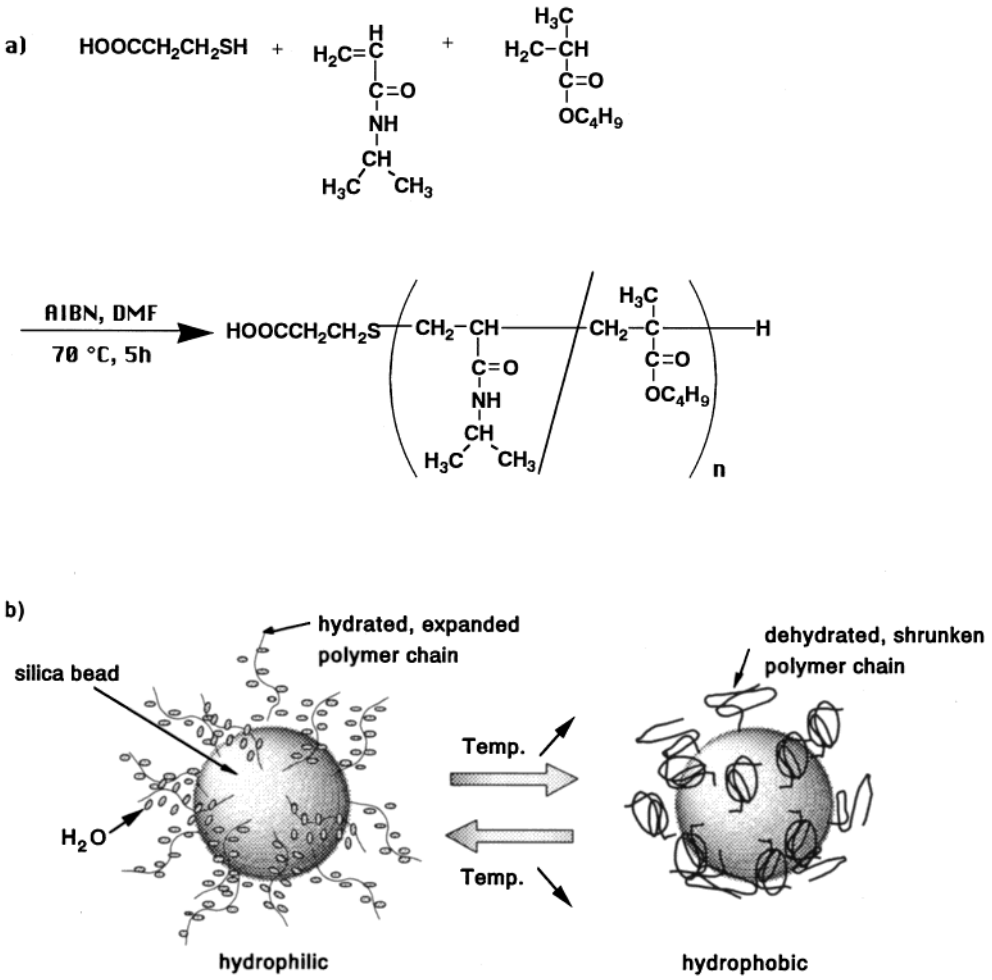

Figure 2. (a) Preparation scheme for the synthesis of thermosensitive polymer. (b) Schematic illustration of temperature-responsive packing materials.

(0.124 mg/mL), (6) Glu (0.471 mg/mL), (7) Gly (0.103 mg/mL), (8) His (1.000 mg/mL), (9) Ileu (0.006 mg/mL), (10) Leu (0.014 mg/mL), (11) Lys (0.005 mg/mL), (12) Met (0.063 mg/mL), (13) Phe (0.038 mg/mL), (14) Pro (0.038 mg/mL), (15) Thr (0.019 mg/mL), (16) Trp (0.019 mg/mL), (17) Tyr (0.077 mg/mL), and (18) Val (0.024 mg/mL).

RESULTS AND DISCUSSION

Figure 2 schematically illustrates preparation of the packing materials. PIPAAm chains are covalently grafted onto aminopropyl-derivated silica beads (average diameter, 5 μm) to yield the thermoresponsive stationary phase. PIPAAm's LCST can be modified by copolymerization of IPAAm with other monomers. Because the phase transition of PIPAAm results from the stability of hydrophobic groups along the polymer chain in aqueous media, the LCST for the polymer should decrease with increasing polymer hydrophobicity.[18] We have reported the regulation of LCST values for PIPAAm using carboxyl end groups by telomerization copolymerization with the hydrophobic comonomer, *n*-butyl methacrylate (BMA).[17] We have used copolymers of IPAAm and BMA having single carboxyl terminal groups as silica modifiers and have produced chromatographic stationary phases with a variety of different LCST values. Semitelechelic IPAAm copolymers (MW, 6300) were synthesized by radical polymerization of IPAAm and BMA in DMF. 2,2′-Azobisisobutyronitrile and 3-mercaptopropionic acid were used as an initiator and a chain transfer agent, respectively. In contrast to the PIPAAm homopolymer, copoly(IPAAm−BMA) containing 3.2 mol % BMA (IBc−3.2) exhibited a lower LCST at 24 °C, which indicates stronger hydrophobic chain aggregation than PIPAAm at lower temperature. IBc−3.2 bearing carboxyl terminals was then grafted onto aminopropyl silica using standard active ester−amine coupling methods.[19]

Figure 3 shows temperature-dependent retention profiles for PTH−amino acids that were separated on IBc−3.2-modified chromatography columns in water. Milli-Q water was used as the sole mobile phase. Eighteen PTH−amino acids were dissolved in Milli-Q water (total, 20 mL) and 100 μL of the sample mixture solution was injected into the IBc−3.2-modified column that was connected to HPLC system. Retention times for each PTH−amino acid were retarded with increasing temperature. Increased retention times for samples are clearly demonstrated with increasing column temperature. The separation of the 18 PTH−amino acids was achieved by selecting the appropriate column temperature

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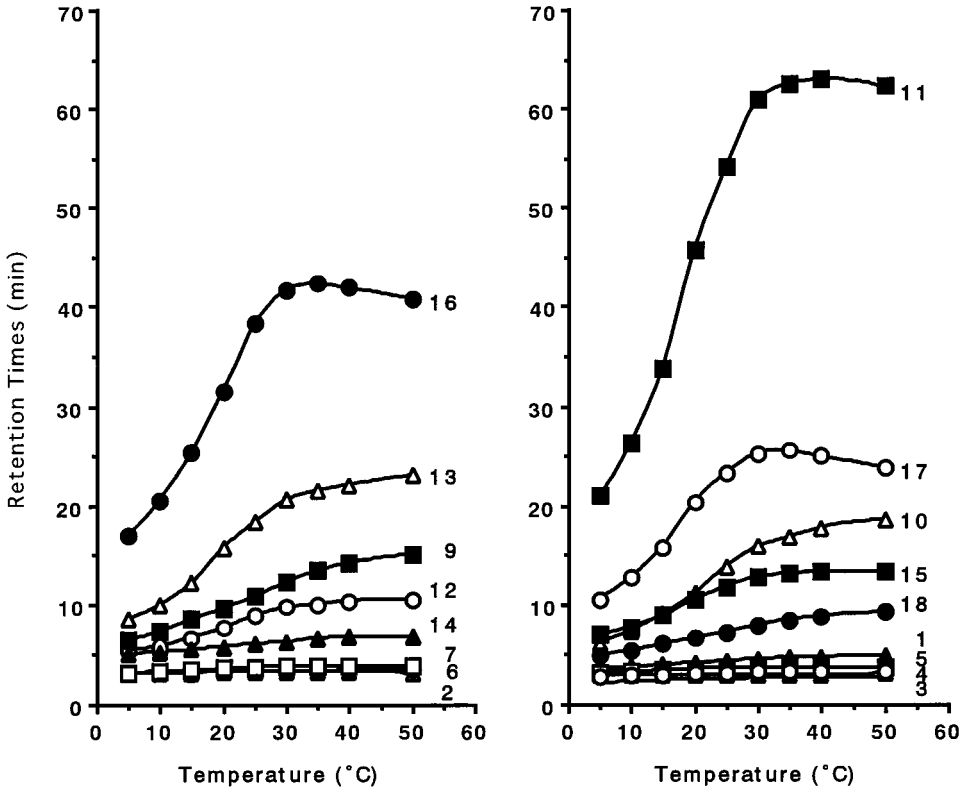

Figure 3. Temperature-dependent retention profiles for PTH−amino acids. HPLC conditions: eluent, pure water; flow-rate, 1.0 mL/min; monitoring, absorption at 254 nm. Numbers are the same as listed in Experimental Section and Table 1.

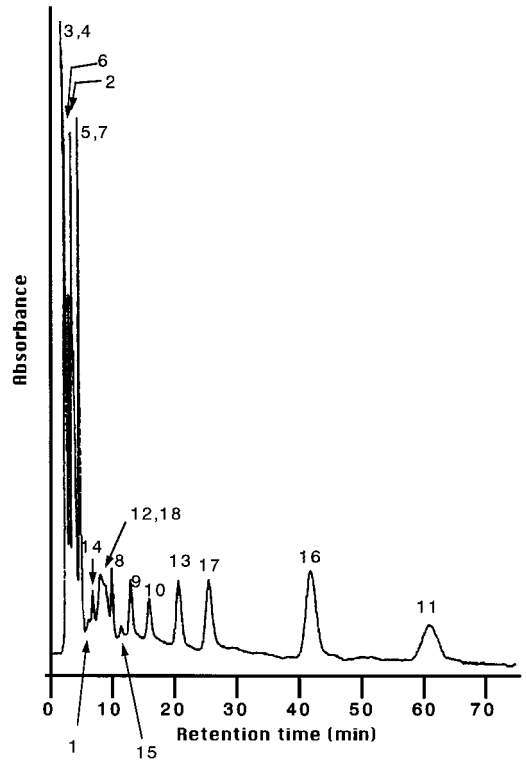

Figure 4. Chromatograms obtained for mixture of PTH−amino acids using pure water as a mobile phase at 30 °C. HPLC conditions: flow-rate, 1.0 mL/min; monitoring, absorption at 254 nm. Peak numbers are the same as listed in Table 1.

Table 1. Precision of PTH−Amino Acids

amino acid	retention time (min)[a]	retention time CV (%)	peak area ($\times 10^5$)[a]	peak area CV (%)
1. PTH−alanine	5.05	0.39	2.28	3.56
2. PTH−asparagine	3.17	0.35	2.76	9.89
3. PTH−aspartic acid	1.61	1.02	3.88	4.64
4. PTH−cysteine	1.74	0.75	16.49	2.97
5. PTH−glutamine	3.34	0.21	2.31	0.71
6. PTH−glutamic acid	1.85	0.59	1.88	4.80
7. PTH−glycine	3.80	0.35	1.58	4.54
8. PTH−histidine	11.11	0.51	6.06	3.65
9. PTH−isoleucine	15.27	0.07	11.81	0.80
10. PTH−leucine	16.89	0.02	3.29	0.66
11. PTH−lysine	57.61	0.05	1.64	2.48
12. PTH−methionine	9.69	0.06	6.44	1.02
13. PTH−phenylalanine	20.91	0.47	3.63	2.29
14. PTH−proline	6.67	0.28	3.73	2.19
15. PTH−threonine	12.59	0.13	1.81	9.93
16. PTH−tryptophan	37.81	0.06	3.16	1.93
17. PTH−tyrosine	22.21	0.14	7.78	5.92
18. PTH−valine	9.81	0.06	3.53	0.81

[a] Average of $n = 5$. HPLC conditions: column, (PNIPAAm-*co*-BMA)-modified column; column temperature, 25 °C; eluent, water; flow rate, 1 mL/min; detection, UV 254 nm.

without any changes in the mobile phase composition, as shown in Figure 4. Reproducible retention times were obtained for all of the PTH−amino acids that were examined. Coefficient of variation (CV) values of the retention times were within 1%, as shown in Table 1. The retention times observed for PTH−amino acids depended largely on temperature, as shown in Figure 3. Retention times significantly increased above the transition temperature of grafted IBc−3.2. This is explained in terms of the increased partitioning of PTH−amino acids into hydrophobized surfaces of IBc−3.2 due to increased dehydration and hydrophobic aggregation of grafted copolymer chains above the transition temperature.

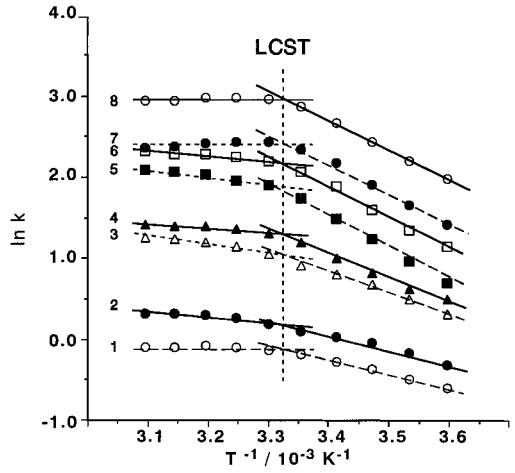

Figure 5. The van't Hoff plots for PTH−amino acids on the IBc−3.2-modified HPLC column. 1, Gly; 2, Ala; 3, Val; 4, Met; 5, Leu; 6, Phe; 7, Tyr; and 8, Trp.

Figure 5 shows the van't Hoff plots for PTH−amino acids on the copolymer-modified separation column. A nonlinear relationship between reciprocal temperature and ln *k* values is apparent for each PTH−amino acid. This provides evidence for the copolymer phase transition on the stationary phase surface. Generally, these plots should be linear for conventional chromatographic processes on commercially available reversed-phase columns under conditions where retention mechanisms do not change. On the temperature-responsive polymer-modified column, however, a deviation from linearity between ln *k* values and reciprocal temperature (1/*T*) is observed. These deviations are consistent with the known phase transition of the grafted copolymer stationary phase. Additionally, the slope of the van't Hoff plots on the copolymer column is negative, which is the opposite of that seen for conventional chromatography. This provides additional evidence that the interaction between PTH−amino acids and temperature-responsive surfaces becomes stronger at elevated temperatures. PTH−amino acids can be generally classified into two groups: apolar species that show retarded retention, and polar species whose retention time remains unchanged with temperature. As reported previously,[12] ln *k* values exhibit a linear relationship with log *P* values for a series of steroids having different hydrophobicities. As is also seen in Figure 5, more hydrophobic PTH−amino acids show higher ln *k* value at higher temperatures, which suggests that stronger hydrophobic interaction is the primary driving force partitioning PTH−amino acids into IBc-modified matrixes. Consequently, it is possible to experimentally determine the optimum separation conditions using thermo-responsive column matrixes based on an appropriate column temperature without modifying mobile phase eluent composition.

In isocratic elution of samples containing solutes with a wide range of polarity, it is sometimes difficult to achieve the desired resolution in a reasonable time. It is effective to use gradient elution where volumes of an organic solvent, composition of mobile phase, or other properties of the solvent (e.g., pH or ionic strength) are changed during the separation.

On HPLC columns packed with temperature-responsive polymer-modified silica, temperature programming is used in lieu of gradient elution. Figure 6 shows typical chromatograms of a mixture of PTH−amino acids for below and above the LCST of the IBc copolymer. The 5 °C peaks shown in Figure 6 are not properly resolved. At 35 °C, the peaks were well-resolved; however, the analytical time was increased too much. To move strongly retained components of the mixture faster, we used a technique of temperature programming. Figure 7 shows chromatograms of a mixture of PTH−amino acids with step gradients by changing column temperature. With a single mobile phase of water and by controlling external temperature, the analytical time was reduced, and the least retained component was well-resolved. With the elevated temperature, the longer the hydrophobic components were retained, the wider their peaks became. In temperature programming (gradient), the resultant peaks are narrow. This was because as the surface property of the stationary phase changed to hydrophilic at decreased temperature, the hydrophobic interaction between component and stationary phase was decreased. Thus, molecules on the tail of the chromatographic

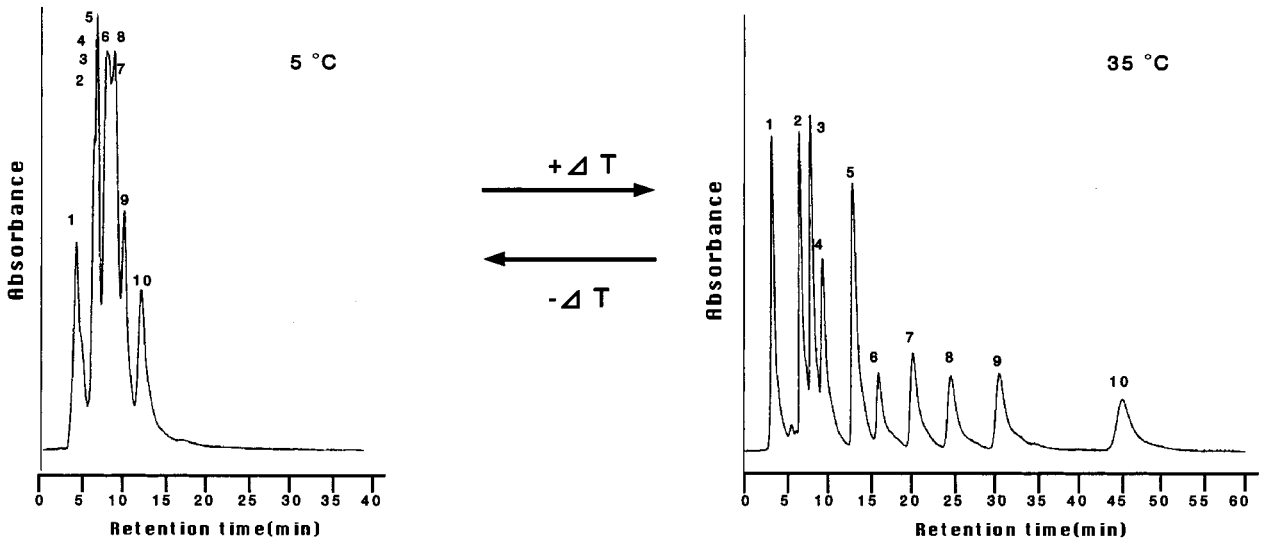

Figure 6. Chromatograms obtained for a mixture of PTH−amino acids using pure water as the mobile phase at 5 and 35 °C on IBc−3.2-modified HPLC columns. HPLC conditions: flow-rate, 1.0 mL/min; monitoring, absorption at 254 nm. Peaks: 1, Cys; 2, Gly; 3, Ala; 4, Pro; 5, Met; 6, Thr; 7, Leu; 8, Phe; 9, Tyr; and 10, Trp.

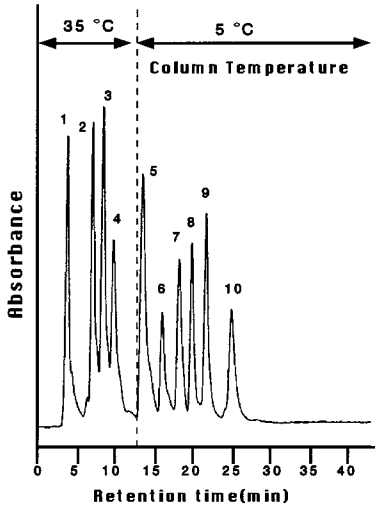

Figure 7. Chromatograms obtained for a mixture of PTH−amino acids using pure water as the mobile phase with a step gradient achieved by changing the column temperature. HPLC conditions: flow-rate, 1.0 mL/min; monitoring, absorption at 254 nm. Peaks: 1, Cys; 2, Gly; 3, Ala; 4, Pro; 5, Met; 6, Thr; 7, Leu; 8, Phe; 9, Tyr; and 10, Trp.

peak will move faster. These features are due to rapid changes in polymer conformation that are attributed to the mobility of grafted polymers.

CONCLUSIONS

We have succeeded in demonstrating reversed-phase HPLC-like separation selectivity of PTH−amino acids by simply changing column temperature and using pure water as the mobile phase. In the proposed chromatographic system, elution of the target substances is controlled isocratically by only a small change in column temperature without any further modification of the aqueous mobile phase by gradient mixtures with organic solvents. The driving force for retention in this system appears to be the hydrophobic interactions between the solute molecules and the hydrophobized polymer chains on the stationary phase surfaces. We can control the LCST of the stationary phase surface modifier by changing the grafted polymer composition and, thus, modulate the hydrophobicity of the stationary phase surfaces. Application of the temperature switch to modulate partitioning is reversible and versatile. Spatially localized temperature gradients along the column length, mixed stationary phases containing two more distinctly different LCST grafted polymers, or kinetically modulated stepwise temperature transients should all produce interesting and useful separation profiles.

Temperature-responsive chromatography should provide highly useful for peptide, protein, and even cell separations, as well as for PTH−amino acids. The ability of the proposed reversible, reusable copolymer-modified stationary phase to separate multiple solutes without the use of an organic solvent is advantageous for retaining solute biological activity, for environmental reasons as a new “green” technology, and for reducing the cost of handling and disposing of mobile phases.

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