New Method for Preparation of Hydrophobic Interaction Chromatographic Stationary Phases Based on Polymer-Grafted Silica and Their Chromatographic Properties

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ABSTRACT: A new "grafting to" method for the preparation of polymer-grafted silica hydrophobic interaction (HIC) packings was used in following steps. Firstly, 3-mercaptopropyltrimethoxysilane was bonded on silica to obtain SH-group modified silica. Secondly, glycidylmethacrylate (GMA) was coated on the surface of SH-silica to obtain polymer-grafted epoxide-silica. Finally, the polymer-grafted epoxide-silica was dispersed in polyethylene glycol of dioxane solution to produce HIC stationary phase. The prepared HIC packings have advantages for biopolymer separation, high column efficiency and good resolution for proteins. The dynamic protein loading capacity of the synthesized packings was 36.0 mg/g. Six proteins were fast separated in less than 12.0 minutes using the synthesized HIC stationary phases. Then the stationary phase was evaluated in detail to determine the effects of pH of mobile phase, concentration of ammonium sulfate and the temperature on the retention of proteins. The thermodynamic parameters of these proteins are consistent with the entropically driven nature of hydrophobic interactions, The HIC column was used for purification of trypsin from a crude extract solution with only one step. The purity of the purified trypsin was more than 95%. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 118: 1513–1519, 2010

Key words: polymer-grafted silica; hydrophobic interaction chromatographic stationary phase; application; thermodynamic parameters

Hydrophobic interaction chromatography (HIC) has been employed extensively for protein separations and purifications since its introduction, as they operate with eluents compatible with bioactive compounds and prevent denaturation of sensitive bioanalytes. These packings are often polysaccharides, polyacrylamides or polyalcohols. However, the pressure resistance of these packings is generally not sufficient for HPLC separations.

TSK gel Phenyl-5PW[1] is the first commercial HPLC support, which is a resin-based weakly hydrophobic support, prepared by introducing phenyl groups into TSK gel G5000PW. The HIC packings based on monodisperse poly(glycidylmethacrylate-ethylenedimethacrylate) beads were used in the separation of biopolymer.[2] But it has weak pressure resistance and swelling for organic solvents. Many attempts have been made to combine the excellent chemical properties of hydrophilic polymers with the outstanding mechanical stability of silica.[3,4] HPLC stationary phase based on polymer-grafted silica has better mechanical strength, better column efficiency, and do not swell in organic eluents. Polyvinylpyrrolidone-grafted silica HIC packings have been employed by Kurganov et al.[5] for separation of proteins and peptides. However, it needs longer time for biopolymer separation.

In this article, we report a new "grafting to" method for the preparation of HIC stationary phase based on polymer-grafted silica. The chromatographic properties of the HIC stationary phase for proteins separation are discussed in detail. Six proteins were fast separated in less than 12.0 min using the synthesized HIC stationary phase. The thermodynamic properties were investigated. The HIC column was also used for purification of trypsin from a crude extract solution. The purity of the purified trypsin was more than 95%.

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EXPERIMENTAL

Instrumentation

All chromatographic tests were carried out using a LC-20A chromatography system (Shimadzu) including two pumps and a multiple-wavelength UV

detector. Samples were injected through a Rtheodyne 7725 valve and detected at 280 nm.

Materials

Macroporous silica purchased from Lanzhou Institute of Chemical Physics (Lanzhou, China), (particle diameter, 6 μm, nominal pore size, 300 Å). Glycidyl methacrylate (GMA) (Aldrich) was distilled under vacuum. Azobisisobutyronitrile (AIBN) was bought from Shanghai Chemical Reagent, (Shanghai, China), polyethylene glycol (PEG, Mr 400) was purchased from Beijing Chemical Reagent, (Beijing, China), 3-mercaptopropyltrimethoxysilane and boron trifluoride ether complex were obtained from Debang Chemical, (Yingcheng, China), Lysozyme (chicken egg white, Lys, Mr 14,000), Ribonucease A (bovine pancreatic, RNase-A,Mr 13,500), Conalbumin (chicken egg white, Con, Mr 77,000), Trypsin, (bovine pancreatic, Mr23300) α-Chymotrypsin (bovine pancreas, α-Chy, Mr 25,000), α-Chymotrypsinogen A (bovine pancreas, α-Chy-A, Mr 25,000), myoglobin (horse skeletal muscle, Myo, Mr 17,000), cytochrome C(horse heart, Cyt-C, Mr12200) and Insulin (bovine pancreas, Ins, Mr 6000) were purchased from Sigma (St. Louis). All other chemical reagents are analysis grade.

Preparation of hydrophobic interaction chromatographic packings

Macroporous silica (3.0 g) were placed in a 250 mL beaker, 50 mL toluene and 2.6 mL 3-mercaptopropyltrimethoxysilane were subsequently added, stirred at 110°C for 24 h under a nitrogen atmosphere. The silica was then filtered, washed with methanol, and dried under vacuum to obtain the SH-group modified silica; 2.0 mL GMA and 0.02 g AIBN were dropped into the suspension of 2.5 g SH-group modified silica in 50 mL toluene under reflux for 24 h to obtain the GMA-grafted silica. 2.0 g of the GMA-grafted silica was then placed in a 250 mL beaker, and 50 mL 1,4-dioxane, 4.0 g polyethylene glycol were subsequently added and the mixture was stirred at 80°C for 3.0 h. Thus, the designed HIC stationary phase was obtained.

Determination of epoxy group

The polymer-grafted epoxide-silica was dispersed in 0.1mol/L tetraethyl ammonium bromide in acetic acid solution and titrated with 0.1mol/L perchloric acid solution until the crystal violet indicator changed to blue-green. The amount of epoxy group was calculated according to the following equation:[6]

$$\text{The amount of epoxy (mmol/g)} = \frac{(V_1 - V_0)C}{W} \quad (1)$$

V_1: the volume of consumed perchloric acid in titration; V_0: the volume of consumed perchloric acid in the blank experiment; C: the concentration of perchloric acid; W: the weight of the sample.

Determination of SH group

The SH-silica was dispersed in methanol including silver nitrate solution and titrated with ammonium thiocyanate until ammonium ferric sulfate indicator changed to red, then titrated with silver nitrate solution until the red disappeared, finally, titrated with ammonium thiocyanate until the indicator changed to red again. The amount of SH group was calculated according to the following equation;[7]

$$SH\,(\text{mmol/g}) = \frac{\mathrm{V_1C_1 - V_2C_2}}{\mathrm{W}} \quad (2)$$

V_1: the volume of silver nitrate; C_1: the concentration of silver nitrate; V_2: the volume of consumed ammonium thiocyanate in titration; C_2: the concentration of ammonium thiocyanate; W: the weight of the sample.

Chromatographic conditions

Ammonium sulfate (3.0 mol/L) in 50 mmol/L phosphate buffer (pH 7.0) was used as A solvent in gradient elution. Phosphate buffer (50 mmol/L, pH 7.0) was B solvent. All mobile phases were previously degassed by aspirator vacuum before use. Column eluents were monitored at 280 nm, and a flow rate of 1.0 mL/min was used in all experiments.

Purification of trypsin by the HIC column

Fresh pig heart tissue (1.0 kg) was grinded away, then was disrupted with 400 mL water and 1.0 mol/L sulfuric acid (pH 4.0), and then was stirred for 2 h at room temperature. Supernatant was adjusted to pH 7.0 with 1.0 mol/L ammonia. After incubation at 4°C for 24 h, the supernatant of trypsin was obtained by centrifugation at 20,000 rpm, then the crude extract was obtained by filtering the supernatant.[8] The crude trypsin was purified by the synthesized HIC column with a single-step, and the purified of trypsin was assayed by sodium dodecylsulfate -polyacrylamide gel eletrophoresis (SDS-PAGE). The purity of the purified trypsin was determined by a dual-wavelength flying-spot scanner.

RESULTS AND DISCUSSION

Preparation of HIC packings based on polymer-grafted silica

Usually, the polymer-grafted silica for HPLC stationary phase is prepared by introducing the vinyl-

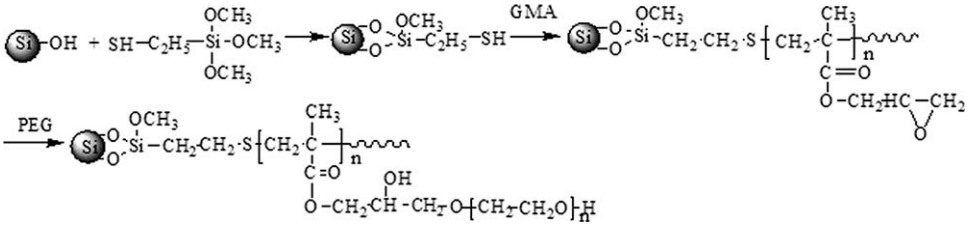

Figure 1 Synthetic route for preparation of the HIC packings.

group on silica, and then the monomer is polymerized with vinyl-silica. However, the polymeric grafting yield is low. Figure 1 shows the chemical modification scheme for the preparation of the new HIC packings, it includes the following three steps. The first step involves a reaction between silica and 3-mercaptopropyltrimethoxysilane to produce the SH-group modified silica, the amount of SH groups were determined to be 0.66 mmol/g. The second step is a reaction of SH-group modified silica and GMA to obtain GMA-grafted silica. The epoxy groups were determined to be 0.41 mmol/g, and the amount of SH groups was reduced to be 0.11 mmol/g. These results prove that the GMA was really bound to the surface of the silica. The third step is a reaction of GMA-grafted silica and polyethylene glycol to obtain the designed HIC stationary phase. After this step reaction, the epoxy groups were decreased to be only 0.12 mmol/g, it can be calculated that 0.29 mmol/g epoxy groups of the GMA-grafted silica reacted with polyethylene glycol, so the amount of PEG (Mr 400) in the HIC packings is 116 mg/g.

Table I shows The FTIR spectrum data of the SH-silica, GMA-grafted silica, and HIC stationary phase. Comparable with that of the SH-silica, the IR spectra of GMA-grafted silica and HIC packings exhibited clear adsorption peak at 1727–1788 cm^{-1} and 2845–3000 cm^{-1} corresponding to the —C=O and $-CH_2$, and from the data of elemental analysis in the Table II, we know that the polymeric grafting efficiency on the silica is 26.7%. Thermogravimetry (TG) measurements were carried out for the SH modified silica, GMA-grafted silica and the final HIC packings. The absolute weight losses of SH modified silica, GMA-grafted silica and HIC packings are about 6.4, 21.5, and 32.9%, respectively. So the amount of PEG in the HIC stationary phase is 114 mg/g, and grafting efficiency is 26.5%, which are basically consistent with the calculated results.

Separation of proteins by the HIC column

To test the resolution characteristics of the synthesized HIC column, experiment was performed to resolve proteins with differences in their hydrophobicity. The protein mixture consisting of Cyt-C, RNase-A, Lys, Trypsin, α-Chy-A, and Ins was separated on the column. Figure 2 shows the chromatogram for the separation of six proteins on the HIC column. The elution order of proteins correlates with their hydrophobic nature (the order of their hydrophobicity: Cyt-C < RNase-A3 < Lys < Trypsin < α-Chy-A < Ins). This also proves that synthesized polymer-grafted silica is HIC stationary phase. The synthesized HIC packings can separate Ins with strong hydrophobicity, which was not reported in the literature,[9,10] and the six proteins were separated within 12 min on the HIC column, which is shorter than that reported in the literature.[11] These results show that the packings have better hydrophobicity performance. Such a high resolution is comparable to that of silica-base packing materials for protein separations.[12]

Dynamic protein loading capacity

Dynamic protein loading capacity on HIC column (5.0 × 0.46 cm I.D.) was determined by measuring the amount of protein loaded onto the column. Solution with 50 mmol/L phosphate in 1.5 mol/L ammonium sulfate containing 2.0 mg/mL lysozyme was chromatographed at a flow rate of 1.0 mL/min. The dynamic protein loading capacity was 36 mg/g, which was higher than those of TSK gel and monolithic column[10,13]

TABLE I
FTIR Spectrum Data for Synthesized the HIC Stationary Phase

Sample	v/cm^{-1}	Possible structure	v/cm^{-1}	Possible structure	v/cm^{-1}	Possible structure
SH-Silica	2569–2592	—SH			2845–3000	$-CH_2$
GMA-grafted silica			1727–1788	—C=O	2845–3000	$-CH_2$
HIC packings			1727–1788	—C=O	2845–3000	$-CH_2$

TABLE II
Results of Element Analysis for the HIC Stationary Phase

Sample	N %	C %	H %
Silica-SH	0.03	3.67	1.05
GMA-grafted silica	0.028	5.19	1.06
HIC packings	0.03	5.65	1.08

Effect of mobile phase pH on protein retention

As shown in Figure 3, mobile phase pH has an important effect on protein retention on the HIC column. The retention times of proteins except for Cyt-C, Lys, and Rnase-A decrease gradually with increasing pH in the range of 5.0–7.0. The retention times of six proteins increase gradually with increasing pH in the range of 7.0–9.0. This is because the change of mobile phase pH brings about the change of electrostatic charge on proteins surface, so that the hydrophobicity of proteins changes, which is shown by retention time. In the HIC, pH 7.0 is usually chosen as the best separation condition to maintain the biological activity of proteins.

Effect of concentration of ammonium sulfate on the protein retention

The effect of salt concentration in the eluent on the retention of proteins was investigated by using ammonium sulfate. As shown in Figure 4, increasing the concentration of ammonium sulfate from 1.5 to 3.0 mol/L, the retention times of six proteins are found to increase gradually. The results indicate that proteins can be desorbed from the surface of the stationary phase in gradient elution mode.The cause is the competing effects of electrostatic and hydrophobic interactions. In HIC, the retention time should be proportional to the molal surface tension increment of the salt. Salts increasing the surface tension of the solvent will favor the reduction in surface area that accompanies adsorption, resulting in increasing retention times.[14–17] So the most convenient and

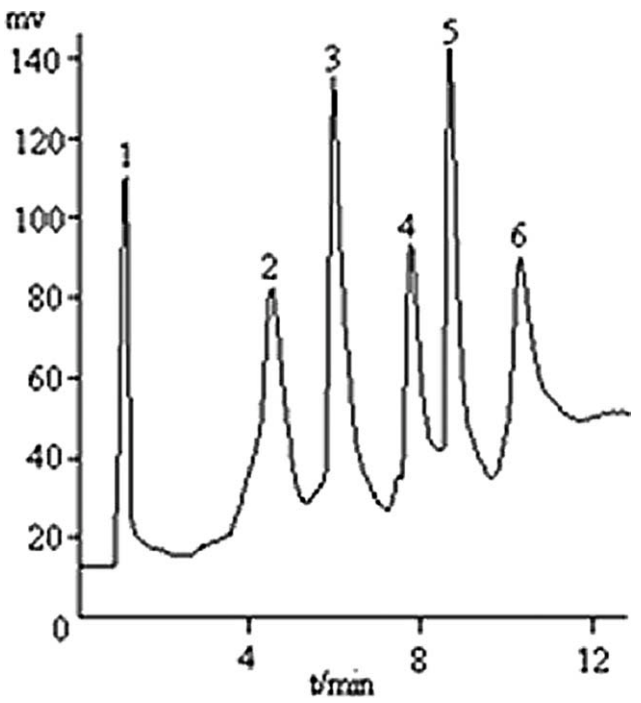

Figure 2 Chromatogram of standard proteins separated by the HIC column (5 × 0.46 cm I.D.) The linear gradient elution was from 100% solution A (50 mmol/L of phosphate + 3.0 mol/L ammonium sulfate, pH 7.0) to 100% solution B (50 mmol/L of phosphate, pH 7.0) at a flow rate of 1.0 mL/min for 15 min with a delay for 5 min. AUFS, 1.00, UV detection at 280 nm. Proteins: 1, solvent + cyt-c; 2, RNase-A; 3, Lys; 4, Trypsin 5, α-Chy-A; 6, Ins.

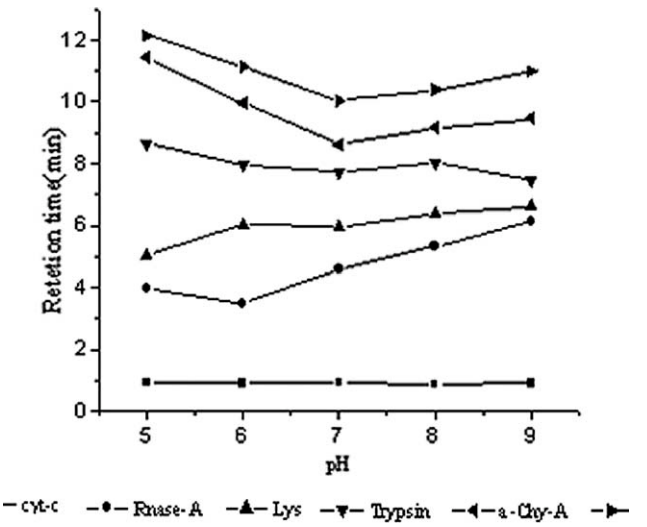

Figure 3 Effect of pH of mobile phase on the protein retention Expect pH of mobile phase being (5.0–9.0), other conditions are the same as that indicated in Figure 2.

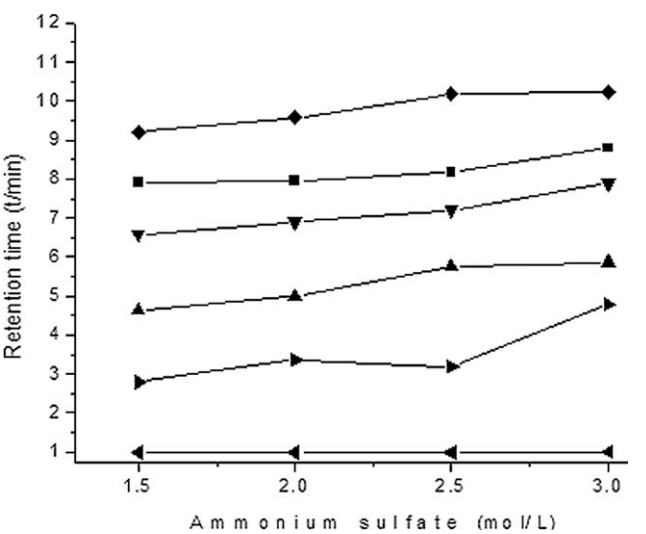

Figure 4 Effect of concentration of ammonium sulfate on the protein retention. Expect concentration of ammonium sulfate being (1.5–3.0), other conditions are the same as that indicated in Figure 2. -◀- Cyt-C, -▶- RNase, -▲- Lys, -▼- 3 Trypsin, -■- α-chy-A, -◆- Ins.

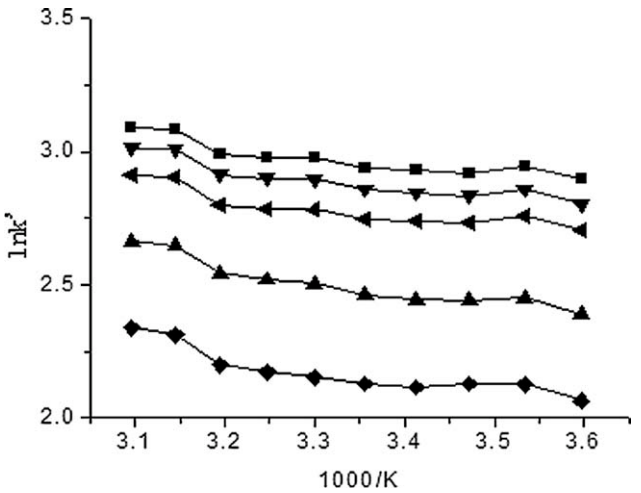

Figure 5 Effect of temperature on the protein retention, Expect temperature being (5–50°C), other conditions are the same as that indicated in Figure 2. -◆- cyt-c -▲- Myo -◀- Con -▼- α-chy -■- α-chy-A.

common elution method in HIC is to reduce ion intensity.

Effect of temperature on the protein retention and calculate the thermodynamic parameters

The retention of the five proteins was measured over the temperature range of 5–50 at 5°C interval with the same concentration of $(NH_4)_2SO_4$ in the mobile phase. Figure 5 shows the Van't Hoff plots of proteins to be nonlinear, indicating maximum retention. From Figure 5, it is apparent that higher the temperature, longer the retention time. This is because the structural change of protein molecules caused by increasing temperature makes the amino acid residues exposed on the surface of proteins, resulting in strengthening the hydrophobicity interaction between proteins and stationary phase.

The thermodynamic quantities associated with the chromatographic process were extracted from the temperature dependence of the retention by using the quadratic equations as follow:[18,19] $\ln k' = a + b/T + c/T^2 + \ln\varphi$ (1), Where a, b, and c are constants, φ is the phase ratio, its value is 1 in this article. By fitting eq. (1) to the experimental data, these four parameters can be evaluated and used to calculate the enthalpy (ΔH°), entropy (ΔS°), heat capacity ($\Delta C^\circ p$) and free energy (ΔG°) changes via the following expressions:

$$\Delta C^\circ \mathrm{p} = 2Rc/T^2 \quad (3)$$

$$\Delta H^\circ = -R(b + 2c/T) \quad (4)$$

$$\Delta S^\circ = R(a - c/T^2) \quad (5)$$

$$\Delta G^\circ = \Delta H - T\Delta S^\circ \quad (6)$$

TABLE III
Thermodynamic Parameters Associated the Retention of Five Proteins Under Different Temperatures in HIC

Proteins	$(NH_4)_2SO_4$ (mol/L)	Temperature (K)	ΔH° (KJ/mol)	ΔS° (J/K/mol)	$\Delta C^\circ p$ (kJ/K/mol)	ΔG° (kJ/mol)
Cyt-C	3.00	283	3.92	31.25	0.025	−4.92
		293	4.16	32.08	0.023	−5.24
		303	4.38	32.82	0.021	−5.56
		313	4.59	33.49	0.02	−5.89
		323	4.78	34.11	0.019	−6.24
Myo	3.00	283	3.63	32.94	0.092	−5.69
		293	4.52	36.05	0.086	−6.04
		303	5.36	38.86	0.081	−6.41
		313	6.14	41.41	0.076	−6.82
		323	6.88	43.72	0.071	−7.24
Con	3.00	283	1.69	27.01	0.151	−5.95
		293	3.14	32.06	0.141	−6.25
		303	4.50	36.62	0.131	−6.6
		313	5.77	40.75	0.123	−6.98
		323	6.97	44.51	0.116	−7.41
α-chy	3.00	283	2.25	31.49	0.25	−6.66
		293	4.67	39.8	0.23	−7.01
		303	6.93	47.45	0.22	−7.44
		313	9.04	54.31	0.20	−7.96
		323	11.02	60.54	0.19	−8.53
α-chy-A	3.00	283	1.43	29.71	0.157	−6.98
		293	2.94	34.97	0.146	−7.31
		303	4.35	39.72	0.137	−7.69
		313	5.68	44.02	0.128	−8.10
		323	6.92	47.92	0.12	−8.56

The thermodynamic parameters obtained by eqs. (3)–(6) are illustrated in Table III for five proteins in the same mobile phase.

As shown in Table III, both Δ° and ΔH° are positive in the temperature range from 283 to 323 K, increase with increasing temperature. ΔG° are negative, decrease with increasing temperature. $\Delta C^\circ p$ are positive, decrease with increasing temperature. This demonstrates that proteins retention in HIC is entropy-driven. The phenomenon can be explained in HIC, as the protein mixture flows through the HIC column hydrophobic patches on the surfaces of the proteins contact the hydrophobic ligands on the packings. The total wetted area decreases, and water is released. Water molecules in contact with hydrophobic areas, unlike those near charged or polar surfaces or in solution with other water molecules, do not experience a highly favorable enthalpy of interaction. Thus the liberation of water from hydrophobic regions increases the entropy of the system without significantly changing its enthalpy, causing the free energy to decrease on adsorption.

Purification of trypsin

The crude trypsin was injected directly on to the HIC column for further separation and purification. Figure 6 shows the chromatogram obtained. In Figure 7, SDS-PAGE analysis shows one main band of purified trypsin from the crude solution; the purity of the purified trypsin is more than 95% after only a 10 min single-step separation by the HIC column.

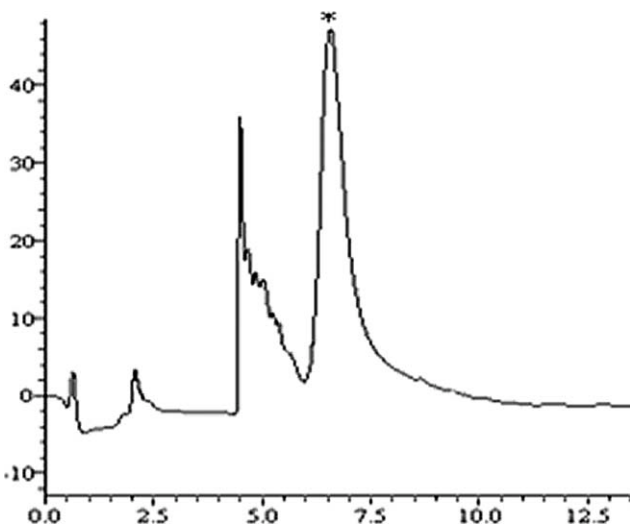

Figure 6 Chromatogram for purification of trypsin by the HIC column,the conditions are the same as that indicated in Figure 2. * trypsin.

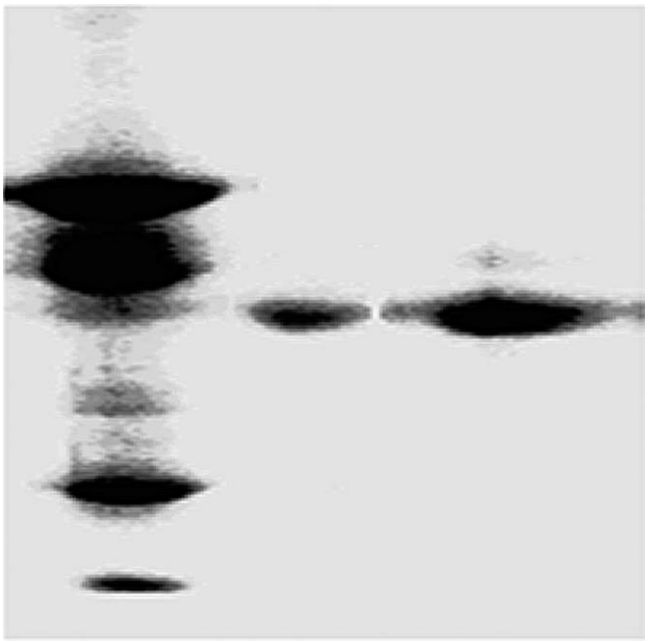

Figure 7 SDS-PAGE analysis of the purified trypsin on the HIC column 1. The crude trypsin 2. The standard trypsin 3. The purified trypsin.

CONCLUSION

We applied a new "grafting to" method to prepare a kind of hydrophobic interaction packings which was based on the macro porous silica. It was found to have a hydrophobic interaction chromatographic retention mechanism. Six proteins were separated within 12.0 min using the synthesized HIC column that is shorter than that of other HIC packings. The highest dynamic protein loading capacity of the synthesized HIC packing for Lys was 36.0 mg/g. The thermodynamic parameters of these proteins determined by using nonlinear Van't Hoff equation illuminates that the retention of proteins in HIC is driven by entropy, The HIC column was also used for purification of trypsin from a crude extract solution with only one step. The purity of the purified trypsin was more than 95%.

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