Side Chain Functionality Dominated the Chromatography of N-Protected Amino Acid on Molecularly Imprinted Polymer

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ABSTRACT: Molecularly imprinted polymer (MIP) with N^{α}-protected amino acid as the print molecule was prepared and used as the stationary phase for the chromatographic study of molecular recognition. Particles of MIP were prepared by photopolymerization of 4-vinylpyridine in the presence of *tert*-butyloxycarbonyl-L-tyrosine (Boc-L-Tyr) and packed into a column for the chromatographic resolution of Boc-L-Tyr and *tert*-butyloxycarbonyl-L-phenylalanine (Boc-L-Phe). These two N^{α}-protected amino acids that differ from each other in the side chain with one hydroxyl group on the benzene ring could be well separated on the MIP. A separation factor of about two was achieved by using a mixture of acetonitrile (99.5 v/v %) and acetic acid (0.5 v/v %) as the mobile phase. Results suggest that the interaction between hydroxyl group in the side chain of amino acid and pyridine in the polymer dominated the selective adsorption of print molecule on the MIP. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 105: 3519–3524, 2007

Key words: molecular imprinting; molecular recognition; high performance liquid chromatography; adsorption; templates

INTRODUCTION

Molecularly imprinted polymers (MIPs) have been proven useful as the separation materials because of their highly selective recognition properties. The technique for preparing MIPs through casting the molecule of interest (print molecule, or referred as template) to create recognition sites is molecular imprinting, also referred as template polymerization. The applications of MIPs for separation media in liquid chromatography and capillary electrophoresis,[1–3] catalysis,[4,5] mimic antibody,[6,7] drug assay,[8] and chiral separation[9,10] have been extensively reviewed. When application, the MIPs allow us to pick up the print molecule in the solution through the molecular recognition based on shape, much like a lock and key. To test the selective recognition properties resulting from molecular imprinting, many N^{α}- or C^{α}-protected amino acid[11–14] and short peptides[15–17] have been used as templates to prepare MIPs. The protection at either N or C terminus is intended to avoid the electrostatic interaction between the terminal charged group and the opposed charged group on the monomer. The recognition of molecule based on the complementary structure can be elucidated. Also, the protected group could increase the solubility of print molecule in organic solvent for the casting by template polymerization.

Chiral recognition has been of concern using the MIPs imprinted with protected amino acids. In addition to the shape selectivity,[18] the orientation of functional groups at the recognition sites can primarily contribute to the molecular recognition.[19] Results from the printed N^{α}-protected amino acids suggest that noncharged functional groups (carbamate and amide in the backbone) of the print molecule may interact via hydrogen bonds with positioned carboxyls of the polymer.[20] To further investigate the mechanism of chiral recognition, Sun et al.[14] synthesized MIPs using N^{α}-protected Boc-L-Tyr as the template and employed them as the stationary phase of liquid chromatography. On the basis of the experimental data, Sun et al.[14] proposed a stoichiometric displacement model that can simulate the retention behavior on the MIPs with a space-effect parameter n to characterize the hydrogen-bonding interaction between solutes and MIPs. This article focuses on the influence of the functions in the side chain of protected amino acid on the molecular recognition. N^{α}-protected Boc-L-Tyr was used as the template to prepare selective material for the recognition of Boc-L-Phe and Boc-L-Tyr, which differ from each other in the side chain with one hydroxyl group on the benzene ring. The polymer was tested for selectively recognizing and rebinding the print molecule. In addition to the strong hydrogen-bonding interaction, other weak interac-

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tions, which were involved especially under less polar mobile phase conditions, were investigated. The results could demonstrate how the interaction between side chain functional group of printed amino acid and functional group on the polymer dominated the selectivity.

MATERIALS AND METHODS

Materials

4-Vinylpyridine (99%) and ethylene glycol dimethacrylate (EGDMA, 98%) were obtained from Acros (USA) and Merck (Germany), respectively, and both were used as received. 2,2′-Azobisisobutyonitrile (AIBN) was obtained from TCI (Japan). Acetone and acetonitrile were from Baker (USA); methanol was from Merck (Germany); glacial acid and tetrahydrofuran (THF) were from TEDIA (USA); all solvents were of HPLC grade. Tert-butyloxycarbonyl-L-tyrosine (Boc-L-Tyr) and *tert*-butyloxycarbonyl-L-phenylalanine (Boc-L-Phe) were purchased from Fluka (USA). All chemicals were used without further purification.

Preparation of molecularly printed polymer

The MIP was prepared by bulk polymerization at a low temperature. Briefly, Boc-L-Tyr (0.281 g), 4-vinylpyridine (1.32 mL), EGDMA (11.67 mL), and AIBN (0.118 g) were dissolved in 18 mL of THF in a conical Erlenmeyer flask. After degassing and nitrogen purging for 3 min, the flask was sealed and allowed to polymerize at 4°C for 6 h under UV-irradiation (365 nm, 100 W lamp). Boc-L-Tyr was used as the template, while 4-vinylpyridine was used here as the functional monomer (Fig. 1). EGDMA and AIBN were employed as the crosslinking monomer and free radical initiator, respectively. THF was used here as the solvent and porogen because of its nonpolarity and its ability to dissolve all the polymerization ingredients.[21] After polymerization, THF was removed and the polymerized product in the form of a white solid was dried in a vacuum oven for 12 h at room temperature. The resultant bulk polymer was finally ground and sieved. The fraction of particles having an average size ranging from 25 to 44 μm was collected for packing in chromatographic column. Referenced nonimprinted (blank) polymer was prepared with the same procedure except no print molecule was present.

Column packing and HPLC

MIP particles were suspended in methanol by sonication and then slurry packed into a 15 cm × 0.46 cm I.D. stainless steel column using an air-driven fluid pump with acetone as the solvent. Template molecule was removed from the column by continuously washing with THF-acetic acid (7 : 3, v/v) until a stable baseline was reached. For the HPLC analysis, a 20-μL sample solution was injected and eluted isocratically at a flow rate of 0.5 mL min^{-1}. The effluent was constantly monitored by measuring the absorbance at 258 nm. Acetone was used as the nonretained component for the determination of the void fraction. Capacity factors (k'_1 and k'_2) were calculated according to the standard chromatographic theory as $k'_1 = (t_1 - t_0)/t_0$ and $k'_2 = (t_2 - t_0)/t_0$, where t_1 and t_2 are the retention times of Boc-L-Tyr and Boc-L-Phe, respectively, and t_0 is the retention of the nonretained component. The separation factor (α) was defined as the ratio of two capacity factors k'_1 and k'_2.

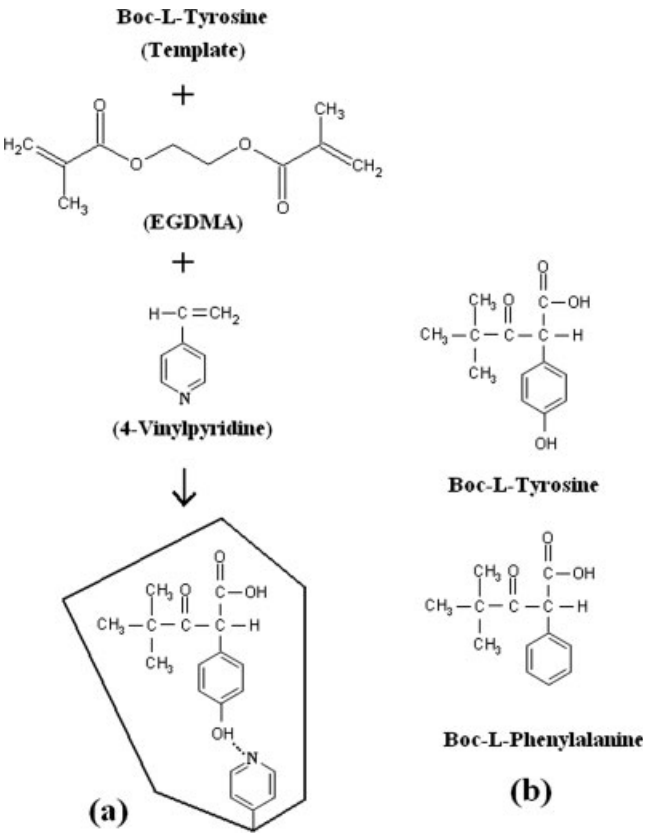

Figure 1 (a) Schematic representation of MIP using Boc-L-Tyr as the template and (b) chemical structures of Boc-L-Tyr and Boc-L-Phe.

RESULTS

Synthesis of MIP

The interactions involving in molecular casting, including hydrogen bonding and electrostatic interaction between the acid and the heteroaromatic base, are subjected to collapse at elevated temperatures. Free radical polymerization was thus carried out at a low temperature using UV-irradiation (365 nm) in this work. On the prepared MIP, 4-vinylpyridine was

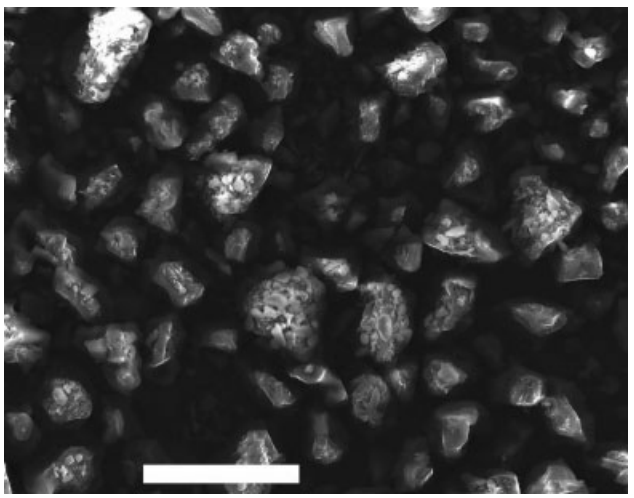

Figure 2 Scanning electron micrographs of the polymers imprinted with Boc-L-Tyr. The bar stands for 40 μm.

incorporated as the host molecule to the template Boc-L-Tyr. Potential interactions for the formation of a complementary shape for template recognition in the chromatographic process included hydrogen bonding between polymerized 4-vinylpyridine and the template molecule (Fig. 1). Although the MIP particles for packing were irregular in shape and had a wide distribution (25–44 μm) (Fig. 2), the 15-cm long column possessed a linear curve of volumetric flow-rate versus pressure. The linearity was proved up to a pressure of $\sim$ 110 kg cm^{-2}, at which the flow-rate was 0.8 mL min^{-1}.

Resolution of Boc-L-Phe and Boc-L-Tyr

In comparison with Boc-L-Phe, the structure of Boc-L-Tyr contains an additional hydroxyl group on the benzene ring (phenol group). Figure 3 shows that individual Boc-L-Tyr and Boc-L-Phe and their mixture could be well recognized and separated using the MIP with Boc-L-Tyr as the template. Results showed that two resembling compounds, which differ from only one hydroxyl group, could be well separated by chromatography on molecularly imprinting polymer. The mixtures of Boc-L-Tyr and Boc-L-Phe with different concentrations were well isolated, as shown in Figure 4. The chromatographic column prepared in this study was effective for the quantitative analysis by the evaluation of peak areas. A linear calibration up to a load of 80 μg of Boc-L-Tyr and Boc-L-Phe in the sample (20 μL) could be obtained (data not shown).

Influence of mobile phase composition on the efficiency of separation

Since the MIP prepared in this work, using a template of simple structure, the mobile phase played a very important role on the resolution of the samples. A mobile phase containing polar substances was used to weaken the binding of target (template) molecules and consequently to release them from the imprinting cavity of the stationary phase. For the chromatography on MIPs prepared by using acrylamide as the functional monomer, the mobile phase containing strong hydrogen-bond competitive solvents such as methanol, alcohol and isopropyl alcohol was useful

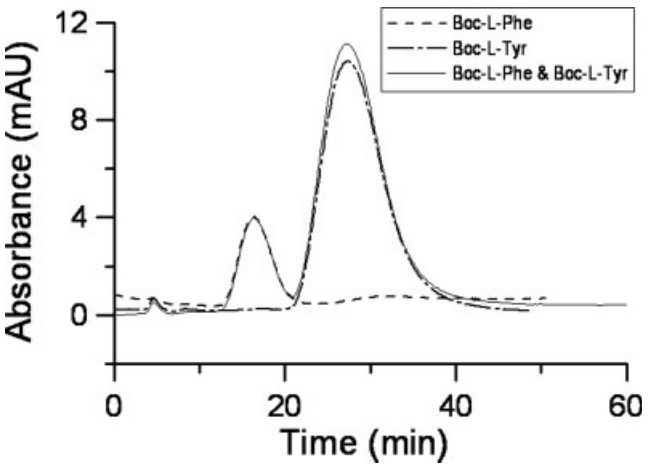

Figure 3 Chromatograms of the samples of Boc-L-Tyr, Boc-L-Phe, and their mixture on Boc-L-Tyr-imprinted polymer. Conditions: sample concentration, 1.0 g L^{-1}; mobile phase, 0.5% of acetic acid, and 99.5% of acetonitrile by volume.

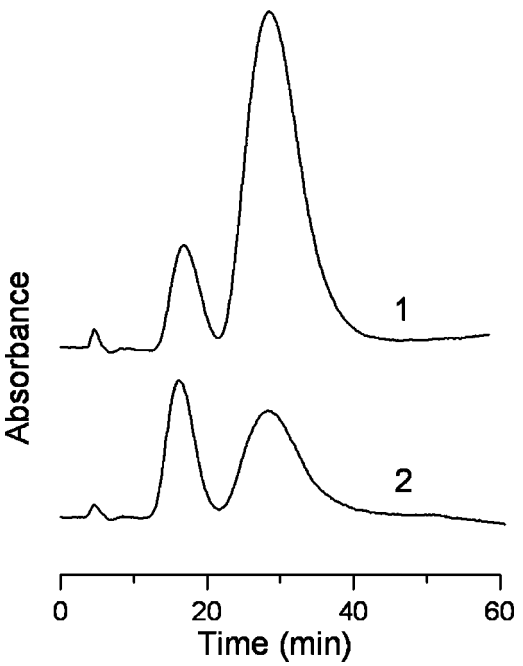

Figure 4 Chromatograms of different concentrations of Boc-L-Phe and Boc-L-Tyr on Boc-L-Tyr-imprinted polymer. Conditions: sample concentration, 0.6 g L^{-1} Boc-L-Phe, and 0.6 (1) or 0.2 (2) g L^{-1} Boc-L-Tyr; others are the same as Figure 2.

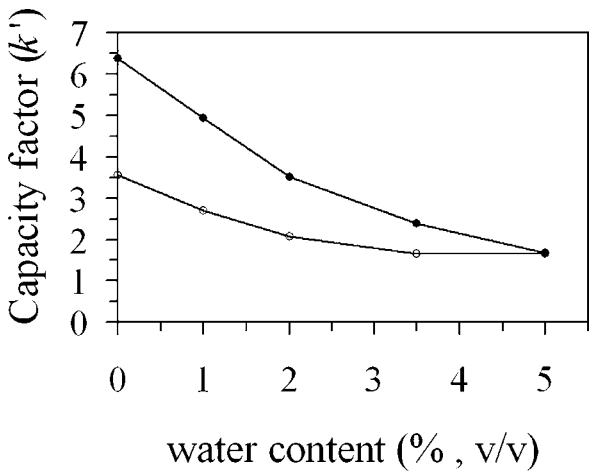

Figure 5 Capacity factors of the imprinted polymer for Boc-L-Tyr and Boc-L-Phe versus water content (v/v) in the mobile phase. Each sample contains 0.2 g L^{-1} of Boc-L-Tyr (●) and Boc-L-Phe (○). The mobile phase also includes acetonitrile and 1 v/v % acetic acid.

for diminishing template adsorption by the binding of strong solvents to the cavity sites.[14] In this work, the mobile phase consisted of deionized water, acetic acid, and acetonitrile. As the acetic acid content in the mobile phase was kept at 1 v/v %, Figures 5 and 6 indicate that the capacity factors for Boc-L-Tyr and Boc-L-Phe decreased with increasing water composition ranging from 0 to 5 v/v %; so did the separation factor. The capacity factor of Boc-L-Tyr decreased from 6.4 to 2.4, and from 3.6 to 1.6 for Boc-L-Phe when the mobile phase of water content was increased from 0 to 3.5%. Also, the separation factor decreased from 1.8 to 1.5 with increasing water composition ranging from 0 to 3.5%. The separation factor was even down to 1 when 5% was employed. In the presence of 5% water, both Boc-L-Tyr and Boc-L-Phe were eluted in a single peak by any mobile phase selected from several combinations of acetic acid and acetonitrile concentrations.

The nonimprinted polymer was also packed into a column (25 cm × 0.46 cm I.D.) for the blank test. As the nonimprinted polymer was employed for the resolution of Boc-L-Tyr and Boc-L-Phe, the retention factors for these two compounds were much smaller than that when they were chromatographied on MIP column, even though the column packed with nonprinted polymer was much longer. When the chromatography was on the column of nonprinted polymer, the separation factor due to reversed-phase effect varied slightly from 1.3 to 1.4 as the water content increasing from 0 to 1%, and then went down gradually to 1.1 as the water content increasing up to 5%.

After a series of tests, the composition of the mobile phase to achieve the best resolution was found to be acetonitrile–acetic acid (99.5 : 0.5, v/v). Using this mobile phase, the resolution of Boc-L-Tyr and Boc-L-Phe was nearly unchanged by the flow rate. As the flow rate increased from 0.5 to 1 mL min^{-1}, the value of separation factor remained unchanged at two. The results (as shown in Fig. 7) indicate that using a higher concentration of acetic acid in the mobile phase could significantly reduce the capacity factor of template molecule. Furthermore, when the mobile phase contained 0.5% acetic acid, a larger separation factor and separation efficiency were obtained, as shown in Figure 8. To investigate the role that acetic acid played in the recognition and binding of template molecule to MIP, chromatographic runs were carried out using different acetic acid levels in the mobile phase without water. The capacity factor of Boc-L-Tyr changed from 16.4 to 3.6, and from 8.4 to 2.1 for Boc-L-Phe when the mobile phase of acetic acid content was increased from 0.1 to 2 v/v %, as shown in Figure 7. The separation factor increased slightly from 1.9 to 2.0 as acetic acid composition increasing from 0.1 to 0.5%, and went down to 1.7 as the acetic acid content was 2%. When the nonimprinted polymer was employed for the resolution of Boc-L-Tyr and Boc-L-Phe, the separation factor varied from 1.7 to 1.0 as the acetic acid content increasing from 0.1 to 2%.

DISCUSSION

The retention of Boc-L-Tyr and Boc-L-Phe on the column packed with MIP was mainly based on the contribution of 4-vinylpyridine functionality on the stationary phase. In addition to the recognition by shape complementary, the interaction between heteroaromatic bases (pyridine) and the functional groups

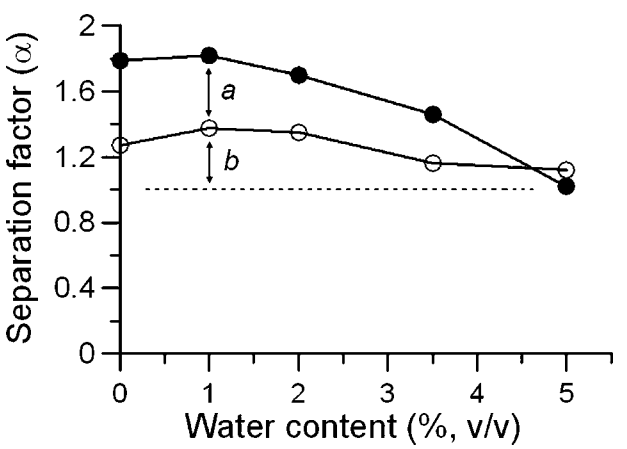

Figure 6 Influence of water content (v/v) in the mobile phase on the separation factor of Boc-L-Tyr and Boc-L-Phe chromatographied on imprinted (●) and nonprinted (○) polymers. Conditions are the same as for Figure 5. Keys *a* and *b* indicate the contributions of molecular recognition and other interaction.

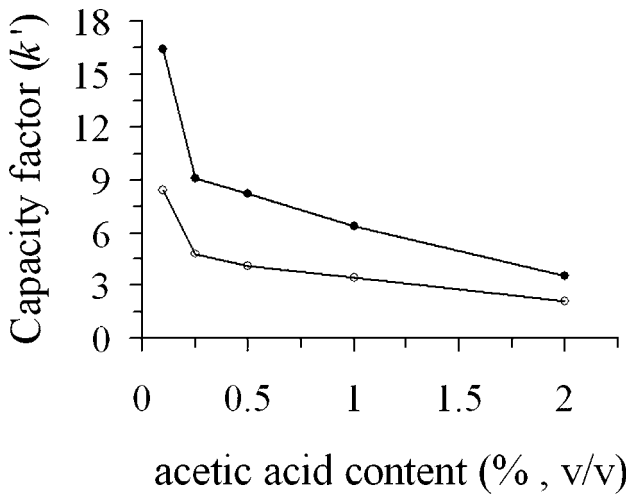

Figure 7 Capacity factors of the imprinted polymer for Boc-L-Tyr and Boc-L-Phe versus acetic acid content (v/v) in the mobile phase. Each sample contains 0.2 g L^{-1} of Boc-L-Tyr (●) and Boc-L-Phe (○). The mobile phase also includes acetonitrile.

in the template were thus the most important factors for the recognition of the template molecule by MIP. The structure of Boc-L-Tyr contains an additional hydroxyl group on the benzene ring in comparison with Boc-L-Phe. This means that in addition to the interaction between pyridine and carboxyl group on the *N*-protected amino acid, there was a hydrogen bond between phenol in Boc-L-Tyr and the functional group of 4-vinylpyridine. This hydrogen bond was believed to play a dominated role in molecular recognition. The conclusion was supported by the well separation of Boc-L-Phe and Boc-L-Tyr in the mixture with different combinations of concentrations on the MIP. In the case of using methacrylic acid as the functional monomer for molecular imprinting of protected amino acid, N^{α}-protecting group and the carbamate and amide functions of the backbone of amino acid were of great importance.[20] In this work, however, these functionalities in the amino acid, which could result in weak interactions, were relatively less important. Our retention peak of print molecule was found to be much narrow than chromatography of print molecule on MIP prepared from the polymerization of methacrylic acid.

As shown in Figure 3, the adsorption and desorption of print and nonprint molecules, Boc-L-Tyr and Boc-L-Phe, were nearly independent. The chromatogram of the mixture of Boc-L-Tyr and Boc-L-Phe was almost identical to the summation of two separate chromatograms of these compounds, suggesting that the competition of Boc-L-Phe for the imprinted sites was very weak in comparison with the print molecule Boc-L-Tyr at the mobile phase conditions of acetic acid:acetonitrile = 0.05 : 0.95 (v/v). Referred to Figure 8, molecular recognition (marked by key *a*) was dominated at these elution conditions. The well resolution of print and onprint molecules proved that the MIP was an ideal separation media for quantitative analysis.

The experimental results (Figs. 5 and 7) showed that a small amount of either acetic acid or water in mobile phase would weaken the interaction between the protected amino acid and the polymer, therefore, the retention of the protected amino acid was lowered to a large extent. The water in the mobile phase was considered to weaken or eliminate the noncovalent interactions (hydrogen bond, Van der Waals force, etc.) and played the very negative role for the retention of both Boc-L-Tyr and Boc-L-Phe. However, the reduction in the strength of noncovalent interactions between print molecule and MIP was less than that for nonprint molecule at low water content (up to 1%), so that the separation factor between Boc-L-Tyr and Boc-L-Phe remained as 1.8 (Fig. 6). The resolution of these two compounds vanished at 5% of water. Figure 6 also indicates that reversed-phase effect (key *b* in the figure) contributed almost the same as the true molecular recognition (key *a*) for the resolution of these two N^{α}-protected amino acids under poor water conditions. Since the Boc-L-Phe is more hydrophobic than Boc-L-Tyr, the later was more retentive than the former in the reversed-phase mode.

The hydrogen bonding between the phenol of Boc-L-Tyr and the pyridine of imprinted polymer was shown to be strong enough to dominate the recognition. The polar acetic acid played the same role as water to weaken this hydrogen bond. However, the presence of acetic acid was necessary for the resolution of these two N^{α}-protected amino acids. If acetonitrile was solely used as the mobile phase, hydrophobic interaction would be dominated for the retention of solutes in MIP. In this instance, the chromatography of these two compounds was likely in the

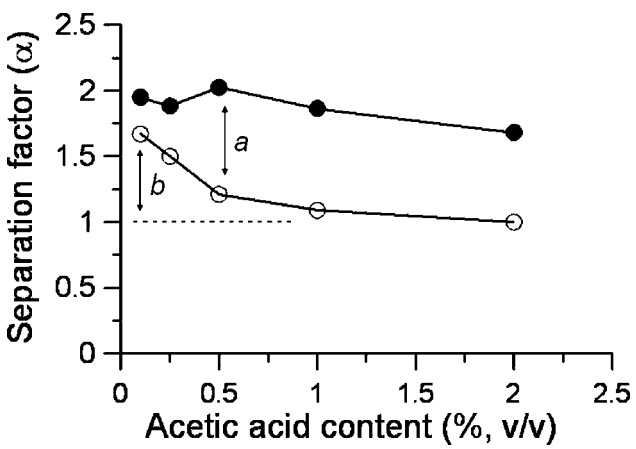

Figure 8 Influence of acetic acid content (v/v) in the mobile phase on the separation factor of Boc-L-Tyr and Boc-L-Phe chromatographied on imprinted (●) and nonprinted (○) polymers. Conditions are the same as for Figure 7. Keys *a* and *b* are the same as in Figure 6.

reversed-phase mode. As shown in Figure 8, the reversed-phase mechanism would be overtaken by increasing acetic acid content to 0.5% (volume ratio). As soon as acetic acid concentration was 0.5 v/v % or higher, the molecular recognition dominated.

CONCLUSIONS

The present article describes the preparation of MIP using Boc-L-Tyr as the template and application of the resultant MIP for the chromatographic study of molecular recognition. Two N^{α}-protected amino acids, Boc-L-Tyr and Boc-L-Phe, which differ from each other in the side chain with one hydroxyl group on the benzene ring, could be well separated on the MIP. In conclusion, the hydrogen bonding between the phenol group of Boc-L-Tyr and the pyridine group of casting monomer 4-vinylpyridine was believed to play the key role in the preparation of MIP as well as in the rebinding of print molecule onto imprinted polymer during the chromatography. The interaction between C terminal carboxyl group and pyridine was less important. The hydrophobicity of the protected amino acids could play a role at less polar mobile phase conditions and functioned as a reversed-phase mode. However, the resolution of Boc-L-Tyr and Boc-L-Phe was clearly dominated by the side chain functionality.

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