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Preparation of hydrophobic interaction chromatographic packings based on monodisperse poly(glycidylmethacrylate-*co*-ethylenedimethacrylate) beads and their application☆

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Abstract

The monodisperse, poly(glycidylmethacrylate-*co*-ethylenedimethacrylate) beads with macroporous in the range of 8.0–12.0 μm were prepared by a single-step swelling and polymerization method. The seed particles prepared by dispersion polymerization exhibited good absorption of the monomer phase. The pore size distribution of the beads was evaluated by gel permeation chromatography and mercury instrusion method. Based on this media, a hydrophobic interaction chromatographic (HIC) stationary phase for HPLC was synthesized by a new chemically modified method. The prepared resin has advantages for biopolymer separation, high column efficiency, low column backpressure, high protein mass recovery and good resolution for proteins. The dynamic protein loading capacity of the synthesized HIC packings was 40.0 mg/ml. Six proteins were fast separated in less than 8.0 min using the synthesized HIC stationary phase. The HIC resin was firstly used for the purification and simultaneous renaturation of recombinant human interferon-γ (rhIFN-γ) in the extract solution containing 7.0 mol/l guanidine hydrochloride with only one step. The purity and specific bioactivity of the purified of rhIFN-γ was found more than 95% and 1.3×10^8 IU/mg, respectively.

Keywords: Stationary phases, LC; Poly(glycidylmethacrylate-*co*-ethylenedimethacrylate); Interferon; Proteins

1. Introduction

Hydrophobic interaction chromatography (HIC) is an important method for separating and purifying proteins. The method is based on the interfacial interaction between biomaterial and hydrophobic ligands which are chemically attached to matrices. Owing to HIC having a weaker interaction than affinity, ion-exchange or reversed-phase chromatography, the structural damage to the proteins is assumed to be minimized, and the biological activity of the proteins is maintained [1,2]. Therefore, HIC has been widely applied separate and purify biopolymers [3]. Silica is usually used as support for preparation of HIC matrix [26]. However, silica-based packings are less stable under high pH conditions, they cannot, sometimes, satisfy the requirement for the separation of biopolymers, this led us to the development of various polymer-based resins in HPLC, such as ion exchanges chromatographic packings [4,5] and metal chelating affinity chromatographic packings [6]. In most cases, polymer-based packings can be employed for biopolymer separations even in the pH range from 1 to 14.

In the search for uniformly sized beads as chromatographic stationary phases, Ugelstad [7] developed a technique named "activated multi-step swelling and polymerization" method. Uniform beads are prepared by Ugelstad's method from a great variety of monomers, such as styrene [8], methylmethacrylate [9], 2-hydroxyethylmethacrylate [10], glycidylmethacrylate [11,12], vinylphenol [13], and chloromethylstyrene [14]. This method is excellent, but it seems rather complex because at least two steps are needed in the swelling process. The first step is the activation of the seed beads by the absorption of a water-insoluble compound. The subsequent step is the absorption of monomer, cross-linker and diluent.

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Ogino et al. [15] reported the preparation of the uniform styrene-*co*-divinylbenzene (PS-DVB) beads by a single-step swelling and polymerization method in 1995, but only the conditions of synthesis and properties of the PS-DVB beads have been reported in some detail by the single-step swelling and polymerization or dynamic swelling method [16–18]. In this paper, we report a preparation of poly(glycidylmethacrylate-*co*-ethylenedimethacrylate) ($P_{GMA/EDMA}$) beads by single-step swelling and polymerization method in the presence of solvents, linear polystyrene as a porogens, and a new method for chemical modification of $P_{GMA/EDMA}$ for the synthesis of HIC stationary phase. The chromatographic properties of the HIC stationary phase for biopolymers separation are discussed in detail. The synthesized HIC resin was firstly used for the purification and simultaneous renaturation of rhIFN-γ from the extract solution. The purity of the purified rhIFN-γ was more than 95%. The specific bioactivity of the purified rhIFN-γ was determined to be 1.3×10^8 IU/mg.

2. Experimental

2.1. Materials

Glycidyl methacrylate (GMA) (Aldrich, USA) was distilled under vacuum. Ethylene dimethacrylate (EDMA) (Aldrich, USA) was extracted three times with 10% aqueous sodium hydroxide and distilled water, and then dried with anhydrous magnesium sulfate. Poly(vinylpyrrolidone, k-30) (PVP, k-30) was purchased from Aldrich (USA). Azobisisobutyronitrile (AIBN), dibutyl phthalate and cyclohexanol were bought from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China). Polyvinyl alcohol (PVA) and sodium dodecyl sulfonate (SDS) were obtained from Beijing Chemical Reagent Co. Ltd. (Beijing, China). Benzyl peroxide (BPO) was obtained from Xi'an Chemical Reagent Co. Ltd. (Xi'an, China). All chemicals were purified by normal methods.

Lysozyme (chicken egg white, Lys), ribonuclease A (bovine pancreatic, RNase-A), myoglobin (horse skeletal muscle, Myo), α-amylase (porcine pancreas, α-Amy), cytochrome C (horse heart, Cyt-C) and Insulin (bovine pancreas, Ins) were purchased from Sigma (St. Louis, USA).

2.2. Instrumentation

All Chromatographic tests were carried out by using a LC-10A chromatograph system (Shimadzu, Japan) including a pump and a multiple-wavelength UV detector. Samples were injected through a Rheodyne 7125 valve and detected at 280 nm.

2.3. Synthesis of polymer seeds

According to the method reported by Pane et al. [19], monodisperse polystyrene seed beads with low molecular weight were prepared by dispersion polymerization of styrene in alcohol media in the presence of the inhibitor, AIBN and the stabilizer, PVP (k-30) under a nitrogen atmosphere. After centrifugal separation, the seed beads obtained were dispersed in an aqueous solution containing 1.0% (w/w) PVA such that the content was 0.1 g/ml. The size of the prepared beads was measured to be 3.2 μm. The conditions for preparing the seed beads are listed in Table 1.

Table 1
Conditions for preparing the seed of polystyrene beads[a]

Substances	Concentrations (%, w/w) [b]	Amount (g)
Styrene	20	5.0
Alcohol	80	20.0
PVP, k-30	2	0.5

[a] Polymerization temperature, 70 °C; polymerization time, 24 h; initiator, AIBN; 0.2 g (4%, w/w, based on styrene).
[b] Based on total recipe (25 g).

2.4. Preparation of uniform porous beads

The monodisperse polystyrene seed beads were swollen by emulsifying mixture of glycidyl methacrylate, ethylene dimethacrylate, benzyl peroxide and diluents in an aqueous solution containing PVA and SDS at room temperature. After the monomer mixture was completely absorbed by the seed beads, the temperature was kept at 70 °C for 24 h. The beads obtained were washed with water and methanol. The porogens were removed by extraction with toluene. The beads were washed with methanol again and dried in air.

2.5. Modification of the beads for hydrophobic interaction chromatographic media

Firstly, the epoxide groups of the $P_{GMA/EDMA}$ beads I were completely hydrolyzed and became diol beads II. Secondly, with the reaction of the hydroxyl group of diol beads II and epichlorohydrin, beads III containing 2.3 mmol/g epoxide groups were obtained. Thirdly, the beads III were then reacted with benzyl alcohol under catalyzed by boron trifluoride-ethyl complex. Thus, the designed HIC HPLC stationary phase is obtained (beads IV). Fig. 1 shows the chemical modification scheme for the preparation of the new HIC packings in this paper. The "P" in the scheme donotes the polymer frame.

2.6. Characterization of polymeric beads

The particle size and surface morphology, and the specific surface area and pore distribution of the synthesized $P_{GMA/EDMA}$ resins were measured by scanning electron microscopy and the mercury intrusion method, respectively. The pore structure of the resins was also characterized by gel permeation chromatography (GPC). The $P_{GMA/EDMA}$ beads were packed in a stainless steel column by the slurry method

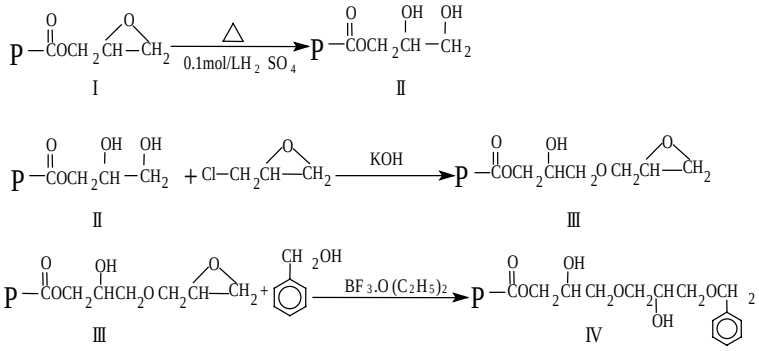

Fig. 1. Chemical modification scheme for preparation of the HIC packings.

with ethanol. The experiments of GPC were carried out in the HPLC instrument with tetrahydrofuran (THF) as eluent and polystyrenes as standard molecular weight and detection at 254 nm at room temperature.

2.7. Determination of epoxy groups

The $P_{GMA/EDMA}$ beads were dispersed in 0.1 mol/l tetraethylammonium bromide in acetic acid solution and titrated with 0.1 mol/l perchloric acid solution until the crystal violet indicator changed to blue-green.

2.8. Purification and simultaneous renaturation of rhIFN-γ by HIC resin

The inclusion bodies of rhIFN-γ were disrupted with buffer consisting of 20 mmol/l phosphate + 1 mmol/l EDTA + 0.2 mg/ml lysozyme (pH = 7.4), then the inclusion bodies were washed three times. Finally, the inclusion bodies were dissolved in 7.0 mol/l guanidine hydrochloride (Gu.HCl) solution. After incubation at 4 °C for 24 h with full agitation, the supernatant of rhIFN-γ was obtained by centrifuging it at 20000 rpm. A 5.0 × 0.8 cm i.d. HIC column was used to purification and simultaneous renaturation of rhIFN-γ in the extract solution.

The bioactivity assay for rhIFN-γ was done by inhibitor with WISH cell and VSV virus [20,21].

3. Results and discussion

3.1. Preparation of monodisperse porous beads

Because of a good miscibility of both solvents of cyclohexanol and dibutyl phthalate with the monomers, the mixture of the two solvents was chosen as the porogen diluents for the preparation of $P_{GMA/EDMA}$ resins in this study. In order to increase the content of the epoxide groups in the polymer which are necessarily for the subsequent chemical modification, a high percentage of GMA must be used. The ratios of monomer to porogen (35/65, v/v) and GMA to EDMA (60/40, v/v) were selected. Such proportions not only provide the resins with macroporous and high mechanic intensity, which is required for protein separation by HPLC, but also provide the resin with a quite high content of epoxide groups.

The size of the final beads was well controlled by the seed diameter and the existing amount of organic phase. As organic phase consisting of GMA, EDMA and diluents is fully adsorbed by the seeds in an effective swelling range, the final particle diameter could be calculated according to the following simple equation [22]:

$$\log D = \log d + \tfrac{1}{3} \log \frac{M+m}{m} \quad (1)$$

where d and D are the diameters of the seeds and the final beads, respectively, M and m are the amounts of organic phase and the seeds, respectively; the value of $(M+m)/m$ is the swelling multiple. The experimental results showed that when 3.2 μm of seed beads were used and the swelling multiple was controlled to be in the range of 20–50, a series of monosized $P_{GMA/EDMA}$ resins with particle diameter in the range of 8–12 μm could be obtained.

Fig. 2a and b shows the scanning electron micrographs of the prepared beads, illustrating that the prepared beads in this study are uniform in size and have macroporous structure.

3.2. Physical properties of the beads

The synthesized $P_{GMA/EDMA}$ resin possess stronger hydrophobicity before chemical modification, so their properties can be characterized by GPC method in THF elution system and by using polymer as samples which does not react with epoxy groups. The pore size distribution of the synthesized resin in dry state, which was also measured by a mercury intrusion method. Table 2 shows the properties of the beads. The volumes of porogen diluents used account for about 65% of the total organic phase. It means that the expected porosity and pore volume of the final beads obtained should be approximately 65% and 1.84 ml/g, respectively. The value of the pore volume in Table 2 is very close to the calculated one. It was found that the backpressure of the column packed with the synthesized resin is only about 3.5 MPa at the flow-rate of 4.0 ml/min. This result indicates

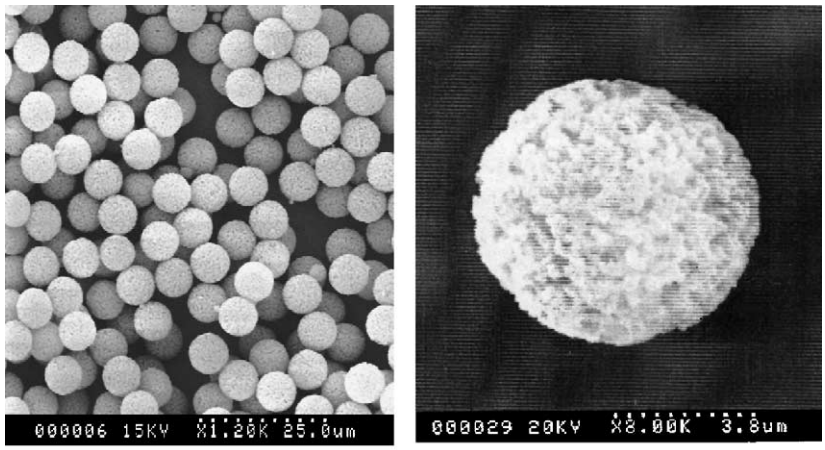

Fig. 2. Scanning electron micrographs of the monosized porous beads (a) and their surface (b).

the column packed with the synthesized beads possess high permeability, which is very favorable to the chromatographic applications under the condition of high flow-rate.

3.3. Chemical modification of $P_{GMA/EDMA}$ beads

Many reactions can be used for the chemical modification of the epoxide groups existed on the surface of the $P_{GMA/EDMA}$ resin. In Fig. 1, the reaction path was designed for the preparation of the HIC packings includes an additional hydrophilization step by completely hydrolyzed epoxide groups of the $P_{GMA/EDMA}$ beads I to obtained diol beads (beads II). This additional hydrophilization step results in a better shielding of the hydrophobic main chains of the polymer thereby preventing from their contact with the protein molecules.

The IR spectrum of the modified polymer exhibited a large broad adsorption peak at 4333 cm^{-1}, corresponding to hydroxyl; adsorption peaks at 1605, 1585, 1500 and 1450 cm^{-1}, corresponding to phenyl group. These results prove that the phenyl groups were really bonded to the $P_{GMA/EDMA}$ beads.

3.4. Dynamic capacity of the HIC packings

Breakthrough curves provide valuable information for the evaluation of the dynamic binding capacity of the separation medium. This is a very important characteristic for large scale separations. Fig. 3 shows three almost identical breakthrough curves that were monitored for lysozyme at flow-rates of 1.0, 1.5 and 2.0 ml/min. The curves are rather steep and do not depend on the flow-rate. The dynamic capacities at 5% breakthrough for lysozyme at flow-rates of 1.0, 1.5 and 2.0 ml/min were 38.2, 37.6, and 37.1 mg/ml, respectively, and the highest dynamic binding capacity of 40.0 mg/ml for lysozyme was found at flow-rates of 1.0 ml/min by a complete adsorption isotherm for a 5.0 × 0.4 cm i.d. HIC column used. The capacity is much higher than those of common rigid polymer beads and monolith column for HIC [23–25].

Table 2
Properties of porous $P_{GMA/EDMA}$ beads

Particle size (μm)	9.0
Epoxide groups (mmol/g)	2.8
Pore volume (ml/g)[a]	1.74
Median pore diameter of GPC (nm)[a]	89.1
Median pore diameter of mercury porosimetry (nm)	110.0
Polystyrene exclusion limit (MW)[a]	1.8×10^6

[a] According to the gel permeation chromatography (GPC).

3.5. Separation of biopolymer by HIC

In order 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, Myo, RNase-A, Lys, α-Amy and Ins was chromatographed on the column. Fig. 4 shows

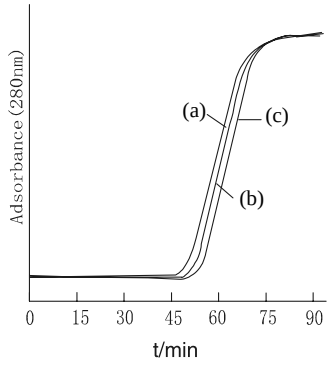

Fig. 3. Breakthrough curves for lysozyme at a flow-rate of: (a) 2.0 ml/min, (b) 1.5 and 1.0 ml/min. Conditions: 5.0 × 0.4 cm i.d. HIC column; mobile phase, 1.5 mol/l ammonium sulfate in 0.05 mmol/l phosphate + 1.0 mg/ml lysozyme.

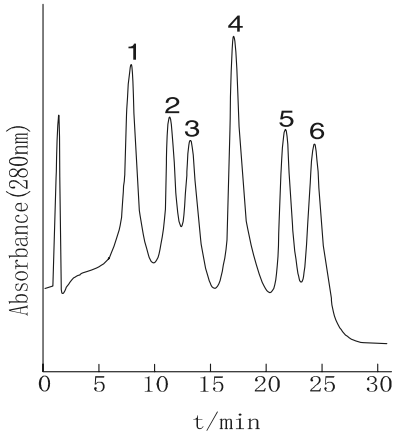

Fig. 4. Chromatogram of standard proteins separated by the HIC column (5 × 0.8 cm i.d.). The linear gradient elution was from 100% solution A (50 mmol/l of phosphate + 3.0 mol/l ammonium sulfate, pH 7.0) to 100% solution B (50 mmol/l of phosphate, pH 7.0) at a flow-rate of 1.0 ml/min for 30 min with a delay for 5 min. AUFS, 0.08, UV detection at 280 nm. Proteins: (1) Cyt-C, (2) Myo, (3) RNase-A, (4) Lys, (5) α-Amy, (6) Ins.

the result the chromatogram of the separation of six proteins on the synthesized HIC column at a flow-rate of 1.0 ml/min. In Fig. 4, elution order of the proteins was according to their hydrophobic nature. This also prove that synthesized resin is HIC stationary phase. Since the breakthrough curves shown did not change with an increase in the flow-rate, the separation can be accelerated using a higher flow-rate of 4.0 ml/min and a steeper mobile phase gradient. This approach shown in Fig. 5 allows the reduction of the separation time to less than 8 min without deterioration in resolution, which demonstrated that the packings can be operated efficiently at high-rates. Such a high resolution is comparable to that of silica-base packing materials for protein separations [26].

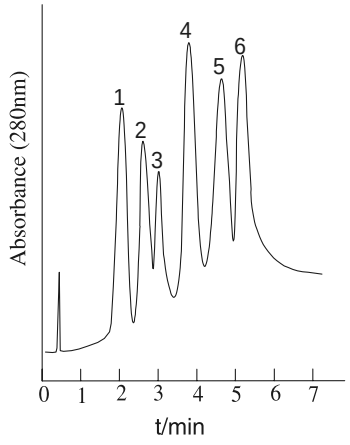

Fig. 5. Chromatogram for a fast separation of standard proteins by the HIC column (5 × 0.8 cm i.d.). Except the linear gradient in 7.0 min and flow-rate of mobile phase being 4.0 ml/min, other conditions are the same as that indicated in Fig. 4.

Table 3
Mass recovery of six proteins by using the synthesized HIC column[a]

Protein	Recovery (%)
Cyt-C	89.8 ± 4.0
Myo	99.1 ± 3.2
RNase-A	97.4 ± 4.1
Lys	99.9 ± 2.7
α-Amy	87.6 ± 2.2
Ins	93.2 ± 2.0

[a] The HIC column (5 × 0.8 cm i.d.). The linear gradient elution was from 100% solution A (50 mmol/l of phosphate + 3.0 M 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 30 min with a delay for 5 min.

3.6. Recovery

High yields in the separation of proteins is an essential requirement for industrial downstream processing. The mass recoveries of six proteins with three continuously individual measurements obtained from the HIC column are listed in Table 3. It is seen that all mass recoveries are greater that 86%. The relative standard deviations of recoveries of six proteins in three parallel tests are all less than ±5%. This result shows that a high mass recovery of proteins by using the HIC column was obtained in this study.

3.7. Effect of loading

Variables such as selectivity and retention factor k' that characterize a separation under non-overload conditions are normally independent of the sample size [27]. Fig. 6 shows the separation of Cyt-C, RNase-A and Lys in an ammonium sulfate gradient. The loading was increased by

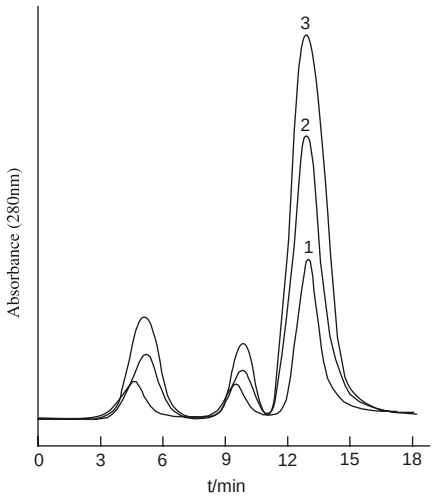

Fig. 6. Effect of column loading on the separation of Cyt-C, RNase-A and α-Amy (order of elution). Except the linear gradient in 18.0 min and flow-rate of mobile phase being 2.0 ml/min, other conditions are the same as that indicated in Fig. 4. Protein loading: (1) 0.40 mg, (2) 1.5 mg, (3) 4.0 mg.

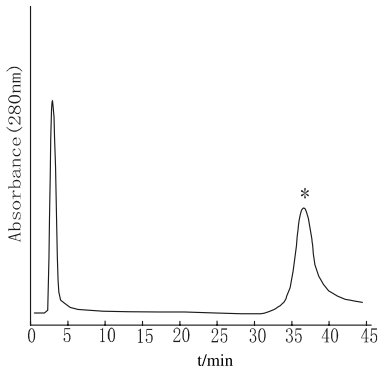

Fig. 7. Chromatogram of the rhIFN-γ extract solution by HIC mode on the prepared HIC column. Except the nonlinear gradient in 45 min, other conditions are the same as that indicated in Fig. 4. (∗) rhIFN-γ.

more than one order of magnitude from 0.4–1.5 to 4.0 mg for each protein. Despite the broad loading range, both retentions times and selectivities are only slightly affected. These almost identical retentions and selectivities document the invariability of the retention characteristics of the HIC column with loadings that span a broad range of protein concentrations.

3.8. Purification and simultaneous renaturation of rhIFN-γ by the HIC column

The rhIFN-γ extract solution was directly injected into the HIC column. Fig. 7 shows the chromatogram of the purification and simultaneous renaturation of the rhIFN-γ extract solution. In Fig. 8 sodium dodecylsulfonate-PAGE analysis shows one main band of purified rhIFN-γ from extract solution and the purity of the purified rhIFN-γ is more than 95% after a single-step purification by the HIC column. The specific bioactivity of the purified rhIFN-γ was determined to be 1.3×10^8 IU/mg.

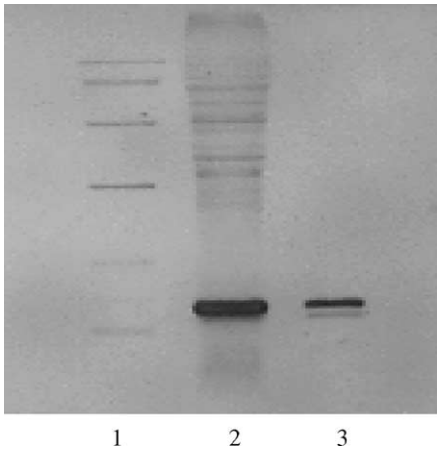

Fig. 8. SDS-PAGE analysis of rhIFN-γ extract solution. (1) Marker (14,400, 20100, 31000, 43000, 66200, 97400 Da). (2) rhIFN-γ extract solution. (3) Purified rhIFN-γ extract solution.

4. Conclusion

The monodisperse poly(glycidylmethacrylate-*co*-ethylenedimethacrylate) resin with macroporous structure was synthesized by a single-step swelling and polymerization method. The physical properties of the resin were measured and discussed in detail. The obtained results show that the beads have the uniformity in particle size, strong particle rigidity and desired macroporousity. Based on this medium, one kind of hydrophobic interaction chromatography resin was synthesized by a new chemically modified method. A better separation, sharpness of the peak shape and fast separation of six biopolymers indicate that the prepared HIC resin in this study is much better than it reported in the literature [23–25]. Because of its better chemically modified method, a high mass recovery of proteins was obtained on the HIC column. The column efficiency obtained by the polymer-based HIC column can be comparable to that of silica-based packing materials [26]. The HIC resin was firstly used for the purification and simultaneous renaturation of rhIFN-γ in the extract solution to obtain a satisfactory result.

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