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High Performance Affinity Chromatography of Antithrombin III Based on Monodisperse Poly (glycidyl methacrylate) Beads

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Abstract: A new approach for the separation of antithrombin III with high performance affinity chromatography (HPAC) was described. A novel monodisperse, non-porous, cross-linked poly (glycidyl methacrylate) beads (PGMA) were used as the affinity support. With the water-soluble carbodiimide, heparin was linked covalently to amino-PGMA-beads, which was prepared by amination of PGMA. The adsorbent obtained exhibits high binding activity to antithrombin III (ATIII), good resolution and excellent mechanical properties and can be used under high flow rate.

Keywords: High performance affinity chromatography, antithrombin III, heparin.

Antithrombin III, a α_2-glycoprotein and one of the major proteinase inhibitors in mammalian plasma, shows specific interaction with heparin. Therefore, immobilized heparin has been widely used as an adsorbent for the separation and purification of plasma components in blood-coagulation systems. Traditionally, affinity chromatography has been carried out at low pressure using soft gels made of polysaccharide[1,2]. Soft gels, however, is flow rate limited, susceptible to microbial degradation, and gives low resolution. A variety of supports like polyacrylamide[3], synthetic polymers[4] and silica gels[5] have been developed to replace soft gel.

In this paper, we described a procedure for the immobilization of heparin on the monodisperse non-porous poly (glycidyl methacrylate) beads (PGMA). Thus obtained medium, heparin - PGMA possesses high binding activity to antithrombin III and can be used under high flow rate.

Preparation of Amino-PGMA

Poly (glycidyl methacrylate) beads are cross-linked copolymer of glycidyl methacrylate and ethylene dimethacrylate. The preparation procedure was described in our previous work[6]. The beads are hydrophilic and monodisperse with 7 μm of diameter. On the surface of beads there are free epoxy groups where several ligands can be immobilized. The concentration of epoxy groups on the surface of the PGMA beads was 1.34 mmol/g resin, which was determined according to the method of Keen[7]. Amino - PGMA was prepared as described by Matsumoto *et al.*[8]. In the reaction vessel, 0.96 g PGMA was suspended in 3 mL of concentrated ammonia solution. The content was then washed

with water after incubated at 40ºC for 5 hours. The concentration of the amino groups introduced on the surface of the PGMA beads was 0.59 mmol/g resin, which was determined using elemental analysis on nitrogen.

Preparation of Heparin-PGMA

Heparin was coupled to amino-PGMA according to the methods suggested by Danishefsky *et al.*[9]. The pathway of coupling reaction is shown in **Figure 1**. In the reaction container, 0.8 g of amino-PGMA beads was suspended in 1 mL of 0.1 mol/L HCl containing 149 mg of heparin. 1 mL of 234 mg/mL EDC solution was added to the mixture under stirring. The suspension was adjusted to pH 4-5 with 0.1 mol/L HCl. The mixture was then allowed to react at room temperature for 40 hours. The heparin-PGMA thus obtained was washed with water in order to remove excess EDC and heparin.

Figure 1 Preparation of heparin-PGMA

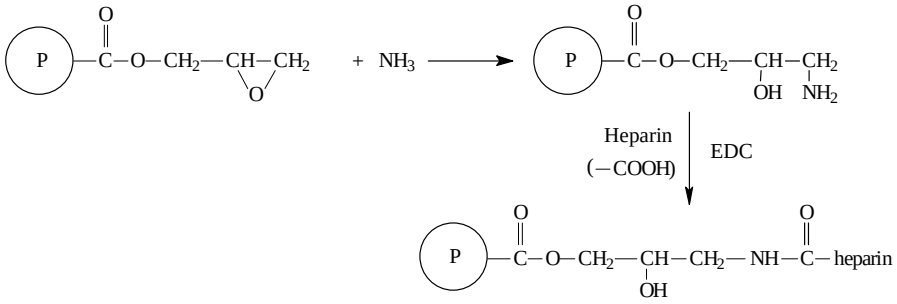
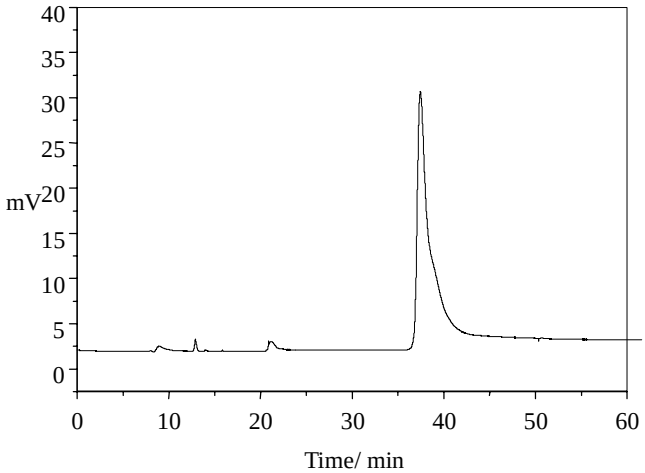


Figure 2 Affinity chromatogram of antithrombin III on heparin-PGMA column



Column: 56×4.0 mm I.D; Sample: 4.5 mg antithrombin III dissolved in 100 μL 0.005 mol/L tris-HCl-0.1 mol/L NaCl, pH = 7.3; Eluent: 0-20 min, 0.005 mol/L tris-HCl-0.1 mol/L NaCl, pH = 7.3; 20-60 min, 0.005mol/L tris-HCl-2.0 mol/L NaCl, pH = 7.3; Flow rate: 0.1 mL/min; Wavelength: 280 nm.

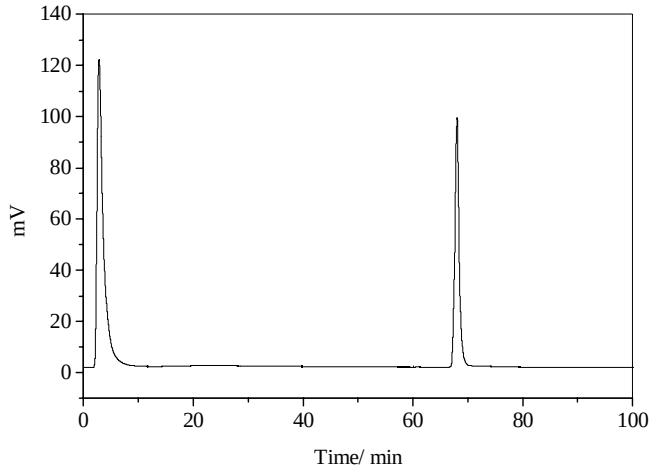
Affinity chromatography of antithrombin III on heparin-PGMA

Approximate 0.8 g heparin-PGMA was packed into a stainless steel column (56×4.0 mm I.D.) under 16 MPa. The sample was 4.5 mg ATIII dissolved in 100 μL 0.005 mol/L tris-HCl-0.1 mol/L NaCl, pH = 7.3. After loading, the column was washed with the same buffer. The bound antithrombin III was then eluted out with tris-HCl buffer containing 2 mol/L NaCl. The chromatogram is shown in **Figure 2**. Heparin-PGMA beads showed high adsorption capacities for antithrombin III. The blank experiments with bare PGMA did not show any binding ability for antithrombin III. Because of the unique monodisperse and mechanical property of the support, the beads can be used under very high flow rate if necessary. At the same time, the column packed with the media displayed lower back pressure, for example, approx 2 MPa at 1.0 mL/min. The non-specific interaction between the support and protein is very weak. The protein recovery was 91% and 94% for antithrombin III and human serum albumin, respectively.

Isolation of ATIII from human plasma with heparin-PGMA column

The starting material for isolation of antithrombin III was human plasma. Donors were screened to ensure the absence of hepatitis B surface antigen and antibody to human immunodeficiency virus. The freezed human plasma was defrozen and centrifuged to remove cryoprecipitate. Vitamin K-dependent coagulation factors were removed by adsorption on DEAE-Sephadex A-50. Then 20 μL of the treated plasma was loaded to the heparin-PGMA column (56×4.0 mm I.D.). Unbound proteins were washed out from the column with 0.005 mol/L tris-HCl buffer (pH 7.3) containing 0.1 mol/L NaCl. The bound antithrombin III was eluted with 0.005 mol/L tris-HCl buffer (pH 7.3) containing 2.0 mol/L NaCl. The result was shown in **Figure 3**.

Figure 3 Affinity chromatogram of human plasma on heparin-PGMA column



Column: 56×4.0 mm I.D; Sample: 20 μL human plasma; Eluent: 0-60 min, 0.005 mol/L tris-

HCl-0.1 mol/L NaCl, pH = 7.3; 60-100 min, 0.005 mol/L tris-HCl-2.0 mol/L NaCl, pH = 7.3; Flow rate: 0.2 mL/min; Wavelength: 280 nm.

Conclusion

Poly (glycidyl methacrylate), PGMA beads were developed as an ideal support for high performance affinity chromatography. Heparin can be immobilized on the surface of PGMA by using EDC. The adsorbent obtained exhibited high flow rate, good resolution and high specific binding activity to antithrombin III and can be used to the preparation and purification of antithrombin III.

Acknowledgments

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