N-Acetyl-D-galactosamine-Specific Lectin Isolation from Soyflour with Poly(HPMA-GMA) Beads

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ABSTRACT: Soybean lectin was purified from seeds of *Glycine max* L.Merrill SA88. Poly(hydroxypropyl methacrylate-glycidyl methacrylate) [poly(HPMA-GMA)] beads were used as an affinity matrix and *N*-acetyl-D-galactosamine (GalNAc) was used as an affinity ligand. Soybean lectin adsorption with GalNAc attached poly(HPMA-GMA) beads from soybean lectin solution (in phosphate buffered saline) was 5.0 mg/g. Maximum adsorption capacity for soybean lectin from the soy flour extract was 26.0 mg/g. Elution of soybean lectin from adsorbent was accomplished by 0.5*M* galactose solution. Purity of soybean lectin was determined by SDS-PAGE. It was observed that soybean lectin could be repeatedly adsorbed and desorbed with GalNAc-attached poly(HPMA-GMA) beads. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 111: 148–154, 2009

Key words: soybean lectin; glycine max; affinity chromatography; polymeric beads

INTRODUCTION

Lectins are multivalent carbohydrate-binding proteins of nonimmune origin that agglutinate cells or precipitate glycoconjugates.[1] They are widely distributed in nature, being found in plants, fungi, viruses, bacteria, insects, and animals.[2] Lectins typically constitute a large fraction of the total protein in most leguminous seeds and plant storage tissues.[3] Although exact physiological functions of most lectins are not completely clear, it is known that most lectins play a crucial role in diverse biological processes, particularly in host defense mechanisms, inflammation, and metastasis, and there is an increasing data suggesting the involvement of lectins in the process of differentiation and development of organisms.[4] Owing to their binding specificities, lectins are employed in numerous biochemical and clinical research areas.[5,6] They are used in the purification of polysaccharides and glycoproteins and in a variety of biological applications including cell separation, mitogenic stimulation of immune cells, identification of blood groups and microorganisms, and monitoring alterations on the surface of normal and abnormal cells.[7–12]

Plant lectins are classified according to their binding affinity for specific carbohydrate residues.[13] *N*-Acetyl galactosamine (GalNAc)-specific lectins are of a great interest, since they have been reported as a detecting agent for tumor associate antigens of malignant cells.[14] Lectins with high affinity to GalNAc have been isolated and characterized from both vertebrates and invertebrates. For the purpose of the anticancer drug production, work on GalNAc-binding lectins has been rapidly increasing. Soybean lectin binds specifically to GalNAc with high affinity and also binds to galactose and its derivatives with less affinity. The lectin is localized in the protein bodies of cotyledon cells in soybean seeds. It is a tetrameric glycoprotein with a molecular weight of 120 kDa and consists of four identical subunits. Each subunit carries an oligosaccharide chain $Man_9(GlcNAc)_2$ and has a molecular weight of 30 kDa.[15]

The potential uses of lectins make it important to obtain them in a pure form. Lectins have been purified by conventional procedures, including salt-induced crystallization, ethanol precipitation, ion exchange chromatography, gel filtration, and affinity chromatography.[16–19] The former methods rely on the physicochemical properties of the proteins for separation, while affinity chromatography depends on the specific interaction between the lectin and a carbohydrate structure attached to an inert matrix that interacts with the corresponding lectin with a high specificity.[20–22]

In the present study, we report the specific isolation of a GalNAc-specific lectin, soybean lectin, from the aqueous solutions and soy flour extract by GalNAc-bound poly(HPMA-GMA) beads that enable ligand coupling through glycidyl methacrylate (GMA) residues without the need for any activation agent.

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EXPERIMENTAL

Materials

Hydroxypropyl methacrylate, ethylene glycol dimethacrylate, glycidyl methacrylate, *N*-acetyl-D-galactosamine (GalNAc) and soybean lectin were all supplied from Sigma Chemical Co (St. Louis, MO). All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany). All water used in the adsorption experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit, with a high-flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion-exchange packed-bed system. The resulting purified water (deionized water) has a specific conductivity of 18.2 mS.

Extraction of protein

Soy flour was obtained from the seeds of *Glycine max* L. Merill SA88 (Çukurova Agricultural Research Institute, Adana, Turkey). Seeds were grounded by a mill, and 15 g of flour was incubated with 100 mL petroleum ether to remove fat. This procedure was repeated thrice, and the resulting flour was filtered and dried. Defatted soy flour was extracted overnight at 4°C with phosphate buffered saline (PBS, pH 7.4). Then the mixture was centrifuged at 7500 rpm for 15 min. Supernatant containing the soluble lectin was collected and subjected to filtration to remove insoluble particles. They were then stored in a refrigerator until use.

Preparation and characterization of poly(HPMA-GMA) beads

Poly(hydroxypropyl methacrylate-glycidyl methacrylate) [poly(HPMA-GMA)] beads were prepared as follows. The stabilizer poly(vinyl alcohol) (60 mg) was dissolved in deionized water (60 mL) for the preparation of a continuous phase. The dispersed phase was prepared by mixing toluene (10 mL), hydroxypropyl methacrylate (HPMA) (6.0 mL), glycidyl methacrylate (GMA) (2.0 mL), and ethylene glycol methacrylate (6.0 mL) in a test tube. Then, benzoyl peroxide (60 mg) was dissolved in this homogeneous solution. The dispersed phase and the continuous medium were mixed within a sealed-glass polymerization reactor (100 mL) placed in a water bath equipped with a thermoregulator. The polymerization reactor sinked in the water bath was heated to 70°C for 6 h, and its temperature was further increased to 90°C for another 2 h to allow the reaction to go to a completion. The polymerization reactor was removed from the water bath and its content was cooled down to the room temperature over time. An extensive washing procedure was applied to remove the diluent and any unreacted monomer from the product. The beads were filtered and resuspended in ethyl alcohol. The new dispersion was stirred for about 2 h at room temperature, and the beads were separated by decanting the liquid part. Poly(HPMA-GMA) beads were washed twice with ethyl alcohol and then four times with deionized water using the same procedure. The beads were kept in 0.02% sodium azide solution at 4°C in the refrigerator to prevent microbial contamination.

The average size and size distribution of the beads were determined by screen analysis using Tyler Standard Sieves. Pore volume and average pore diameter greater than 20 Å were determined by a mercury porosimeter (Carlo Erba model 200) that is capable of 2000 kg/cm^2 pressure. The surface area of the bead sample was determined in Brunauer–Emmett–Teller (BET) isotherm of nitrogen using an ASAP2000 instrument (Micromeritics, USA).

Water uptake ratio of the beads was determined in deionized water. The experiment was conducted as follows: initially dry poly(HPMA-GMA) beads were carefully weighed out and placed in a 50-mL vial containing deionized water. The vial was placed in an isothermal water bath (25°C ± 0.5°C) for 2 h. The beads were then taken out of the vessel, wiped out with a filter paper, and finally weighed out. The weight ratio of dry (W_o) and wet (W_s) beads was recorded. The water content of the beads was calculated by using the following expression:

$$\text{Water uptake ratio } \% = \frac{(W_s - W_o)}{W_o} \times 100 \qquad (1)$$

W_o and W_s are the weights of beads before and after swelling, respectively.

The surface morphology of the poly(HPMA-GMA) beads was examined using scanning electron microscopy (SEM). The polymer sample was initially dried in air at 25°C for 7 days before SEM analyses. A tiny amount of dried beads was mounted on a SEM sample holder and was sputter-coated by gold for 2 min. The sample was then mounted in a SEM (JEOL, JEM 1200EX, Tokyo, Japan), and its surface was scanned on a desired magnification scale to observe the morphology of the beads.

N-acetyl-D-galactosamine attachment on Poly(HPMA-GMA) beads

GalNAc was attached covalently through epoxy groups of GMA. Different amounts of GalNAc (0.5–10 mg/mL) were dissolved in 10 mL of 0.5*M* NaOH in small reaction vessels. Then, 500 mg of beads was added to each vessel. The reaction was conducted for 24 h at room temperature while stirring magnetically at 200 rpm. At the end of this

period, the beads were separated by centrifugation at 3000 rpm for 5 min, and the supernatant was collected to determine the amount of attached GalNAc. The beads were washed with 0.5*M* NaOH solution and distilled water several times and then dried under vacuum at 40°C for 2 days. The amount of attached GalNAc was determined by Somogyi method.[23]

Adsorption studies

Adsorption experiments were carried out in a batch experimental set-up. Effects of GalNAc content and amount of soybean lectin in adsorption medium on adsorption capacity were investigated. At first, 50 μ*M* of Mn^{2+} and Ca^{2+} ions were added to 10 mL of lectin solution, and then it was transferred to reaction vessels including 0.5 g of adsorbent. The mixture was stirred in a rotary mixer at room temperature for 2 h. Soybean lectin adsorption was periodically checked by taking samples from the adsorption media. Protein concentration was determined by Bradford method, and the adsorption capacity was calculated from the difference between the initial and final protein concentration by using the following equation. The same procedure given earlier was followed with soy flour extract.

$$Q = \frac{(C_i - C_f)V}{m} \quad (2)$$

Where C_i and C_f are the initial and final lectin concentrations (mg/mL), respectively, V is the volume of the protein solution (mL) and m is the amount of adsorbent used (g).

Evaluation of lectin purity

Purity of the soybean lectin was assessed using SDS-polyacrylamide gel electrophoresis (PAGE). A native-PAGE analysis was also done to examine the quality of the purified lectin. Samples used for the electrophoretic analyses were the collected and purified soybean lectin, Sigma-grade soybean lectin, and the clarified extract/supernatant obtained after extraction and filtration.

Experiments were performed as follows: A 100 μL amount of each sample was mixed with 100 μL of reducing sample buffer and heated at 100°C for 10 min. Samples were then loaded onto two 4–20% tris-glycine gradient gels, such that each sample was present in both gels. After SDS-PAGE, gels were stained with silver-staining reagents.

RESULTS AND DISCUSSION

Characterization

The suspension polymerization procedure provided poly(HPMA-GMA) beads in the excellent spherical form mostly in the size range of 150–200 μm. According to mercury porosimetry data, the average pore size of the poly(HMPA-GMA) beads was 200 nm. Specific surface area of the beads was found to be 88.6 m^2/g by BET isotherm. The total pore volume was 2.4 mL/g and represented a porosity of more than 45%. These results indicated that the pore volume and pore size were sufficiently large to ensure a modest resistance to the mobile phase.

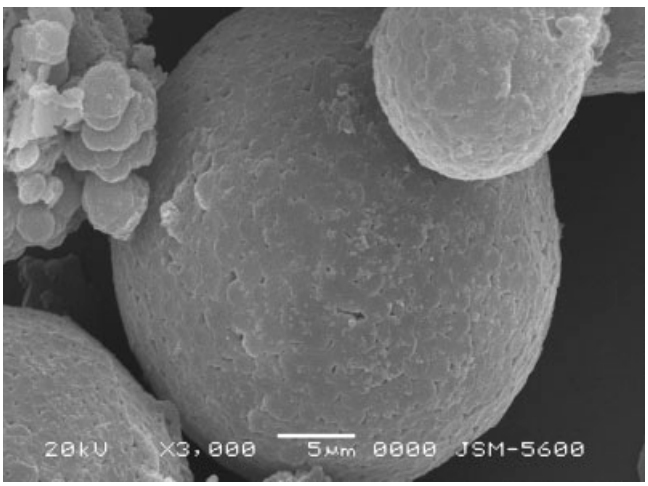

Figure 1 SEM micrograph of poly(HPMA-GMA) gel beads.

Poly(HPMA-GMA) beads are crosslinked hydrogels. They do not dissolve in aqueous media, but do swell, depending on the degree of crosslinking. The equilibrium water uptake ratio of the poly(HPMA-GMA) beads is 75%. The water molecules penetrate into the entanglement polymer chains. It should be also noted that these hydrophilic beads are quite rigid and strong enough because of crosslinked structure; therefore, they are suitable for possible packed-bed applications.

The surface morphology of poly(HPMA-GMA) beads are exemplified by the electron photograph in Figure 1. As clearly seen here, the beads have a spherical form and rough surface because of the pores which formed during the polymerization procedure. The poly(HPMA-GMA) beads have a porous structure in the dry state. The roughness of the surface should be considered as a factor providing an increase in the surface area. In addition, the pores in the polymer structure reduce diffusional resistance and facilitate mass transfer because of high internal surface area. This also provides higher soybean lectin adsorption amount.

GalNAc attachment studies

To determine the optimum amount of GalNAc attached to the beads, the ligand (i.e., GalNAc) concentration was changed between 0.5 and 10 mg/mL.

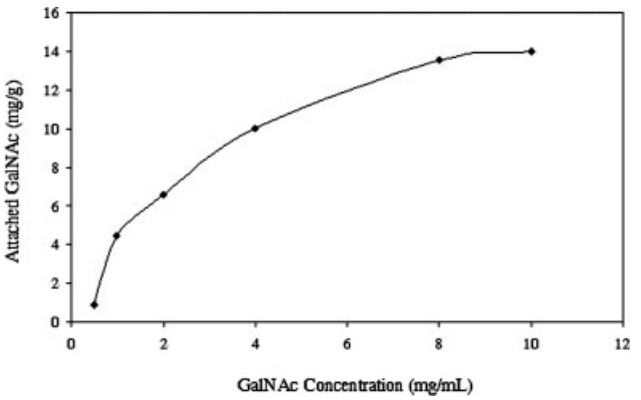

Figure 2 Effect of GalNAc concentration on GalNAc attachment. pH, 10.0; *T*, 20°C.

The results were given in Figure 2. As seen in the figure, with the increase in the GalNAc concentration, the amount of attached GalNAc increased linearly first and reached a plateau level at 8.0 mg/mL GalNAc concentration. This shows that all the binding sites (i.e., GMA groups) were saturated up to the point where steric hindrances allow. Maximum amount of attached GalNAc was found as 14.0 mg/g.

Soybean lectin adsorption studies

Effect of GalNAc content

Figure 3 shows the effects of GalNAc content on soybean lectin adsorption. Poly(HPMA-GMA) beads having different amounts of GalNAc on their surfaces were used in the adsorption experiments. As expected with the increase in ligand loading, soybean lectin adsorption has increased. However, above 4.0 mg/mL initial GalNAc concentration (i.e., 10 mg/g), adsorption amount decreased slightly. When considering the size of soybean lectin molecule (118.6 × 88.9 × 165.9 Å and molecular weight of 120,000 Da),[24] this is an expected situation. Maximum amount of soybean lectin that can be adsorbed onto GalNAc-loaded surface is dependent on the protein size rather than the amount of GalNAc loading. Excess ligand loading on surface is both economically undesirable, and it can cause conformational changes and undesired interactions/repulsions between adsorbed proteins.

Effect of soybean lectin concentration

For these experiments, 10 mg/g ligand containing (4.0 mg/mL GalNAc initial concentration) beads were used. Obtained results are shown in Figure 4. As seen in this figure, soybean lectin adsorption increased with increasing soybean lectin concentration and saturation was achieved at about 0.75 mg/mL soybean lectin initial concentration. Maximum adsorption amount of soybean lectin was 15.1 mg/g. The steep slope in the initial part shows the high affinity between the ligand (i.e., GalNAc) and the protein molecules (i.e., soybean lectin). Soybean lectin adsorption onto naked poly(HPMA-GMA) beads is negligible (0.1 mg/g).

An adsorption isotherm is used to characterize the interactions of each molecule with the adsorbents. This provides a relationship between the concentration of the molecules in the solution and the amount of protein adsorbed on the solid phase when the two phases are at equilibrium. The Langmuir adsorption model assumes that the molecules are adsorbed at a fixed number of well-defined sites, each of which is capable of holding only one molecule. These sites are also assumed to be energetically equivalent and distant from each other, so that there are no interactions between molecules adsorbed on adjacent sites. The Langmuir adsorption isotherm is expressed by eq. (3). The corresponding transformations of the equilibrium data for soybean lectin gave rise to a linear plot, indicating that the Langmuir

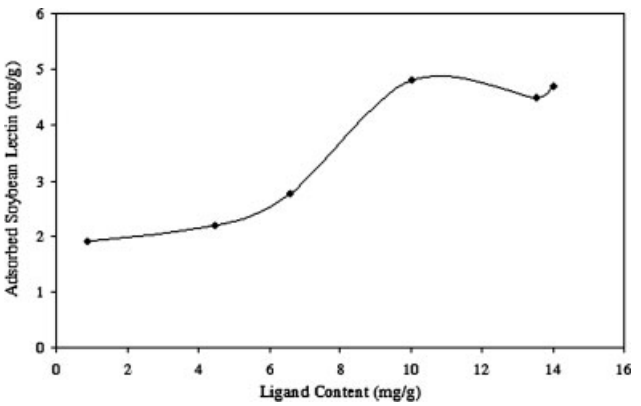

Figure 3 Effect of GalNAc contents on soybean lectin adsorption: Soybean lectin concentration, 0.1 mg/mL; pH, 6.8 (PBS); *T*, 20°C.

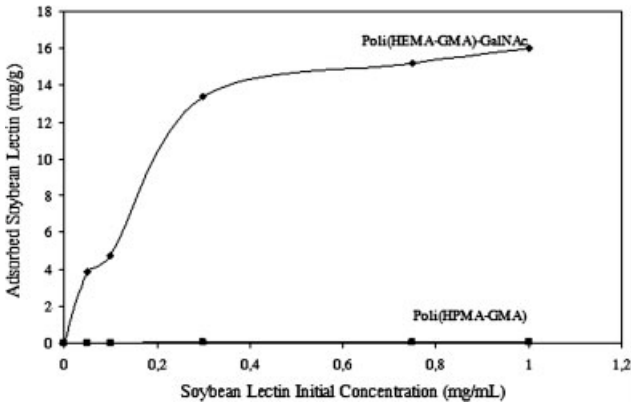

Figure 4 Effect of soybean lectin concentration on soybean lectin adsorption: GalNAc content, 10 mg/g; pH, 6.8 (PBS); *T*, 20°C.

model could be applied in these systems and described by the equation:

$$Q = \frac{Q_{\max} b C_e}{1 + bC_e} \tag{3}$$

where Q is the concentration of adsorbed soybean lectin on the adsorbent (mg/g), C_e is the equilibrium soybean lectin concentration in solution (mg/mL), b is the Langmuir constant (g/mL), and $Q_{\max}$ is the maximum adsorption capacity (mg/g). This equation can be linearized:

$$\frac{1}{Q} = \frac{1}{Q_{\max} b}\frac{1}{C_e} + \frac{1}{Q_{\max}} \tag{4}$$

The plot of $1/C_e$ versus $1/Q$ was employed to generate the intercept of $1/Q_{\max}$ and the slope of $1/Q_{\max}b$.

The maximum adsorption capacity ($Q_{\max}$) data for the adsorption of soybean lectin was obtained from the experimental data. The correlation coefficient (R^2) was 0.9235. The Langmuir adsorption model can be applied in this affinity-adsorbent system. It should also be noted that the maximum adsorption capacity ($Q_{\max}$) and the Langmuir constant were found to be 17.95 mg/g and 5.16 g/mg, respectively.

To quantify the extent of uptake in adsorption kinetics, a pseudofirst-order was used[25]:

$$\frac{dq_t}{dt} = k_1(q_{\text{eq}} - qt) \tag{5}$$

where k_1 is the rate constant of pseudofirst-order adsorption (min^{-1}) and q_{eq} and q_{t} (mg/g) denote the amounts of adsorbed protein at equilibrium and at time t (min), respectively. After integration by applying boundary conditions, $q_{\text{t}} = 0$ at $t = 0$ and $q_{\text{t}} = q_{\text{t}}$ at $t = t$ gives

$$\log\left(\frac{q_{\text{eq}}}{q_{\text{eq}} - q_{\text{t}}}\right) = \frac{k_1 t}{2.303} \tag{6}$$

Equation (6) can be rearranged to obtain a linear form

$$\log(q_{\text{eq}} - q_{\text{t}}) = \log(q_{\text{eq}}) - \frac{k_1 t}{2.303} \tag{7}$$

a plot of log ($q_{\text{eq}} - q_{\text{t}}$) versus t should give a straight line to confirm the applicability of the kinetic model. In a true first-order process log(q_{eq}) should be equal to the interception point of plot of log ($q_{\text{eq}} - q_{\text{t}}$) via t.

In addition, a pseudosecond-order equation based on adsorption equilibrium capacity may be expressed in the form

$$\frac{dq_t}{dt} = k_2(q_{\text{eq}} - q_{\text{t}})^2 \tag{8}$$

TABLE I
The First- and Second-Order Kinetic Constants for Soybean Lectin Adsorption

	Q_{eq} (mg/g)	k	R^2
1	9.32	0.0599	0.9293
2	6.68	4.23×10^{-3}	0.9832

where k^2 (g/mg min) is the rate constant of pseudosecond-order adsorption process. Integrating eq. (8) by applying boundary conditions, $q_t = 0$ at $t = 0$ and $q_t = q_t$ at $t = t$ leads to

$$\frac{1}{(q_{\text{eq}} - q_{\text{t}})} = \frac{1}{q_{\text{eq}}} + k_2 t \tag{9}$$

or equivalently for linear form:

$$\frac{t}{q_{\text{t}}} = \frac{1}{k_2 q_{\text{eq}}^2} + \frac{1}{q_{\text{eq}}} t \tag{10}$$

A plot of t/q_t versus t should give a linear relationship for the applicability of second-order kinetics. The rate constant (k^2) and adsorption at equilibrium (q_{eq}) can be obtained from the intercept and slope, respectively.

According to the values in Table I, the optimum results are for both the second- and first-order models, with the second-order mechanism R^2 values being the highest. These results suggest that the pseudosecond-order mechanism is predominant and that chemisorption might be the rate-limiting step that controls the adsorption process. The rate-controlling mechanism may vary during the course of the biosorption process, and three possible mechanisms may be occurring.[26] There is an external surface mass transfer or film-diffusion process that controls the early stages of the biosorption process. This may be followed by a reaction or constant rate stage and finally by a diffusion stage where the biosorption process slows down considerably.[27]

Soybean lectin purification from soy flour

Table II shows soybean lectin purification from soy flour extract by GalNAc-attached poly(HPMA-GMA) beads. As seen from the table, soybean lectin adsorption from the extract (diluted with PBS, pH 6.8) is higher than the adsorption from aqueous protein solution. This behavior can be explained as the protein concentration in the extract is much higher, and soybean lectin molecules are in the proper conformation to interact with the attached ligand molecules (i.e., GalNAc) in their native medium.

The purity of the soybean lectin purified from the extract was determined with SDS-PAGE. Figure 5

shows the gel stained by silver nitrate. The purified soybean lectin was compared with standard protein markers. The bands numbered by 1 and 5 represents the bovine serum albumin (67,000 Da) and ovalbumin (45,000 Da), respectively. Band 3 is pure soybean lectin obtained from Sigma. Eluted fractions are in the wells 2 and 4. Purified soybean lectin samples displayed in an expected position on the gel because of the soybean lectin consists of four subunits, molecular weight of each 30,000 Da. Further, the single band confirms the affinity of soybean lectin molecules to GalNAc ligand and the success of purification procedure.

Desorption and reusability

In the last step of the purification procedure, the main concern was to desorb the adsorbed protein in the shortest time and at the highest amount possible. It was thus necessary to evaluate the regeneration efficiency of the affinity adsorbents after each cycle. Elution of soybean lectin from GalNAc attached poly(HPMA-GMA) beads was also carried out in batch system, using 0.5*M* galactose. Soybean lectin adsorbed beads were placed within the elution media, and the amount of soybean lectin desorbed in 2 h was determined. More than 98% of the adsorbed soybean lectin molecules were eluted easily from the affinity beads in 2 h. With the elution data obtained, we concluded that galactose is a suitable elution agent. To show the reusability of the GalNAc-attached poly(HPMA-GMA) beads, the adsorption–elution cycle was repeated four times using the same beads. There was no significant loss in the adsorption capacity of the beads. Moreover, no obvious changes of the morphology of the beads were found in the recycling process when examined visually. These results demonstrated the suitability of the present solid support as an affinity adsorbent for soybean lectin purification from aqueous solutions and soy extracts.

CONCLUSIONS

Biospecific affinity chromatography plays a unique role in the purification of proteins. Lectins are important proteins that are employed in a wide range of applications. Their one-step purification with high purity in a shortest and cheapest way by the use of their specific sugars as a ligand is therefore advantageous. In this study, we obtained soybean lectin from soy flour with high purity and high yield (about 90% recovery from the crude extract). It can be concluded that our GalNAc-bound poly(HPMA-GMA) beads were efficient tools for soybean lectin purification from the soy flour, and it can be used many times without noticeable loss in the adsorption capacity.

TABLE II
Soybean Lectin Purification from Soy Extract

	Adsorption amount (mg/g)	Recovery (%)
Extract (undiluted)	26.01	90
1/2 dilution	8.28	77
1/4 dilution	3.06	57
1/6 dilution	1.36	38
1/10 dilution	0.06	3

Figure 5 Eight percent SDS-PAGE image. (1) Bovine serum albumin, BSA (67,000 Da), (2) purified lectin, (3) pure lectin (4), concentrated lectin, and (5) ovalbumin (45,000 Da). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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