PHEMA Based Cryogel For Lectin Purification From Soybean Flour

Soya Unundan Lektin Saflaştırılması için PHEMA Bazlı Kriyojel

Research Article

Işık Perçin*, Erol Aksöz
Department of Biology, Moleculer Biology Division, Hacettepe University, Ankara, Turkey

ABSTRACT

In this study poly(hydroxyethyl methacrylate) (PHEMA) based cryogel column was prepared and its usability for lectin purification from soybean flour was investigated. PHEMA cryogel was characterized by swelling test and scanning electron microscope (SEM). N-acetyl-D-galactosamine (GalNAc) was immobilized to PHEMA cryogel as a ligand by cyanogen bromide activation. Soybean flour extract obtained from soybean seeds was used as source of lectin. Maximum lectin binding from soybean flour was 261 mg/g. Lectin elution from the N-acetyl-D-galactosamine immobilized PHEMA (GalNAc-PHEMA) cryogel was achieved by 0.5 M galactose. Purity of the lectin obtained from soybean flour was investigated by SDS-PAGE. Finally binding-elution cycle was performed for 5 times and no significant decrease was observed at column capacity.

Key Words
PHEMA, Cryogel, Lectins, Protein Purification, Soybean Flour.

ÖZET

Bu çalışmada poli(hidroksietilmetakrilat) (PHEMA) temelli kriyojel kolon hazırlanmış ve soya unundan lektin saflaştırılmasında kullanılabilirliği araştırılmıştır. PHEMA kriyojel taramalı elektron mikroskobu (SEM) ve şişme testi ile karakterize edilmiştir. PHEMA kriyojele ligand olarak N-asetil-D-galaktozamin siyanojen bromür (CNBr) aktivasyonu ile bağlanmıştır. Soya tohumlarından elde edilen soya unu özütü lektin kaynağı olarak kullanılmıştır. Soya unundan en fazla lektin bağlama miktarı 261 mg/g'dır. N-asetil-D-galaktozamin immobilize edilmiş PHEMA kriyojelden lektin elusyonu 0.5 M galaktoz ile gerçekleştirilmiştir. Soya unundan elde edilen lektinin saflığı SDS-PAGE ile araştırılmıştır. Son olarak, bağlanma ve elusyon döngüsü 5 kez tekrarlanmış ve kolon kapasitesinde önemli bir azalma gözlenmemiştir.

Anahtar Kelimeler
PHEMA, Kriyojel, Lektinler, Protein Saflaştırma, Soya Unu.

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Correspondence to: Işık Perçin, Department of Biology, Moleculer Biology Division, Hacettepe University, Ankara, Turkey
Tel: +90 312 297 80 12 Fax: +90 312 299 20 28 E-Mail: ipercin@hacettepe.edu.tr

INTRODUCTION

Lectins are a group of glycoproteins found in many organisms from bacteria to animals. They specifically bind sugar chains, so they are valuable tools in biochemistry [1-3]. Carbohydrate moiety of cancer cells may be different from normal cells. So lectins can be used for identification of glycoproteins on the surface of cells and for diagnosis of cancer [4]. Furthermore, immobilized lectins are very useful tools for purification of glycoconjugates, enzymes, immunoglobulins and cells [5,6].

Plants especially legume seeds are an important source of lectins [7]. Some methods such as ethanol precipitation, gel filtration and ion-exchange chromatography have been used for purification of lectins [8,9]. But affinity chromatography is the most effective technique to purify lectins in a pure form. There are many affinity chromatography studies aim to purify lectins using sugars as ligands. Lectin-sugar specifity presents an advantage for purification of lectins from complex seed crude extract [10-12].

Cryogels used in this study are new generation polymeric systems. They have interconnected macropores enabling viscous medias such as blood and seed extracts to flow easily through cryogel [13]. As a consequence, cryogels are advantageous for lectin purification from seed extracts. This study presented here describes a method for lectin purification from soybean flour using GalNAc-PHEMA cryogel.

EXPERIMENTAL

Materials

N-acetyl-D-galactosamine,N,N′-methylene-bis(acrylamide) (MBAAm) and ammonium persulfate (APS) were supplied from Sigma Chemical Co. (St. Louis, MO USA). N,N,N′,N′-tetramethylene diamine (TEMED) and hydroxyethyl methacrylate (HEMA) were obtained from Fluka A.G. (Buchs, Switzerland). Cyanogen bromide (CNBr) was purchased from Aldrich (Munich, Germany). All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany). All water used in the binding 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

Soybean seeds were obtained from Çukurova Agricultural Research Institute, Adana, Turkey. Soybean seeds were crushed and soybean flour was obtained. Then soybean flour treated with petroleum ether to remove fat and stirred with %0.9 NaCl overnight. The obtained solution centrifuged at 7500 rpm for 15 min. Supernatant was collected and labeled as soybean crude extract. Crude extract was divided into equal parts and stored at -20°C until use.

Preparation of PHEMA Cryogel

The preparation of PHEMA cryogel described previously by our group [14]. In summary, 1.3 ml HEMA and 0.283 g MBAAm were dissolved in 5 ml deionised water in a beaker which is placed in an ice bath. Then free radical polymerization was initiated by adding TEMED (25 μl) and APS (20 mg) into reaction mixture. The mixture immediately allocated into syringes (volume: 5ml; internal diameter: 0.8 cm) closed at the bottom. The polymerization mixture in the syringe was frozen at -16°C for 24 h. After polymerization period, cryogel was thawed at room temperature and washed with water. PHEMA cryogel was stored in distilled water containing 0.02% sodium azide at 4 °C until use.

Characterization of PHEMA Cryogel

Characterization of the PHEMA cryogel was achieved as reported before by our group [15]. The swelling degree of the cryogel (S) was calculated as in equation 1 and determined as follows: firstly, the cryogel was washed with water and excess water on its surface was removed. Then it was transferred to a pre-weighed vial and weighed (mwet gel). After drying to constant mass in the oven at 55°C, the mass of dried sample was determined (mdry gel).

$$S = (m_{wet\ gel} - m_{dry\ gel}) / m_{dry\ gel} \quad (1)$$

The morphology of a cross-section of the cryogel was examined by SEM. After the cryogel was freeze dried, it was coated with gold-palladium (40:60) and examined using a JEOL JSM 5600 SEM.

CNBr Activation

Firstly, 0.5 M sodium carbonat buffer (pH 10.5) was pumped to the PHEMA cryogel for 1 h. Then 50 mg/ml CNBr solution was circulated through the PHEMA cryogel for 3 h. The pH of this solution was kept stable in a range of 10.5-11.5 during activation procedure. After the activation reaction, the PHEMA cryogel was washed with 0.5 M NaCl solution to remove excess of CNBr. In the end, the PHEMA cryogel was washed with sodium citrate buffer (pH 6.5). All the activation procedures were achieved at room temperature.

N-Acetyl-D-Galactosamine Immobilization on PHEMA Cryogel

GalNAc was dissolved in sodium citrate buffer at a final concentration of 4 mg/ml. Then this solution was pumped through the PHEMA cryogel overnight at 1 ml/min flow rate. After this process, the GalNAc-PHEMA cryogel was washed with distilled water to remove excess of ligand. The amount of immobilized ligand was determined by Dinitrosalicylic acid (DNS) method [16].

Lectin purification from soybean flour

Lectin purification from soybean flour was achieved in a recirculating system including a peristaltic pump for a continuous flow. Firstly, GalNAc-PHEMA cryogel was equilibrated with pH 7 phosphate buffer. Crude extract was thawed at room temperature and diluted with 0.9% NaCl in different dilution ratios. After dilution process, 1 mM Ca^{+2} and 1 mM Mn^{+2} ions were added into the extract. Then crude extract was pumped to the cryogel column for 2 h. Binding of lectin was controlled periodically by determining the protein concentration in the soybean extract. Also effect of temperature on lectin binding to the GalNAc-PHEMA cryogel was investigated. Bradford method was used to determine the protein concentrations of the samples. Binding capacity of the GalNAc-PHEMA cryogel was determined using equation 2.

$$Q = (C_i - C_f)V / m \quad (2)$$

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). All the experiments were replicated at least three times.

Elution and Repeated Use

Lectin elution was performed using 0.5 M galactose. Galactose solution was circulated through the cryogel column for 2 h and the elution ratio was determined based on the ratio of lectin eluted and lectin bound. Lectin binding and elution cycle was repeated 5 times to investigate the reusability of GalNAc-PHEMA cryogel. Then it was washed with 50 mM NaOH solution to regenerate the column after each binding-elution cycle.

SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis)

Purity of lectin purified from soybean seeds evaluated by SDS-PAGE under denaturating conditions [17]. Samples were mixed with sample buffer and loaded on 8% trisglycine gel. Electrophoresis was carried out using the SE 600 Ruby electrophoresis system (GE Healthcare Bio-Sciences Corp., USA). After SDS-PAGE, gel was stained with silver nitrate.

RESULTS AND DISCUSSION

Characterization of GalNAc-PHEMA Cryogel

Surface morphology of the GalNAc-PHEMA cryogel was investigated by SEM and presented in Figure 1. The GalNAc-PHEMA cryogel have interconnected pores and sizes of the pores are in the range of about 10-200 μm in diameter. Interconnected large pores allow soybean seed extract to flow easily from the GalNAc-PHEMA cryogel. As a result, back pressure in the GalNAc-PHEMA cryogel column is low. The GalNAc-PHEMA cryogel is white, opaque and its structure is elastic like a sponge. The equilibrium swelling degree of the GalNAc-PHEMA cryogel was 9.5 g

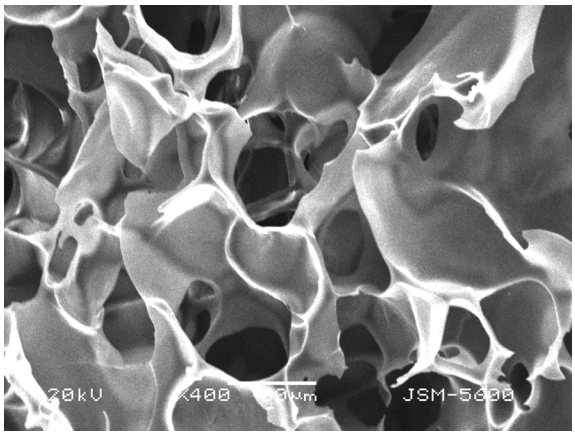

Figure 1. SEM image of GalNAc-PHEMA cryogel.

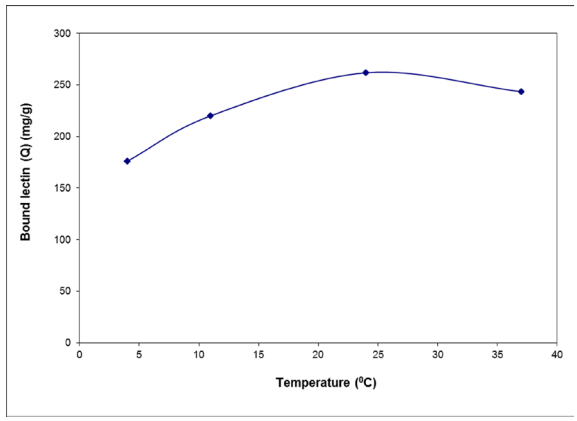

Figure 2. Effect of temperature on lectin binding to the GalNAc-PHEMA cryogel. Experimental conditions: equilibration buffer: 0.9% NaCl, flow rate: 1 ml/min.

H_2O/g cryogel. Also, water inside the pores can be easily removed with compressing by hand. GalNAc was covalently attached on the PHEMA cryogel by cyanogen bromide activation, via the reaction between the hydroxyl groups of the GalNAc molecules and the hydroxyl groups of the PHEMA cryogel. 490 mg GalNAc was loaded on one gram PHEMA cryogel. After ligand immobilization, the GalNAc-PHEMA cryogel was washed with 0.9% NaCl until no ligand leakage was observed.

Effect of temperature

Figure 2 shows the effect of temperature on lectin binding to the GalNAc-PHEMA cryogel. According to these results, maximum lectin binding was observed at room temperature and lectin binding capacity of the GalNAc-PHEMA cryogel decreased at lower temperatures. Interactions between lectins and sugar chains depends on mostly hydrophobic interactions and Van der Waals attraction forces [18]. Consequentially, increasing of lectin binding capacity with increasing temperature is an expected result.

Lectin purification from soybean flour

Soybean flour extract was diluted with 0.9% NaCl in different dilution ratios (1/2, 1/4, 1/8) and it was recirculated through the GalNAc-PHEMA cryogel. As clearly seen in Figure 3, lectin binding was decreased at more diluted soybean flour extract. Maximum lectin binding capacity was 261 mg/g cryogel. Elution of lectin was observed with 0.5 M galactose. Purity and molecular weight of eluted lectin were evaluated by SDS-PAGE. In figure 4 presenting the SDS-PAGE results, lane 1 is the soybean extract and lane 4 is the marker including bovine serum albumin (BSA) and soybean lectin (SBL). The amount of lectin was decreased in lane 2 (soybean extract after binding) compared to lane 1. The eluted lectin in lane 3 shows only one band and its molecular weight is the same with marker SBL. SBL has four subunits and each subunit has a molecular weight of 30 kDa. As a result, lectin purified in this study shows the same molecular weight profile with commercial SBL. The single band in lane 3 proves the purity of eluted lectin.

Elution and Reusability

Reusability of polymeric materials is an important factor to provide economic advantage. Lectin was eluted from the GalNAc-PHEMA cryogel with 0.5 M mannose in 2h with 90% yield. Then

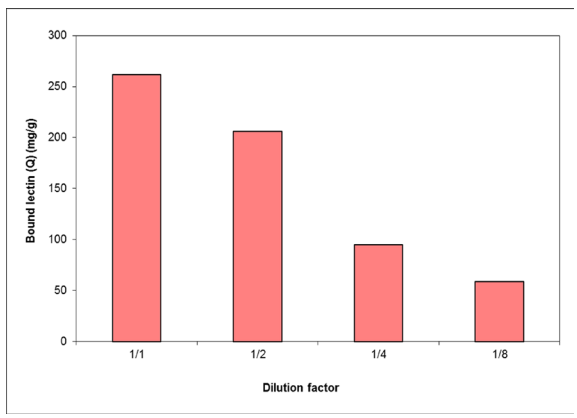

Figure 3. Lectin purification from soybean flour extract. Experimental conditions: equilibration buffer: 0.9% NaCl, T: 24°C, flow rate: 1ml/min.

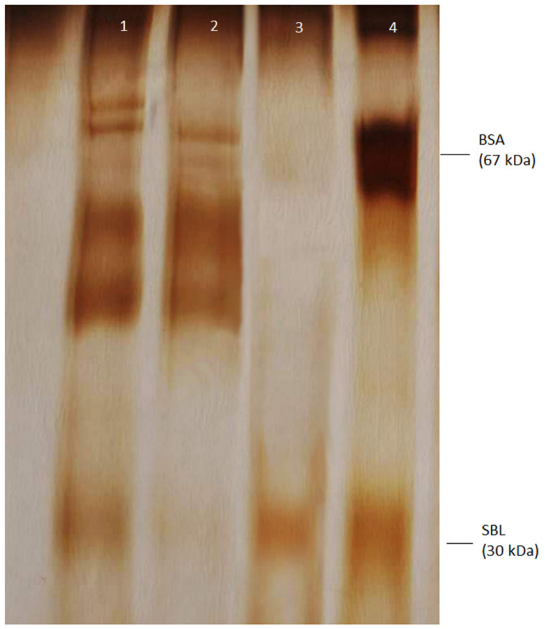

Figure 4. SDS-PAGE image of soybean flour extract fractions. Lane 1: Soybean flour extract before binding Lane 2: Soybean flour extract after binding. Lane 3: Eluted lectin from GalNAc-PHEMA cryogel. Lane 4: Commercial BSA and SBL protein standarts. Equal amounts of samples were applied to each lane.

binding-elution cycle was repeated five times and no significant decrease in column capacity was observed. In conclusion, 0.5 M mannose is a suitable elution agent and the GalNAc-PHEMA cryogel can be used several times with a very little decrease in lectin binding capacity.

CONCLUSION

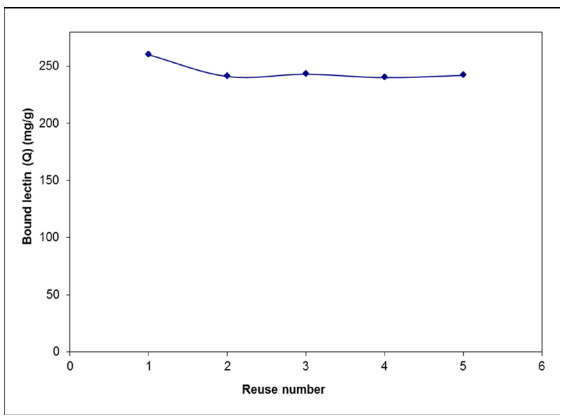

Figure 5. Reusability of the GalNAc-PHEMA cryogel. Experimental conditions: equilibration buffer: 0.9% NaCl, T: 24°C, flow rate: 1ml/min.

Lectins have many applications including purification of polysaccharides, glycoproteins, cell separation and identification of microorganisms. So purification of lectins from natural resources is very important. In this study, a lectin specific to GalNAc was purified with a 90% yield using the GalNAc-PHEMA cryogel. In conclusion, the GalNAc-PHEMA cryogel is an efficient material for purifying lectins from seed extracts and it can be used several times.

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