JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

N-Acetyl-D-Galactosamine-Specific Lectin Isolated from the Seeds of *Carica papaya*

Teng-Hsu Wang,[†] Yen-Lun Kung,[†] Min-Hsiung Lee,[*,‡] and Nan-Wei Su[*,†]

[†]Department of Agricultural Chemistry, National Taiwan University, Taipei 10617, Taiwan

[‡]Department of Nutrition and Health Science, Chung Chou Institute of Technology, Changhua County 51003, Taiwan

ABSTRACT: *N*-Acetyl-D-galactosamine (GalNAc)-specific lectins are of great interest because they have been reported to detect tumor-associated antigens of malignant cells. We isolated a novel lectin from *Carica papaya* seeds, named *C. papaya* lectin (CPL). Purification of the lectin involved ammonium sulfate fractionation and DEAE anion exchange and repeated gel filtration chromatography. Inhibition of CPL causing hemagglutination on human erythrocytes showed that the lectin shows specificity to GalNAc and lactose. Surface plasmon resonance further revealed that the lectin possesses high specificity toward GalNAc with a dissociation constant of 5.5×10^{-9} M. The lectin is composed of 38- and 40-kDa subunits with a molecular mass of ~804 kDa estimated by size-exclusion high-performance liquid chromatography. Incubation of CPL with Jurkat T cells showed significant induction of IL-2 cytokine, which suggests that CPL has potent immunomodulatory effects on immune cells.

KEYWORDS: Lectin, *Carica papaya*, purification, *N*-acetyl-D-galactosamine, surface plasmon resonance, immunomodulation

INTRODUCTION

Lectins are a group of proteins or glycoproteins of nonimmunological origin that can recognize specific carbohydrate structures. They can bind reversibly with free sugars or with sugar residues of polysaccharides, glycoproteins, or glycolipids.[1] Lectins are typically divalent or polyvalent, that is, they contain 2 or more carbohydrate binding sites, and thus can cross-link with corresponding erythrocyte receptors of different blood types. Lectins are present in all types of organisms and are ubiquitous in nature. However, the most early and comprehensively investigated lectins are from plants such as mistletoe or legumes.[2,3]

Lectins can be categorized by their origins, structures, functionalities, or, by the most intuitive way, carbohydrate specificities.[4] Among them, *N*-acetyl-D-galactosamine (GalNAc)-specific lectins are of great interest because they have been reported to detect tumor-associated antigens of malignant cells.[5,6] Studies of the molecular structure and sugar specificity of soybean agglutinin (SBA) and peanut agglutinin (PNA) have led to substantial progress in biochemistry, glycobiology, and medical breakthroughs.[7] Mistletoe lectins (MLs) have been shown to possess marked cytotoxic effects and induce apoptosis of tumor cells in vitro,[8,9] as well as initiate profound immunomodulating responses in patients with malignant diseases.[10,11] ML-I has shown a synergism with interleukin 2 (IL-2) in inducing enhanced lymphokine-activated killer-cell cytotoxicity.[12] Several Gal/GalNAc-specific fungal lectins, such as those from *Agaricus bisporus*, *Volvariella volvacea*, *Grifola frondasa*, and *Tricholoma mongolicum*, possessed antitumor and immunomodulating activities.[13−16] The intake of lectins in the human diet may be significant.[17] Many lectins can resist digestion, survive gut passage, and bind to gastrointestinal cells and/or enter the circulation intact, thus maintaining full biological activity. Therefore, plant lectins have been investigated for use in cancer treatment.[18]

The demand on novel lectins for deciphering sugar codes is a continuing quest in the emerging field of glycobiology.[19] In our previous study, we established a sugar polymer-based adsorbent assay to screen novel lectins.[20] We found an extract with specific GalNAc binding activity from the seeds of papaya (*Carica papaya*). In this study, we report on the purification, molecular mass characterization, and sugar specificity of the novel GalNAc-specific lectin from *C. papaya* seeds (CPL). The affinity kinetics of the purified lectin toward GalNAc was determined by surface plasmon resonance. In addition, we investigated the effects of papaya lectin on the viability of immune cell lines and modulation of cytokine secretion.

MATERIALS AND METHODS

Materials. Multivalent α-*N*-acetyl-D-galactosamine polyacrylamide conjugates (GalNAc-PAA) and biotinylated GalNAc polyacrylamide conjugates (GalNAc-PAA-biotin) were purchased from GlycoTech Corp. (Gaithersburg, MD, USA). Standard lectins, SBA and *Phaseolus vulgaris* agglutinin (PHA-L), were from Sigma (St. Louis, MO, USA). Inhibition sugars (i.e., α-D-mannose, α-D-galactose, *β*-*N*-acetyl-D-glucosamine, α-*N*-acetyl-D-galactosamine, and α-lactose) were from Sigma (St. Louis, MO, USA). Phosphate buffered saline (PBS), 137 mM NaCl, 15 mM phosphate, and 2.7 mM KCl, pH 7.4, was freshly diluted from a 10-fold stock and stored at 4 °C for use within 2 weeks. All other chemicals were of analytical grade and obtained from local suppliers in Taiwan.

Preparation and Extraction of Papaya Seed Protein. Fresh fruits of *C. papaya* were obtained from a local farmer in Kaohsiung, Taiwan. Papaya seeds were freeze-dried and ground into fine powder to pass through a 40-mesh sieve. An amount of 200 g of powder was extracted with 4 L of PBS at 4 °C overnight, then clarified on a 0.22-μm microfilter. Clear extracts were subjected to an ultrafiltration cassette (Pellicon XL, Millipore) to remove small molecules and concentrate the

Received: December 27, 2010
Accepted: March 15, 2011
Revised: March 14, 2011
Published: March 15, 2011

ACS Publications © 2011 American Chemical Society

fractions of molecular weights greater than 50 kDa to a final volume of 200 mL. The lectin activity of papaya seed extracts was monitored by the hemagglutination assay (HA) during fractionation processes. The protein content of each fraction was determined by the use of the Pierce bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL, USA). The colorimetric formation was detected at 562 nm absorbance, and the calibration curve was established by serially diluted bovine serum albumin.

Fractionation by Ammonium Sulfate Precipitation. Concentrated crude extracts of papaya seeds were sequentially precipitated by adding ammonium sulfate to 30%, 50%, 70%, and 90% saturations in the solution. Each precipitate was centrifugated (10,000*g*, 10 min), redissolved in minimal PBS, then thoroughly dialyzed in PBS for 24 h. The sugar binding activity of papaya seed extracts was monitored by both HA and the sugar-polymer adsorbent assay[20] before subsequent fractionation. The precipitates of up to 70% ammonium sulfate saturation of papaya seed extracts were pooled, dialyzed, and freeze-dried for preservation.

Fractionation by Ion Exchange Chromatography. The freeze-dried samples were redissolved in deionized water and centrifugated (10,000*g*, 2 min) to remove insoluble pellets. An amount of 2 mL supernatants with about 5 mg/mL protein was injected into a DEAE-Sepharose Fast Flow column (i.d. 26 mm × 65 mm) pre-equilibrated in 50 mM phosphate buffer, pH 7.0, with 15 mM NaCl at a flow rate of 1.0 mL/min. After elution for 30 min, the unbound fraction was collected (fraction a). The resins were eluted with 50 mM phosphate buffer, pH 7.0, containing 150 and 500 mM NaCl. The eluates of 150 and 500 mM NaCl were collected as fractions b and c, respectively. Each fraction was concentrated, and the buffer was exchanged with 50 mM phosphate buffer, pH 7.0, containing 150 mM NaCl before HA tests for lectin activity. The active fractions were pooled and concentrated by a 10-kDa MWCO centrifugal filter for stock. During preservation, pasteurized glycerol was used as the antifrozen reagent and added to 50% final concentration to fraction **b** concentrates, with 25 mM DTT, then preserved at −20 °C for subsequent purification within 30 days.

Fractionation by Gel Filtration Chromatography. Fraction **b** (eluates of 150 mM NaCl in the DEAE-Sepharose column) was concentrated by centrifugal filters (MWCO 10 kDa, Amicon Ultra-15, Millipore), reconstituted to 1.4 mg/mL in elution buffer and 1 mL injected into a Superdex 200 10/300 GL gel filtration column. Samples were eluted with 50 mM phosphate buffer, pH 7.0, with 150 mM NaCl at a 0.4 mL/min flow rate. The fractions were collected every 2 min, and the sugar binding activity of CPL was monitored by HA assay. Active fractions were pooled and concentrated for SDS−PAGE and subsequent characterization assays.

The native molecular size of CPL was determined by size-exclusion high-performance liquid chromatography (HPLC) with a Shodex KW-804 column (Showa Denko Co., Japan). The column was equilibrated in 50 mM phosphate buffer, pH 7.0, with 150 mM NaCl. Purified lectin was subjected to HPLC via a 20 μL sample loop. The elution was carried out at 1.2 mL/min flow rate in the same equilibration buffer, and the eluted proteins were monitored by absorbance at 280 nm. The calibration curve was established by the use of the HMW gel filtration calibration kit (Cat. No. 28-4038-42, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with the following proteins with high molecular weight: thyroglobulin, 669 kDa; ferritin, 440 kDa; aldolase, 158 kDa; conalbumin, 75 kDa; ovalbumin, 44 kDa.

The purified lectin was subjected to 10% SDS−PAGE under denatured conditions.[21] After electrophoresis, the gels were stained with 0.1% Coomassie brilliant blue (CBB, R-250) or by the periodate-acid-Schiff (PAS) staining method (Pierce Glycoprotein Staining Kit, Thermo Scientific, Rockford, IL, USA), according to the manufacturer's instructions. Native PAGE at pH 8.3 and 10.2 in 6% polyacrylamide gel was also conducted.[22]

Hemagglutination Assay. The HA assay followed a 2-fold serial dilution procedure with glutaraldehyde-stabilized human erythrocytes prepared by previously described methods.[23] In brief, the erythrocytes from the peripheral blood of O type healthy donors were prepared with EDTA as an anticoagulant, washed with 5 volumes of PBS, and centrifuged at 600*g* for 10 min. After 3 repeated washes, the erythrocytes were resuspended in PBS containing 0.25% glutaraldehyde and incubated at 37 °C for 15 min, then washed with PBS a further 3 times. Finally, the glutaraldehyde-fixed erythrocytes were resuspended at 2×10^8 cells/mL in PBS (approximately 4% of whole blood) and stored at 4 °C for up to 30 days. HA tests involved mixing 50 μL of serially diluted standard lectin solutions with 50 μL of 4% glutaraldehyde-stabilized erythrocytes in round-button microplates and incubating at 37 °C for 30 min.

The assay of sugar inhibition of lectin-induced HA with various sugars was similar to the HA assay. Serial 2-fold dilutions of test sugar samples were prepared in PBS, pH 7.4. Each dilution was mixed with an equal volume (25 μL) of a solution of the lectin with 16 HA units. The mixture was allowed to stand for 30 min at 4 °C before mixing with 50 μL of 4% glutaraldehyde-stabilized erythrocyte suspension. The minimum concentration of the test sugar in the final reaction mixture, which completely inhibited 16 HA units of the lectin, was recorded.

Effects of Temperature, pH, and EDTA on CPL Stability. The pH stability of CPL was assessed by incubating the protein with buffers from pH 3 to 10. The buffers used were 50 mM glycine−HCl (pH 2−3), 50 mM Na-acetate−acetic acid (pH 4−5), 50 mM maleic acid−NaOH (pH 6.0), 50 mM Tris−HCl (pH 7−8), and 50 mM glycine−NaOH (pH 9−10). In total, 20 μg (10 μL) of lectin was incubated with 90 μL of buffer in a microtiter plate and allowed to stand at 4 °C overnight. The relative binding activity was determined by the enzyme-linked carbohydrate polymer adsorbent assay to GalNAc as described previously.[20] The absorbance at pH 7.0 was considered 100%. The experiment involved 3 replications. For determining the heat stability of the lectin, 20 μg of lectin in 100 μL of PBS was incubated in a water bath from 25 to 100 °C for 30 min. The remaining lectin activity was determined as described above.

To investigate the effect of metal cations on the binding activity of CPL to GalNAc, lectin solution (40 μg in 100 μL of PBS) was added to 100 μL of 20 mM EDTA and incubated for 24 h at 4 °C. After extensive dialysis against PBS, aliquots of 100 μL each of the samples was incubated individually with 100 μL of 2 mM $CaCl_2$, $MgCl_2$, $MnCl_2$, and $ZnCl_2$ for 2 h. The remaining lectin activity was determined by an enzyme linked adsorbent assay and compared to the sample without EDTA treatment or without restoration of the cations.

Surface Plasmon Resonance Analysis. Real-time detection of CPL binding to GalNAc conjugates was recorded by using a Biacore T100 (Biacore, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). A certified sensor chip SA (BR-1005-31) was applied for immobilizing the ligand for CPL binding. The surface of the sensor chip SA carries a dextran matrix to which streptavidin has been covalently attached. The glycoconjugate GalNAc-PAA-biotin was immobilized via the biotin−streptavidin interaction to the sensor chip SA according to the manufacturer's specifications. Briefly, after the chip was conditioned with 3 consecutive 1-min injections of 1 M NaCl in 50 mM NaOH, GalNAc-PAA-biotin (in 50 mM phosphate buffer, pH 7.0, containing 150 mM NaCl) at 6.5 μg/mL was passed through the flow cells at 10 μL/min. After immobilization, the chip was equilibrated with 50 mM phosphate buffer containing 150 mM NaCl and 0.005% surfactant P-20, pH 7.0 (PBS-P). All analyses were performed at a flow rate of 30 μL/min. Each analyte, purified CPL, at various concentrations in the same buffer was injected over the immobilized ligand. After injection of the analyte, PBS-P was introduced onto the sensor surface to start dissociation. After 3 min of dissociation, the chip was regenerated by repeat injections of 30 μL of 200 mM GalNAc, followed by equilibrating in PBS-P before the next analysis.

Table 1. Purification of *N*-Acetyl-D-galactosamine Binding Lectin from *C. papaya* Seeds

step	protein (mg)	hemagglutination titers (HA units/mg protein)	HA recovery (%)	purification fold
crude extracts of dried papaya seeds (200 g)	5588	3	100	
>50 kDa retentate	730	18	85	6
0−70% ammonium sulfate fractionation	580	22	85	7.3
DEAE 150 mM NaCl eluent	72	89	43	29.7
Superdex 200 f.22-26	1	2560	17	853.3

Association and dissociation rate constants (K_a and K_d) were calculated by the use of Biacore T100 v2.0.3 software (Biacore, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The affinity constant (K_D) was calculated from the K_a and K_d.

Effect of CPL on Cell Viability and Cytokine Secretion in Jurkat and THP-1 Cells. Human leukemia cell lines, Jurkat T cells and THP-1 monocytes, were inoculated with 2×10^6 cells per 1.8 mL in each well of a 6-well plate. PHA-L and SBA were used as control lectins for the evaluation of cytotoxicity and stimulation of cytokine production. Serial concentrations of the control lectins and purified lectin (200 μL/well) were added, followed by incubation at 37 °C for 24 h, in an atmosphere of 5% CO_2. At the end of incubation, cell suspensions were carefully agitated, and aliquots of cells (100 μL) were transferred to 96-well plates for viability analysis. Metabolic indicator WST-1 solution (10 μL) was added to each well, followed by further incubation at 37 °C for 1 h. Cell viability was measured by the absorbance at 450 nm in a microplate reader. Cytotoxicity was assessed according to controls with the absence of cells or lectin treatment.

The remaining culture suspensions were centrifuged to collect supernatants for the cytokine assay. The assay involved a human IL-2 or IL-10 analyzing kit (R&D Systems, Minneapolis, MN, USA), which was based on a solid-phase sandwich ELISA. A monoclonal antibody specific for human IL-2 or IL-10 was coated onto the wells of the microplates. The antigen (or samples) and a biotinylated monoclonal antibody specific for target cytokines were, respectively, added and washed to remove unbound analytes. Results were revealed with an incubation step with streptavidin-horseradish peroxidase and 3,3′,5,5′-tetramehylbenzidine (TMB) as the chromogen. Results were read immediately at 450 nm by the use of a microplate reader.

Statistical Analysis. Statistical analysis involved the use of SPSS v13.0 for Windows (SPSS Inc., Chicago, IL) with one-way ANOVA. Statistical significance was established at $p < 0.05$.

RESULTS

Pretreatment and Extraction of Papaya Seeds. Each batch of papaya seeds was extracted and subjected to 50 kDa ultrafiltration . After monitoring the agglutination of 2% human O-type erythrocytes by the papaya crude extracts, the HA activities were retained in the fractions of >50-kDa retentates. The retentates were gently dispensed with ammonium sulfate until 70% saturation. Precipitated proteins were separated by centrifugation and redissolved in minimal PBS, and desalted by dialysis. The precipitated fraction was brought to a final volume 100 mL that derived from each batch of 200 g papaya seeds (2-fold concentration, w/v). Tests of the 0−70% ammonium sulfate-precipitated fraction showed the HA titer units increased to 22 U/mg protein, which was 7.3-fold purification as compared with the initial crude extracts. The total HA unit recovery until this step was 85% (Table 1). The sample was freeze-dried as the raw material for the following purification.

Fractionation by DEAE Anion Exchange Chromatography. The above freeze-dried materials were redissolved in deionized water and destined for DEAE anion exchange chromatography. For each injection, 2 mL of redissolved sample with a protein concentration of 5 mg/mL was injected. After the unadsorbed components were eluted by phosphate buffer with 15 mM NaCl (fraction a, Figure 1), further fractionation was carried out by a stepwise increase of NaCl in the elution buffer to 150 mM and 500 mM. Three protein peaks were collected and designated as fraction a (15 mM NaCl eluted), fraction **b** (150 mM NaCl), and fraction **c** (500 mM NaCl eluted) as shown in Figure 1A. By monitoring the HA activity of each eluent, fraction **b** exhibited the most agglutination of erythrocytes. Fraction **a** possessed trivial agglutination activities, and fraction **c** had no observable agglutinations. In our preliminary tests, pooled fraction **a** was reinjected into the DEAE column and eluted by the same stepwise process to ensure that the column reached maximum capacity for each injection. Therefore, fractions of lectin activity in papaya seed extracts can be adsorbed to DEAE-Sepharose under minimal NaCl concentration (15 mM) in 50 mM phosphate buffer, pH 7.0, then successfully eluted with the buffer containing 150 mM NaCl. Active fraction **b** was pooled and concentrated by a 10-kDa MWCO centrifugal filter and added to 50% glycerol and 25 mM DTT for preservation at −20 °C.

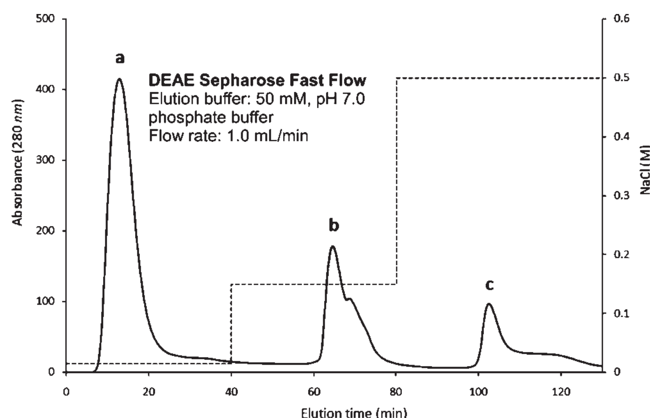

Figure 1. DEAE-Sepharose ion-exchange chromatography of *Carica papaya* seed extracts. The lectin was adsorbed on a DEAE-Sepharose column (50 mL) equilibrated with 50 mM phosphate buffer, pH 7.0, containing 15 mM NaCl. Each injection was 2 mL with a protein concentration of 5 mg/mL. Elution was carried out stepwise by increasing the ionic strength as follows: (a) equilibration buffer (15 mM NaCl), (b) 150 mM NaCl, and (c) 500 mM NaCl.

Purification by Gel Filtration Chromatography. Active fraction **b** was further fractionated by gel filtration in a Superdex 200 10/300 GL column. Fractionation involved the use of 50 mM phosphate buffer, pH 7.0, containing 150 mM NaCl at a 0.4 mL/min flow rate. HA tests revealed that the most significant activity among the fractions was from minute 22 to

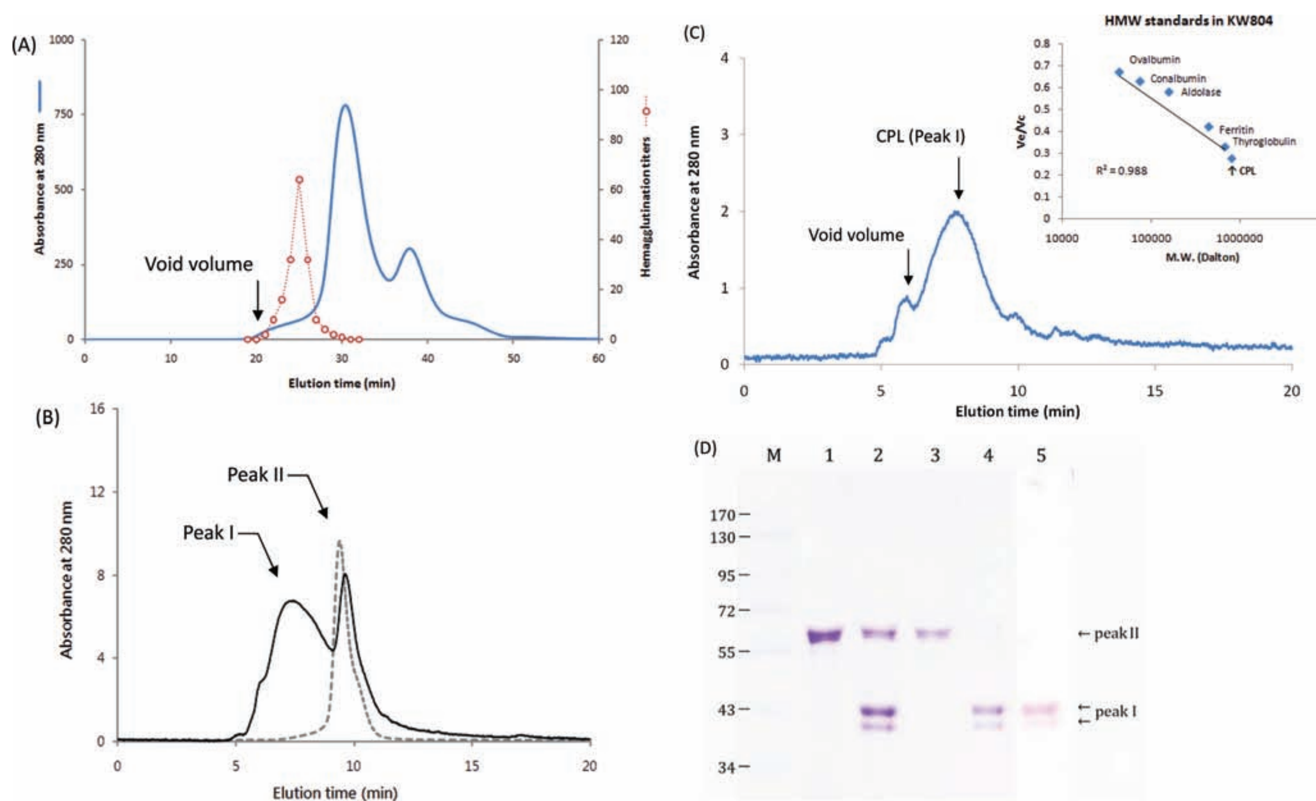

Figure 2. Separation of *C. Papaya* seed lectin by gel filtration chromatography. (A) Previously pooled fraction **b** was fractionated on a Superdex 200 gel filtration column. Each injection was 1 mL with a protein concentration of 1.4 mg/mL. Active fractions (S200f.22-26, dotted line with open circles) were pooled for further purification. (B) Determination of molecular distribution by size-exclusion HPLC on a Shodex KW-804 column. The chromatogram shows the pooled active fractions S200f.22-26 (solid line) and the removed impurity peak II (dotted line) by repeated separation in Superdex 200 gel filtration. (C) Size-exclusion HPLC on a Shodex KW-804 column. CPL was purified by repeated collection of peak I from the active fractions by Superdex 200 gel filtration chromatography. Inset: The calibration curve was obtained by plotting marker proteins with high molecular weight (thyroglobulin, 669 kDa; ferritin, 440 kDa; aldolase, 158 kDa; conalbumin, 75 kDa; ovalbumin, 44 kDa). Ve: elution volume of target protein. Vc: column bed volume. Each injection was 20 μL with a protein concentration of 100 μg/mL. (D) SDS electrophoresis of fractioned proteins. Lane 1, fraction **b** of Superdex 200; Lane 2, pooled active fractions by Superdex 200; Lane 3, peak II impurities; Lane 4, purified CPL; Lane 5, purified CPL stained by the periodic acid Schiff's reagent.

26 (Figure 2A). To obtain a linear correlation curve between molecular mass and elution volume, standard protein markers were eluted in the Superdex 200 column with the same conditions. The putative molecular size of the eluted fractions from minute 22 to 26 exceeded 600 kDa, the upper analytical limit of the column. In addition, the 2000-kDa blue dextran was eluted at around 20 min.

The active fractions eluted from Superdex 200 gel filtration column, denoted as S200f.22-26, were repeatedly collected and analyzed for approximate molecular weight by an HPLC system equipped with a Shodex KW-804 size-exclusion column. The results are shown as Figure 2B and C. Figure 2B shows that S200f.22-26 was composed of 2 major protein peaks: peak I eluted at around 7.5 min and peak II at 10 min. Concentrated S200f.22-26 was eluted in the Superdex 200 column repeatedly to separate these 2 peaks thoroughly. HA tests showed that peak I possessed significant agglutination activity, whereas peak II did not.

The 2 isolated peaks were concentrated and analyzed on 10% SDS−PAGE. Peak II was the major 60-kDa impurity in the DEAE-Sepharose fraction **b**, whereas peak I, despite eluting earlier than peak II, showed 2 major bands at 38 and 40 kDa (Figure 2D). Purified peak I was treated with or without β-mercaptoethanol during staining, then subjected to SDS−PAGE analysis. Both results showed 2 identical bands at 38 and 40 kDa, indicating that CPL was composed of these two fundamental subunits. In light of the high HA activity and exclusive characteristics of purified peak I during the isolation processes, we designated it as CPL.

To determine the native molecular size of CPL, we performed native PAGE at pH 8.3 and 10.2 in 6% polyacrylamide gels but did not visualize specific bands for peak I. Hence, we used an approximate approach for the molecular mass of native CPL by size-exclusion HPLC on a Shodex KW-804 column. The calibration curve with a molecular weight of 44 to 669 kDa was established by use of the HMW gel filtration calibration kit. Nonetheless, the purified CPL was eluted earlier than the 669-kDa marker bovine thyroglobulin and had a broad symmetric peak. To determine the mean peak elution volume of CPL, 6 repeated injections were eluted by size-exclusion HPLC. After extrapolation from the HMW calibration curve, the molecular mass of native CPL was estimated to be 804 ± 30 kDa (Figure 2C).

The developed SDS−PAGE of CPL was oxidized with periodic acid, then conjugated with Schiff reagent dyes to visualize the carbohydrate-containing moieties. The 38- and

Table 2. Hemagglutination Inhibition Assay on CPL by Various Sugars[a]

sugar	MIC (mM)
GalNAc	6.3
galactose	>100
lactose	6.3
mannose	>100
GlcNAc	>100

[a] MIC: minnimum inhibitory concentrations required for the inhibition of 16 hemagglutination titers of papaya seed lectin against 2% human O-type erythrocytes. All sugars are of D configuration.

40-kDa bands were both stained magenta, which suggests that the CPL might be composed of glycoproteins (Figure 2D).

Hemagglutination Assay with Various Sugars. Purified CPL was diluted to 16 HA titers while mixing with various sugar solutions at different concentrations, then added to the suspension of 2% human O-typed erythrocytes in each well. Inhibition of CPL caused agglutination, which indicated the competitive resemblance to the sugar ligand specificity of the lectin. As shown in Table 2, the minimum concentration required for GalNAc and lactose to inhibit the agglutination was 6.3 mM for both, whereas no inhibition was observed for Gal, Man, and GlcNAc at the highest concentration, 100 mM, in our study. Thus, the presence of GalNAc and lactose on the membrane surface of erythrocytes could significantly contribute to CPL-induced hemagglutination.

Effects of Temperature, pH, and EDTA on the Stability of CPL. The effects of temperature, pH, and EDTA on the stability of CPL were evaluated by the GalNAc-conjugate adsorbent assay as previously described. CPL was stable between 30 to 60 °C but was rapidly inactivated at 70 °C or above. At pH 6.0 to 8.0, lectin showed similar binding activity in the sugar polymer adsorbent assay, whereas a marked loss of activity was observed at pH values higher than 9.0 or lower than 5.0. No significant loss of binding activity was found when CPL was incubated with 20 mM EDTA at 4 °C for 24 h. Restoration of bivalent metallic cations, including Mg^{2+}, Zn^{2+}, Mn^{2+}, and Ca^{2+}, did not increase the binding activities.

Surface Plasmon Resonance on Immobilized GalNAc. To investigate the affinity kinetics of purified CPL, the biotinylated GalNAc polyacrylamide conjugate was immobilized on the surface of the SA sensor chip coupled with its streptavidin groups. The analysis was carried out on a Biacore T100 Surface Plasmon Resonance system. Because the molecular weight of CPL was relatively large, the immobilizing ligands should be limited to a lower extent to achieve an adequate binding saturation profile. Here, we immobilized 40 RU of GalNAc conjugates on the sensor chip. The purified CPL was diluted to 1.8, 3.7, 7.5, 15, 30, and 60 nM, then injected onto the sensor chip surface. The acquired sensorgrams were subsequently analyzed by the use of Biacore T100 software to obtain the association and dissociation rate constants for the CPL−GalNAc interaction. The results are shown in Figure 3. When the input of CPL ended in each injection, the complex dissociated with a very shallow curve and required substantial regenerations to return to baseline. Thus, CPL possessed high affinity for GalNAc. According to the computed fitting results, the association rate constant (Ka or Kon) for CPL against GalNAc was 5.5×10^4 (1/MS), and the dissociation rate constant (K_d or K_{off}) was 3×10^{-4} (1/S). Therefore, the equilibrium dissociation constant for CPL and GalNAc was 5.5×10^{-9} M.

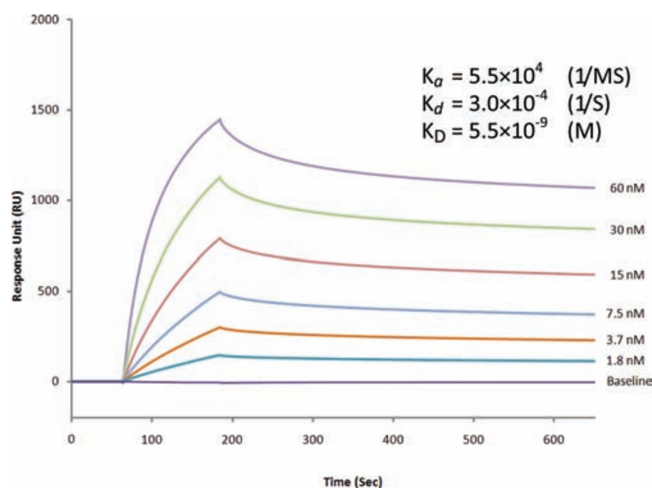

Figure 3. Sensorgrams of the interaction between CPL and immobilized GalNAc residues by surface plasmon resonance. Biotinylated GalNAc-polyacrylamide conjugates were immobilized on a streptavidin sensor chip (40 RU). Five concentrations of CPL were applied (1.8 nM, 3.7 nM, 7.5 nM, 15 nM, 30 nM, and 60 nM). Flow rate, 30 μL/min; temperature, 25 °C; buffer, PBS. The sensorgrams were evaluated by the use of Biacore T100 v2.0 based on the 1:1 binding model.

Effect of CPL on Cell Viability and Cytokine Secretion in Jurkat and THP-1 Cells. To determine the effect of CPL on cell viability and induction of cytokines, Jurkat T cells and THP-1 monocytes were cultured to 2×10^6 cells in 1.8 mL of culture medium. Then, 200 μL of diluted CPL was added to each well to a final concentration of 3.8, 7.5, 15, and 30 nM. Cell viability was analyzed by the use of the metabolic indicator WST-1 after 24 h of incubation. An amount of 3.8 or 7.5 nM CPL had no significant effect on the cell viability of Jurkat cells (Figure 4A); however, 15 nM CPL significantly inhibited the viability of Jurkat cells ($p < 0.05$). With 30 nM CPL in the medium, the viability of Jurkat cells was reduced about 30% as compared with the control. The extent of inhibition was comparable to that of 10 μg/mL SBA. However, CPL had no significant influence against THP-1 cells in the above experimental conditions.

After 24 h of incubation of Jurkat cells with 1 μg/mL of the lectins SBA or PHA, the level of IL-2 in culture medium was close to or lower than 10 pg/mL (Figure 4C and D). After incubation with 10 μg/mL SBA or PHA, the level of IL-2 increased to 377 and 308 pg/mL, respectively. When cells were incubated with 3.8 nM CPL for 24 h, the IL-2 level in the medium was 9.7 pg/mL, which did not differ from that in controls. The level increased to 44.5 pg/mL with 7.5 nM CPL and was further increased to 173 and 274 pg/mL with 15 and 30 nM CPL, respectively, for a dose-dependent effect. Significant differences occurred with the incubation of ≥7.5 nM of CPL ($p < 0.05$). However, incubation with different lectins had no effect on the secretion of IL-10 in THP-1 cells.

DISCUSSION

During the purification of CPL, an interesting phenomenon regarding the selection of ionic exchange resins for protein purification was observed because the active components in papaya seed extracts could be retained with pH 7.0 of DEAE-Sepharose as well as pH 6.0 of CM-Sepharose, both at a minimal NaCl concentration (15 mM). Adsorption at NaCl < 15 mM

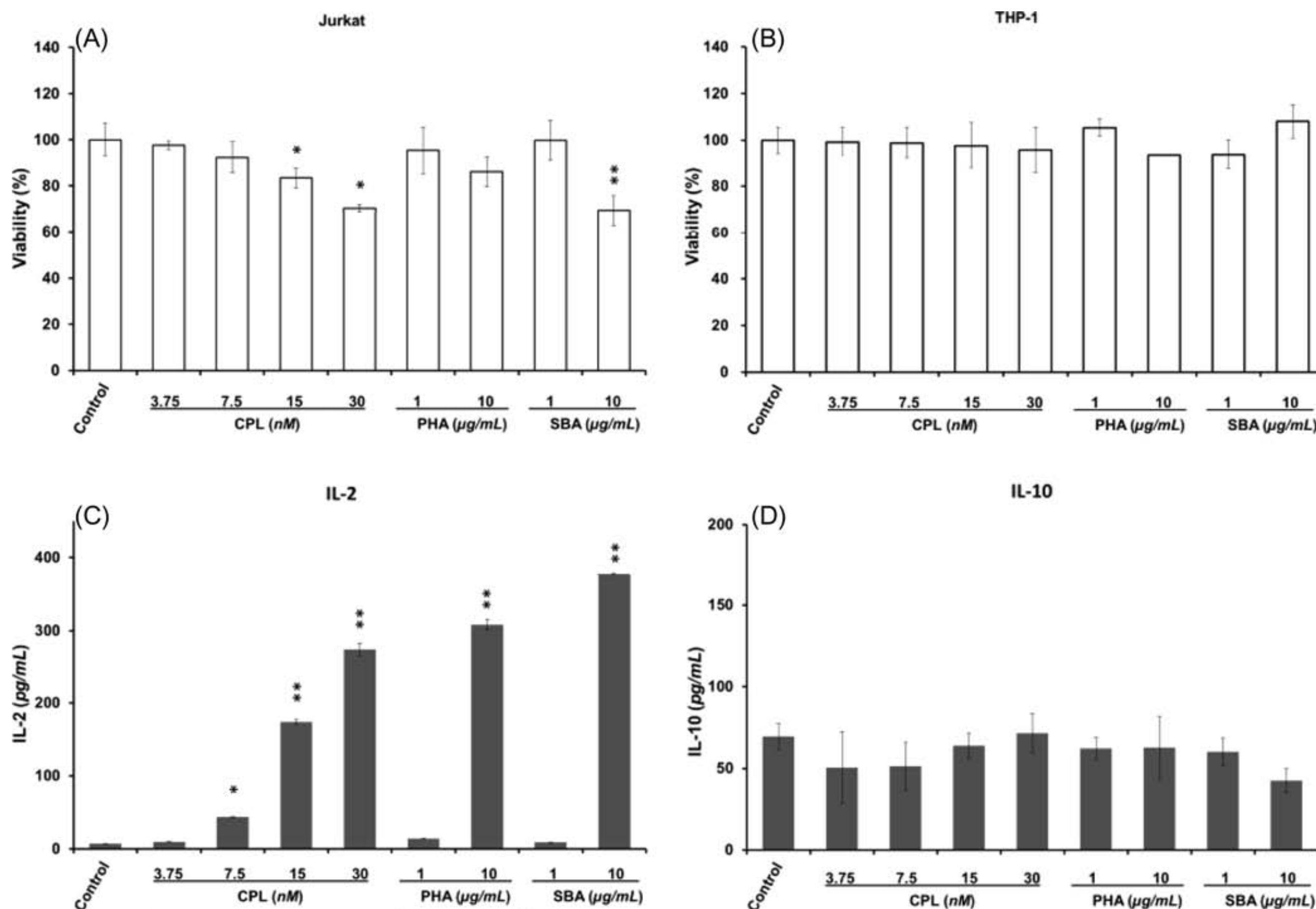

Figure 4. Immunomodulatory effect of CPL on the cytotoxicity and induction of cytokine production in Jurkat T lymphocytes and THP-1 monocytes. (A) and (B) cells were cultured in 6-well plates at 1×10^6/well in the presence or absence of concentrations of CPL, PHA, or SBA at 37 °C for 24 h. Cell viability was assessed by the metabolic indicator WST-1. (C) levels of IL-2 cytokine production after 24 h treatment of lectins in the culture medium of Jurkat T cells. (D) levels of IL-10 production after 24-h treatment of THP-1 cells with lectins. All results are the mean ± SD ($n = 3$). *$p < 0.05$ and **$p < 0.001$ by ANOVA.

could lead to an irreversible loss of HA activity. Increasing the ionic strength could successfully elute the active fractions from both DEAE and CM resins. With pH 7.0 of DEAE-Sepharose, the required NaCl concentration was 150 mM for eluting CPL, although it was 300 mM NaCl with pH 6.0 of CM-Sepharose. Thus, CPL carries abundant positive and negative charges at pH 6.0 and 7.0, respectively, and affords the adsorption of CPL in both resins at minimal NaCl concentration. To maintain the isotonic conditions for the subsequent HA assays and reduce the loss of recovery from buffer exchange, we chose the anionic exchange procedure by DEAE-Sepharose for the initial step of fractionation.

Native CPL is a large glycoprotein (Figure 2C and D); however, the composition and molecular weight of its native form still need to be elucidated. The purified lectin was analyzed by the use of a size-exclusion column, Shodex KW-804, by HPLC. The highest molecular weight marker was the 669-kDa bovine thyroglobulin, yet CPL was eluted earlier than thyroglobulin. The approximate molecular mass of CPL was extrapolated from the calibration curve as 804 ± 30 kDa. We attempted identification by mass spectrometry and N-terminal sequencing. However, some N-terminal blockage might be present in CPL; therefore, we have not yet obtained significant conclusions.

When conducting SDS−PAGE of the purified lectin, we attempted to use 6% acrylamide gels at pH 8.3 and 10.2 but detected no specific band corresponding to the native size. Perhaps CPL exhibits a barrier owing to its massive size relative to the polyacrylamide gels or to the deficiency of charges to mobilize the huge molecule. However, results of SDS−PAGE revealed purified CPL with only 2 major bands at 38 and 40 kDa, and its polymerization degree is estimated to be of 20 subunits. The results were identical both in the presence or absence of the reducing agent β-mercaptoethanol. The subunit configuration of CPL may not involve disulfide linkage. This could be further proven by the reducing treatment prior to Superdex 200 gel filtration. After treatment with 25 mM DTT at 40 °C for 1 h before analysis, the retention time of the active fractions remained the same, and the activity was even slightly higher than that without reducing treatment. Its carbohydrate binding activity was not affected by treatment of 20 mM EDTA either. In light of the multiple valences and abundant charges, it is suggested that ionic and hydrogen bonding would play important roles in the intermolecular forces among the subunits. Metallic ions, however, may not be involved in this case. Many renowned lectins are composed of two subunits with various compositions.[4,24] However, more works are required to disclose whether CPL has isoforms.

Although the massive configuration of CPL is unique and rare as compared with common lectin families, other studies have reported similar results on some lectins from different origins. Nakamura et al. isolated a mannose/glucose-specific lectin from Japanese chestnut (*Castanea crenata*) cytoledons (CCA).[25] The lectin was classified as a member of the Jacalin-related lectin family by sequence alignment. Native CCA was found to be 332.7 kDa, which indicates that it is a decamer of identical subunits of 33 kDa. However, the authors also indicated that the lectin may further aggregate in solution and form an icosamer (20 subunits) with a molecular mass corresponding to 805 ± 7 kDa. Some lectins of animal origin were found with a decameric configuration or more complex structure.[26,27] Therefore, lectin structures in nature are extremely diverse, yet most require tremendous work to resolve their configuration and functionality.

On the carbohydrate binding specificity, GalNAc and lactose showed significant inhibition of the CPL-induced erythrocyte agglutination, whereas galactose showed no inhibition at concentrations as high as 100 mM. Such subtle discrepancies in similar carbohydrate ligand preferences have been found in many well-known model lectins. Some may be contributed by merely a single substitution of amino acid in the carbohydrate recognition sites.[28−30] Despite similar MIC levels for GalNAc and lactose in HA tests, when searching for the suitable regeneration buffer for surface plasmon resonance assay, we found that lactose failed to remove the bound CPL on the sensor chip at concentrations as high as 200 mM. Therefore, CPL might have a higher preference for GalNAc over lactose.

Because native CPL is an oligomeric protein with unresolved heteromeric subunit composition, calculation of its affinity for GalNAc from the SPR data might not be accurate. However, analysis of the sensorgrams was based on a simple 1:1 model for CPL versus GalNAc conjugates to obtain an approximate affinity value. The results indicate that CPL binds GalNAc with high affinity (K_D 5.5 × 10^{-9} M), which is almost comparable to antigen−antibody interaction (K_D 10^{-7}−10^{-11} M). Previous research into lectins with Gal/GalNAc specificity also demonstrated high affinity profiles on complex glycoproteins. For instance, the mushroom *Pleurocybella porrigens* lectin, PPL, showed the highest affinity toward bovine submaxillary mucin (BSM).[31] The K_D for PPL to BSM was 3.05 × 10^{-8} M. Mannose binding lectin from human serum also exhibited a nanomolar scale of K_D against pathogen peptidoglycans.[32] Nonetheless, we consider that the specific affinity of CPL toward GalNAc is unique and noteworthy.

Many GalNAc binding lectins are known to possess mitogenic or immunomodulatory effects. Among them, mistletoe lectin, ML-I, is the most extensively studied.[3] The effects of lectins on T-cell stimulation and proliferation were associated with increased level of IL-2, which was secreted by a particular subset of T cells.[33] IL-2 is one of the fundamental cytokines directing the TH1 immune pathway by helper T cells. It activates naïve T cells and macrophages and stimulates the proliferation of antigen-specific cytotoxic T cells. In particular, research has indicated that inducing TH1 immunity by increasing IL-2 and IFN-γ levels helped to boost natural killer cells and facilitate their recognition and cytotoxicity against malignant cells.[34] In the current study, we demonstrated that CPL was potent in stimulating IL-2 production in Jurkat T cells. Further research into the potent immunomodulatory effects and biological functionalities of CPL are in progress.

In summary, a novel lectin CPL, possessing specific and relatively high binding affinity toward GalNAc, was isolated from the seeds of papaya. Lectin is a glycoprotein which is composed of 38 and 40 kDa subunits and estimated to be around 800 kDa in its native form. No disulfide bonding and metallic ions were found to contribute to its intramolecular linkages. The degree of polymerization and topology of CPL are yet to be further elucidated. CPL is also potent in stimulating the TH1 immune pathway and should be worth further investigations on its potential applications.

■ AUTHOR INFORMATION

Corresponding Author

*(M.-H. Lee) Tel: +886-2-33664806. Fax: +886-2-23632714. E-mail: mhlee@ntu.edu.tw. (N.-W. Su) Tel: +886-2-33664819. Fax: +886-2-23632714. E-mail: snw@ntu.edu.tw.

Funding Sources

This study was supported by the National Science Council of Republic of China, Taiwan (Project Nos. NSC 95-2313-B-002-079 and NSC 96-2313-B-002-056).

Notes

All of the authors have no conflicts of interest to this study.

■ ACKNOWLEDGMENT

We are very grateful to Dr. Rita P.-Y. Chen, Institute of Biological Chemistry, Academia Sinica, Taiwan, and Dr. Shu-Chuan Jao, Biophysics Core Facility, Scientific Instrument Center, Academia Sinica, for providing technical assistance with data acquisition and analysis of SPR experiments.

■ ABBREVIATIONS USED

GalNAc, α-*N*-acetyl-D-galactosamine; BSA, bovine serum albumin; CPL, *Carica papaya* seed lectin; Glc, D-glucose; Man, D-mannose; ELISA, enzyme-linked immunosorbent assay; Gal, galactose; HA, hemagglutination; MIC, minimum inhibition concentration; sugar-PAA, monosaccharide polyacrylamide conjugates; PBS, phosphate-buffered saline; SBA, soybean agglutinin; streptavidin-HRP, streptavidin-labeled horseradish peroxidase; MLs, mistletoe lectins; SPR, surface plasmon resonance; TMB, tetramethylbenzidine.

■ REFERENCES

(1) Goldstein, I. J.; Poretz, R. D.; Liener, I. E.; Sharon, N.; Goldstein, I. J. Isolation, Physicochemical Characterisation, and Carbohydrate-Binding Specificity of Lectins. In *The Lectins: Properties, Functions, and Applications in Biology and Medicine*; Academic Press, Inc.: Orlando, FL, 1986; pp 33−237.

(2) Sharon, N.; Lis, H. How proteins bind carbohydrates: Lessons from legume lectins. *J. Agric. Food Chem.* **2002**, *50*, 6586–6591.

(3) Pryme, I. F.; Bardocz, S.; Pusztai, A.; Ewen, S. W. Suppression of growth of tumour cell lines in vitro and tumours in vivo by mistletoe lectins. *Histol. Histopathol.* **2006**, *21*, 285–299.

(4) Sharon, N.; Lis, H. *Lectins*, 2nd ed.; Springer: Dordrecht, The Netherlands, 2007.

(5) Piller, V.; Piller, F.; Cartron, J. P. Isolation and characterization of an N-acetylgalactosamine specific lectin from *Salvia sclarea* seeds. *J. Biol. Chem.* **1986**, *261*, 14069–14075.

(6) Lee, X.; Thompson, A.; Zhang, Z.; Ton-that, H.; Biesterfeldt, J.; Ogata, C.; Xu, L.; Johnston, R. A.; Young, N. M. Structure of the complex

of *Maclura pomifera* agglutinin and the T-antigen disaccharide, Gal-beta-1,3-GalNAc. *J. Biol. Chem.* **1998**, *273*, 6312–6318.

(7) Sharon, N. Lectins: Carbohydrate-specific reagents and biological recognition molecules. *J. Biol. Chem.* **2007**, *282*, 2753–2764.

(8) Choi, S. H.; Lyu, S. Y.; Park, W. B. Mistletoe lectin induces apoptosis and telomerase inhibition in human A253 cancer cells through dephosphorylation of AKT. *Arch. Pharm. Res.* **2004**, *27*, 68–76.

(9) Lyu, S. Y.; Park, W. B.; Choi, K. H.; Kim, W. H. Involvement of caspase-3 in apoptosis induced by *Viscum album* var. *Coloratum* agglutinin in HL-60 cells. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 534–541.

(10) Heiny, B. M.; Albrecht, V.; Beuth, J. Correlation of immune cell activities and beta-endorphin release in breast carcinoma patients treated with galactose-specific lectin standardized mistletoe extract. *Anticancer Res.* **1998**, *18*, 583–586.

(11) Stein, G.; Henn, W.; von Laue, H.; Berg, P. Modulation of the cellular and humoral immune responses of tumor patients by mistletoe therapy. *Eur. J. Med. Res.* **1998**, *3*, 194–202.

(12) Baxevanis, C. N.; Voutsas, I. F.; Soler, M. H.; Gritzapis, A. D.; Tsitsilonis, O. E.; Stoeva, S.; Voelter, W.; Arsenis, P.; Papamichail, M. Mistletoe lectin I-induced effects on human cytotoxic lymphocytes. I. Synergism with IL-2 in the induction of enhanced lak cytotoxicity. *Immunopharmacol. Immunotoxicol.* **1998**, *20*, 355–372.

(13) Yu, L. G.; Fernig, D. G.; White, M. R. H.; Spiller, D. G.; Appleton, P.; Evans, R. C.; Grierson, I.; Smith, J. A.; Davies, H.; Gerasimenko, O. V.; Petersen, O. H.; Milton, J. D.; Rhodes, J. M. Edible mushroom (*Agaricus bisporus*) lectin, which reversibly inhibits epithelial cell proliferation, blocks nuclear localization sequence-dependent nuclear protein import. *J. Biol. Chem.* **1999**, *274*, 4890–4899.

(14) Wang, H. X.; Ng, T. B.; Ooi, V. E. C. Lectins from mushrooms. *Mycol. Res.* **1998**, *102*, 897–906.

(15) Wang, H. X.; Ng, T. B.; Liu, W. K.; Ooi, V. E. C.; Chang, S. T. Isolation and characterization of 2 distinct lectins with antiproliferative activity from the cultured mycelium of the edible mushroom *Tricholoma mongolicum*. *Int. J. Pept. Protein Res.* **1995**, *46*, 508–513.

(16) Guillot, J.; Konska, G. Lectins in higher fungi. *Biochem. Syst. Ecol.* **1997**, *25*, 203–230.

(17) Nachbar, M. S.; Oppenheim, J. D. Lectins in the United-States diet - a survey of lectins in commonly consumed foods and a review of the literature. *Am. J. Clin. Nutr.* **1980**, *33*, 2338–2345.

(18) Gonz'Alez De Mej'Ia, E.; Prisecaru, V. I. Lectins as bioactive plant proteins: A potential in cancer treatment. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 425–445.

(19) Gabius, H. J.; Siebert, H. C.; Andre, S.; Jimenez-Barbero, J.; Rudiger, H. Chemical biology of the sugar code. *ChemBioChem* **2004**, *5*, 740–764.

(20) Wang, T. H.; Lee, M. H.; Su, N. W. Screening of lectins by an enzyme-linked adsorbent assay. *Food Chem.* **2009**, *113*, 1218–1225.

(21) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.

(22) McLellan, T. Electrophoresis buffers for polyacrylamide gels at various pH. *Anal. Biochem.* **1982**, *126*, 94–99.

(23) Vanblitterswijk, W. J.; Walborg, E. F.; Feltkamp, C. A.; Hilkmann, H. A. M.; Emmelot, P. Effect of glutaraldehyde fixation on lectin-mediated agglutination of mouse leukemia-cells. *J. Cell Sci.* **1976**, *21*, 579–594.

(24) Laitinen, L. *Griffonia simplicifolia* lectins bind specifically to endothelial cells and some epithelial cells in mouse tissues. *Histochem. J.* **1987**, *19*, 225–234.

(25) Nakamura, S.; Ikegami, A.; Matsumura, Y.; Nakanishi, T.; Nomura, K. Molecular cloning and expression of the mannose/glucose specific lectin from *Castanea crenata* cotyledons. *J. Biochem. (Tokyo)* **2002**, *131*, 241–246.

(26) Omori-Satoh, T.; Yamakawa, Y.; Mebs, D. The antihemorrhagic factor, erinacin, from the European hedgehog (*Erinaceus europaeus*), a metalloprotease inhibitor of large molecular size possessing ficolin/opsonin p35 lectin domains. *Toxicon* **2000**, *38*, 1561–1580.

(27) Silva, F. P., Jr.; Alexandre, G. M.; Ramos, C. H.; De-Simone, S. G. On the quaternary structure of a C-type lectin from *Bothrops jararacussu* venom-BJ-32 (BjcuL). *Toxicon* **2008**, *52*, 944–953.

(28) Sharma, V.; Srinivas, V. R.; Adhikari, P.; Vijayan, M.; Surolia, A. Molecular basis of recognition by Gal/GalNAc specific legume lectins: Influence of Glu 129 on the specificity of peanut agglutinin (PNA) towards C2-substituents of galactose. *Glycobiology* **1998**, *8*, 1007–1012.

(29) Hamelryck, T. W.; Loris, R.; Bouckaert, J.; Dao-Thi, M. H.; Strecker, G.; Imberty, A.; Fernandez, E.; Wyns, L.; Etzler, M. E. Carbohydrate binding, quaternary structure and a novel hydrophobic binding site in two legume lectin oligomers from *Dolichos biflorus*. *J. Mol. Biol.* **1999**, *286*, 1161–1177.

(30) Adar, R.; Moreno, E.; Streicher, H.; Karlsson, K. A.; Angstrom, J.; Sharon, N. Structural features of the combining site region of *Erythrina corallodendron* lectin: Role of tryptophan 135. *Protein Sci.* **1998**, *7*, 52–63.

(31) Suzuki, T.; Amano, Y.; Fujita, M.; Kobayashi, Y.; Dohra, H.; Hirai, H.; Murata, T.; Usui, T.; Morita, T.; Kawagishi, H. Purification, characterization, and cDNA cloning of a lectin from the mushroom *Pleurocybella porrigens*. *Biosci. Biotechnol. Biochem.* **2009**, *73*, 702–709.

(32) Nadesalingam, J.; Dodds, A. W.; Reid, K. B. M.; Palaniyar, N. Mannose-binding lectin recognizes peptidoglycan via the N-acetyl glucosamine moiety, and inhibits ligand-induced proinflammatory effect and promotes chemokine production by macrophages. *J. Immunol.* **2005**, *175*, 1785–1794.

(33) Smith, K. A. Interleukin-2: inception, impact, and implications. *Science* **1988**, *240*, 1169–1176.

(34) Davies, F. E.; Raje, N.; Hideshima, T.; Lentzsch, S.; Young, G.; Tai, Y. T.; Lin, B.; Podar, K.; Gupta, D.; Chauhan, D.; Treon, S. P.; Richardson, P. G.; Schlossman, R. L.; Morgan, G. J.; Muller, G. W.; Stirling, D. I.; Anderson, K. C. Thalidomide and immunomodulatory derivatives augment natural killer cell cytotoxicity in multiple myeloma. *Blood* **2001**, *98*, 210–216.