Purification and Identification of a Natural Lectin from the Seed of Peanut *Arachis hypogaea*

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Abstract: A natural lectin from the seed of peanut *Arachis hypogaea* was purified by singlestep affinity chromatography using galactoside-coupled agarose. The native molecular mass of purified *A. hypogaea* lectin (PN-L) was 29 kDa. The lectin PN-L was detected for agglutinating activity, glycoinhibiting action and thermostability. The influence of pH on those activities was also tested. The results showed that PN-L could not agglutinate three kinds of human erythrocytes. But it showed a strong affinity to human A, B and O erythrocytes (RBC) treated by neuraminidase. Agglutinating activity of PN-L to neuraminidase treated human O erythrocytes was inhibited by lactose, raffinose, melibiose and D-galactose. The agglutinating activity of peanut seed lectin was stable up to 55°C and at pH 5.0-11.0. The results of MALDI-TOF-TOF analysis indicated that the protein PN-L showed highly homology with the Peanut Lectin Chain A protein (gi|1942899).

Keywords: Peanut, lectin, agglutinating activity, property.

1. INTRODUCTION

Lectins are proteins or glycoproteins, usually without catalytic activity, that have the ability to bind to specific carbohydrates expressed on different cell surfaces [1]. They are ubiquitously distributed in nature and most abundant in the Plantae kingdom, where they can be found in seeds, leaves, barks, bulbs, nrhizomes, roots and tubers depending on the plant species [2-6]. However, the majority of the studies on lectins have been carried out on legume species [7, 8] particularly in their seeds where they comprise up to 15%of the total protein. They have attracted great interest because of their various biological activities, such as cell agglutination, antiproliferative, antitumor, immunomodulatory, antifungal, and antiviral [9-14]. They have emerged as an important class of proteins having a wide variety of biochemical applications including their use in bioseparation [15] and reversible immobilization [16].

Seeds of legumes such as peas and beans have long been known to represent a rich source of lectins [17]. Legume lectins are the best-studied group of lectins and hundreds of these proteins have been isolated and extensively investigated in relation to their chemical, physicochemical, structural, and biological properties. The present investigation has been devoted to purify and characterize a D-galactose-binding lectin from seeds of peanut. The lectin has been studied with respect to its structure, composition, biological activity, and sugarspecificity. These properties were compared to other reports on legumes lectins.

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2. MATERIALS AND METHODS

2.1. Materials and Reagents

Dried peanut were used for all the experiments in this study. The fruits were collected in 2009 during the September season at the farm of Laixi in China. Neuraminidase N2876 and galactoside-agarose were purchased from Sigma Chemical Company, USA. Typed human blood cells (A, B, and O) were obtained from healthy donors. All other reagents were either of analytical grade or of the highest quality available.

2.2. Extraction of and Purification Lectin

Peanut seed (5.0 g) was broken into pieces (approx 1mm), added to a 10-fold volume (50ml) of 50mM phosphate buffer (pH 7.2) and extracted at 4 °C for 16h. Supernatant obtained by centrifugation (10,000g for 20min) was then used for lectin purification. Proteins in the supernatant were fractionated by ammonium sulfate precipitation (20-60% saturation). The precipitate was resuspended and dialyzed extensively against 50mM phosphate buffer (PBS pH 7.2) before applied to affinity chromatography on a galactoside-agarose column (10 x 1.5 cm) pre-equilibrated and eluted with the same buffer. unbound material was washed from the column with a PB-NaIbuffer (35mM NaCl 50mM PBS, pH 7.2), until the O.D.280 of eluents was below 0.01. Subsequently, the column was washed with a PB-NaIIsalt buffer (500mM NaCl 50mM PBS, pH 7.2) until the O.D.280 of eluents was below 0.01 and then re-equilibrated with 50mM PBS at a 0.2 ml/min flow rate. The lectin was desorbed with with 0.2 M D-Lactose in 50mM PBS at a 0.5 ml/min flow rate. The fractions, which showed absorbance at 280 nm, were collected and dialyzed extensively against 50mM PBS at 4°C

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overnight to remove D-Lactose. The fractions containing haemagglutinating (HA) activity were pooled, lyophilized and stored at -70°C for further analysis. The effluent (4.5 ml fractions) collected after adsorption, washing, and elution was assayed for hemagglutinating activity (HA) [18].

2.3. Preparation of RBC Suspension

For human red blood cells (RBC)preparation, the extracted RBC were centrifuged at 500 g for 5 min, and then washed with PBS three times, and diluted to a 2% RBC suspension. The diluted RBC suspension (in PBS, 10 ml) was treated with 1 ml neuraminidase (100 μg/ml) for 1 h at 37°C. Then the cells were washed by buffer PBS three times [19].

2.4. Hemagglutination Assays

Hemagglutination assays (HA) were performed in V-bottom microtiter plates according to the method described by Murali S *et al.* [20]. The HA titers were recorded as the reciprocal of the highest dilution of the sample causing complete agglutination of RBC. Contol was substituted the sample by 50mM PBS, pH 7.2. The HA assay was repeated three times.

2.5. Hemagglutination-Inhibition Assays

The specificity of the HA-inhibition activity was investigated by using a variety of carbohydrates. The potential inhibitors were serially diluted with 50mM PBS, pH 7.2 (carbohydrates, 0.2 M), and then incubated with an equal volume of the PN-L (4 hemagglutination units or 4 HU, final concentration) for 30 min at RT, prior to the agglutination assays. The inhibitory capacity was expressed as the minimum concentration of the tested substance that completely inhibited 4 HU. The HA-inhibition assays were repeated three times.

2.6. Electrophoresis

The purity of the lectin during the purification steps was tested by SDS-PAGE according to the procedure of Laemmli [21], using 12% total concentration polyacrylamide as the separating gel and 4% polyacryl-amide as the stacking gel. The molecular weight of PN-L was determined by SDS-PAGE under reducing and non-reducing conditions. The separated proteins were stained with Comassie Brilliant Blue R-250. The protein concentration of the different samples was determined through the method of Bradford, using BSA (bovine serum albumin) as standard.

2.7. Effect of pH and Temperature on Hemagglutinating Activity

The effect of pH on the hemagglutinating activity of purified lectin was carried out by preincubating the samples with buffers of different pH: 0.05 M sodium acetate/acetic acid (pH 4-5), 0.05 M sodium phosphate/HCl (pH 6-7), 0.05 M Tris/HCl (pH 8-9) and 0.05 M glycine/NaOH (pH 9-11) for 12 h at 4°C. The solution was subsequently incubated with 50mM PBS at room temperature then neutralized to pH 7.2. The agglutinating activity of samples was checked using a 2% suspension neuraminidase treated human O RBC.

The effect of temperature on the hemagglutinating activity of purified lectin was examined after the lectin was incubated in PBS for 10 min at various temperatures (25, 30, 40, 50, 55, 60, 65, 70, 75°C). The residual agglutinating activity of the lectin was checked as described previously.

2.8. Effect of EDTA and Ca^{2+} on Hemagglutinating Activity

To investigate the role of Ca^{2+} ions on hemagglutination, the lectin was stripped of Ca^{2+} ions by adding 50μl of 25mM EDTA to 50μl of the lectin (63.84 μg/ml). The extent of hemagglutination was determined after 30min incubation at room temperature.

2.9 MALDI-TOF-TOF MS of the Lectin

The MALDI-TOF MS and MALDI-TOF-TOF MS analysis of PN-L were determined by 4700 MS/MS spectrometry in Beijing Proteome Research Center.

3. RESULTS

3.1. Purification of Lectin

Purification of the lectin PN-L from the seed of peanut *A. hypogaea* was achieved by one chromatographic steps, with a galactoside-agarose column. The unretained fraction was eluted with a PB-NaⅠbuffer and a PB-NaⅡbuffer. The elution had no hemagglutinating activity in the presence of tested erythrocytes. The lectin eluted with 0.2 M D-Lactose in 50mM PBS showed hemagglutinating activity. Lectin crude extraction was applied to the column and on elution with lactose in PBS yielded 63.83 μg of pure lectin. The specific activity of lectin was increased by 4032-fold compared to that of the crude extraction. Analysis of purified lectin on SDS-PAGE in the absence of Dithiothreitol (DTT) revealed a single band at 29 kDa. However, upon reduction with DTT this lectin was still a single protein bands whose molecular weights was 29 kDa (Fig. **1**).

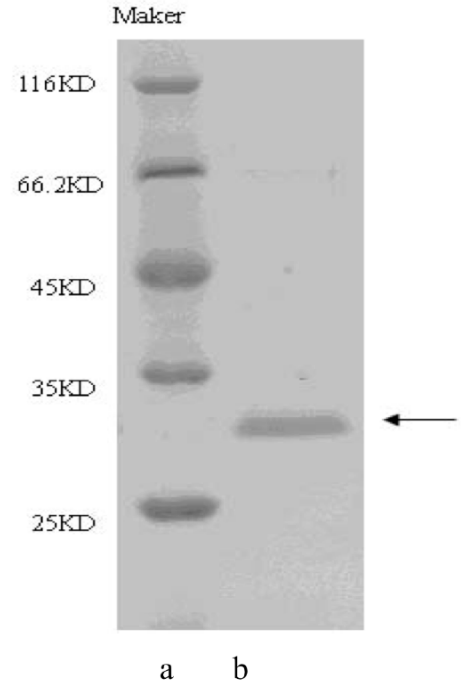

Fig. (1). Electrophoretic analysis of PN-L from *A.hypogaea.* SDS-PAGE of FC-L from Fetuin-agarose affinity chromatography (12%) lane a: Marker, lane b: PN-L.

3.2. Hemagglutination Assays and Hemagglutination-Inhibition Assays

PN-L didn't have the capacity to agglutinate three types of the human A, B, O erythrocytes. But it could intensively agglutinat the RBC which treated by neuraminidase. The HA activity of PN-L against different erythrocytes is given in Table **1**. The strongest agglutination titer was towards treated human O erythrocytes. Neuraminidase-treated erythrocytes were more agglutinated than the untreated ones, suggesting a preferential affinity of the activity towards non-sialoglycoconjugates.

Table1. Hemagglutinating Activity of Purified PN-L

Type of Erythrocytes	Hemagglutinating Titer	
	Untreated	Neuraminidase Treated
Human A	0	$2^{10\pm0.58}$
Human B	0	$2^{10\pm0.58}$
Human O	0	$2^{11\pm1.00}$

The sugar specificity of purified lectin PN-L was investigated by competitive inhibition of various carbohydrates and glycoproteins. Of the 11 carbohydrates tested, only 4 kinds of sugars had binding specificity. None of the three N-acetyl sugars namely, N-Acetylneuraminic acid (NeuAc), N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) inhibited the agglutinating activity of PN-L (Table **2**). In contrast, some monosaccharides or oligosaccharides namely, D-Galactose, Lactose, D-Raffinose, Melibiose inhibited the HA activity of PN-L even at very low concentrations (1.56. or 6.25 mM).

3.3. Effect of pH and Temperature

The optimum pH for purified PN-L to agglutinate human O erythrocytes was5.0-8.0. Its capacity was reduced below pH 5.0 and above pH 9.0. The HA activity of the lectin was inhibited at pH<5.0. HA activity was observed between pH 5.0-11.0 (Table **3**). Purified PN-L retained the full agglutinating activity over the temperature range of 0-40 °C, but there was a dramatic decrease between 50 and 55 °C, and at 55 °C the activity was completely abolished (Table **4**).

3.4. Requirement of Divalent Calcium

The activity of this lectin is dependent on the availability of Ca^{2+}. Treatment of purified PN-L with 20 mM EDTA, the agglutination activity was partly inhibited. Furthermore, the activity was completely restored when Ca^{2+} was added into the reaction system.

3.5.MALDI-TOF-TOF MS of the Lectin

Fig. (**2**) showed the result of MALDI-TOF analysis of PN-L. Fig. (**3**) showed the result of MALDI-TOF-TOF analysis of PN-L. These database search results indicated that the protein PN-L showed highly homology with the Peanut Lectin Chain A protein (gi|1942899). The fitting degree of these two protein was 81%. This result suggested that PN-L and gi|1942899 are same protein in the peanut seed.

Table 2. Agglutinating Activity of Purified PN-L Against Various Carbohydrates

Carbohydrates Tested	Maximum Concentration Tested	Minimum Inhibitory Concentration
Lactose	200 mM	1.56mM
D-Galactose	200 mM	6.25 mM
L-Rhamnose	200 mM	-
D-Xylose	200 mM	-
D-Raffinose	200 mM	12.5 mM
Sucrose	200 mM	-
D-Fructose	200 mM	-
Melibiose	200 mM	12.5 mM
N-Acetylglucosamine (GlcNAc)	200 mM	-
N-Acetylgalactosamine (GalNAc)	200 mM	-
N-Acetylneuraminic acid (NeuAC)	200 mM	-

Note: "-": no agglutinating activity at the concentration of 200mmM.

Table 3. Effect of pH on the Agglutination of Erythrocytes by PN-L

pH	4	5	6	7	8	9	10	11	12	Control
Hemagglutinating activity	0	$2^{5\pm0.00}$	$2^{5\pm0.00}$	$2^{4\pm0.00}$	$2^{4\pm0.00}$	$2^{3\pm0.00}$	$2^{3\pm0.00}$	$2^{3\pm1.00}$	0	$2^{5\pm0.00}$

Table 4. Effect of Temperature on the Agglutination of Erythrocytes by PN-L

Temperature	25	30	40	50	55	60	65	Control
Hemagglutinating activity	$2^{5\pm0.00}$	$2^{4\pm0.00}$	$2^{4\pm0.00}$	$2^{3\pm0.00}$	0	0	0	$2^{5\pm0.00}$

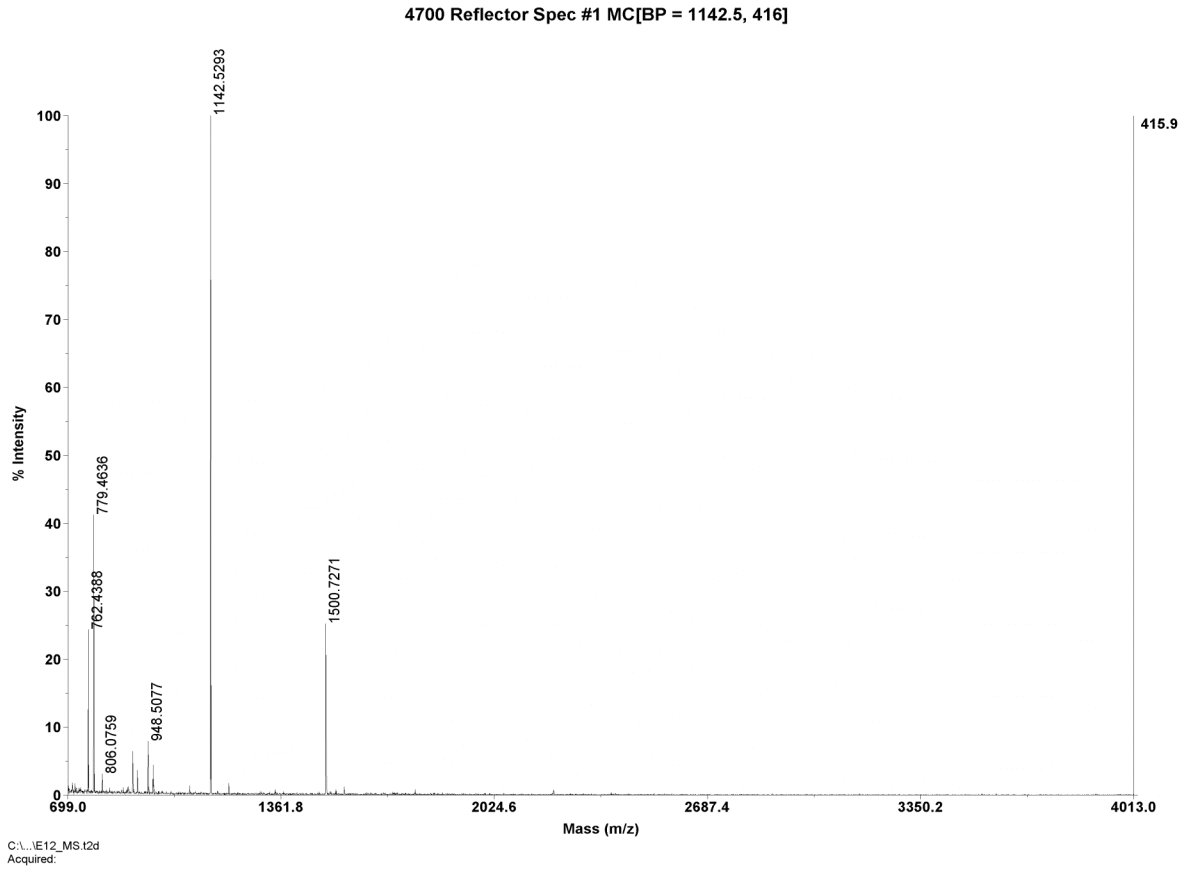

Fig. (2). MALDI-TOF analysis of PN-L from *A.hypogaea.*

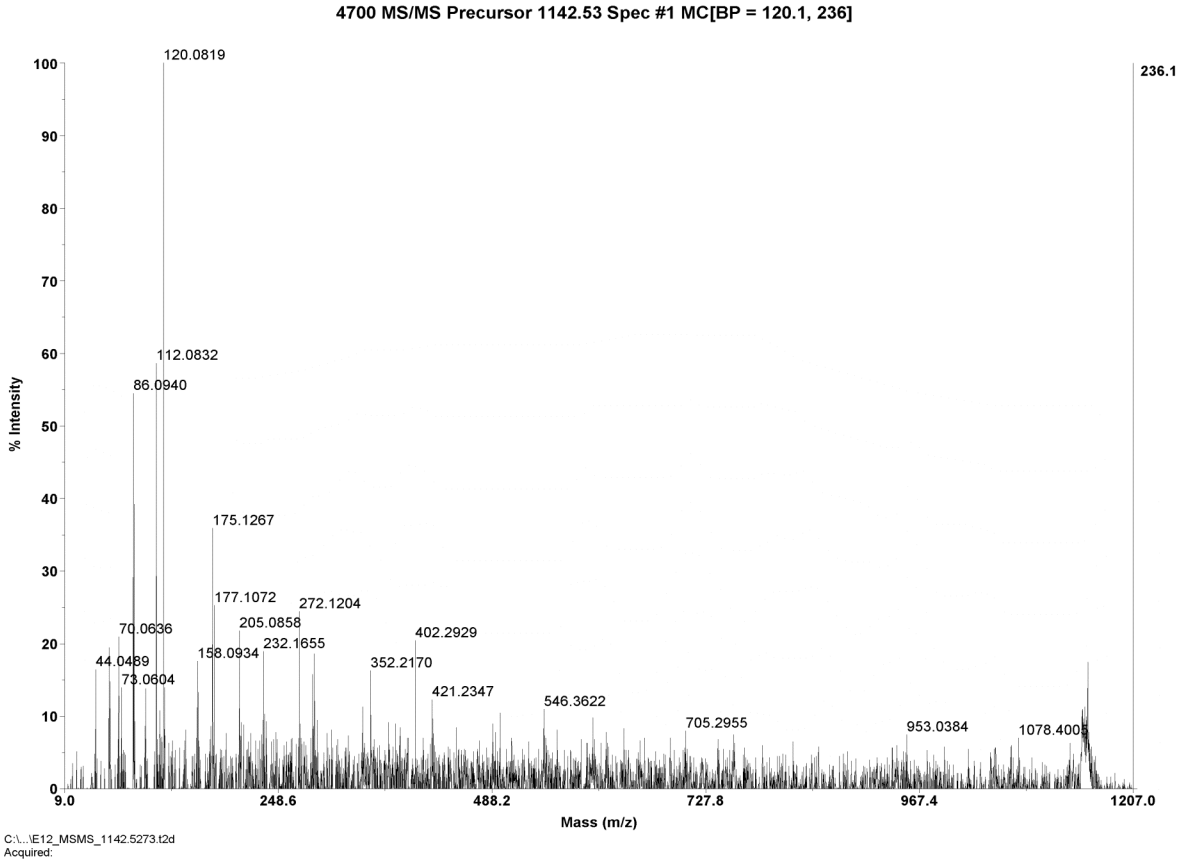

Fig. (3). MALDI-TOF- TOF analysis of PN-L from *A.hypogaea.*

4. DISCUSSION

The presence of naturally occurring lectins in the haemolymph of several plants has been well known since the beginning of the 20th century. Most current research has emphasized the biochemical applications of lectins, such as antitumor, immunomodulatory, antifungal, antiviral. and so on [10-13]. In the present study, a new lectin PN-L was purified from the seed of peanut *A. hypogaea* by affinity chromatography. SDS-PAGE analysis revealed one band with a molecular mass of 29 kDa. This agglutinin appeared to be a C-type agglutinin, as Ca^{2+} is required for it's HA activity [22] and it was reversibly sensitive to EDTA. The lectin specific activity was increased 4032-fold following purification. PN-L didn't have the capacity to agglutinate three types of human erythrocytes and it could not discriminate human A, B and O RBC types. While Neu5Ac is the major type of the sialic acid found in human erythrocytes, which account for 30% of the total sialic acid respectively [23,24]. PN-L could not to combine NeuAc (Table **2**) on the surface of erythrocytes. However, after treated by neuraminidase which removed the NeuAc on the surface of the erythrocytes, PN-L could agglutinate these three types of human erythrocytes. Its agglutination activity is similar to the lectin found in jacalin.

The inhibitory analysis showed that N-acetylated sugars and sialylated glycoproteins had no inhibition on PN-L agglutinating activity. Among the carbohydrates tested, the following sugars had inhibition at 200 mM: D-Galactose, Lactose, D-Raffinose, Melibiose. They were effective inhibitors of haemagglutination. Lactose was the strongest inhibiter, which inhibited the HA activity of PN-L at a concentration of 1.56 mM. These were similar with another peanut lectin PNA and *Dolichos biflorus* agglutinin (DBA), which could distinguish servel cancer cell [25]. Thses result drop a hint that PN-L might a potential distinguish factor on cancer diagnosis. The fitting degree of PN-L and Lectin Chain A protein (gi|1942899) was 81%. These results confirmed that the protein we purified was the translateed protein of Peanut Lectin Chain A protein.

In conclusion, a putative lectin PN-L was successfully purified from peanut *A. hypogaea*. PN-L was able to recognize the terminal D-Galactose and Lactose groups in the oligosaccharide chain of glycoconjugates. Further investigation is required to focus on the biochemical characters and precise applications on cancer diagnosis.

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