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Isolation and characterization of bacterial agglutinating lectin from Lima Bean (*Phaseolus lunatus* L.)

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Abstract Results showed that the isolated lima bean lectin (LBL) exhibited a total protein composition of 0.261 mg/mL and was found to be a glycoprotein containing 0.053 µg of carbohydrates per milligram of protein. The hapten inhibition assay presented that purified LBL is precise to the following sugars such as glucose, N-acetyl-d-glucosamine, d-galactose, xylose, fructose, sucrose, d-maltose, mannose, mannitol, raffinose, arabinose, and lactose. It is also demonstrated that LBL established an affinity toward bacteria causing disease such as *Escherichia coli* and *Bacillus subtilis.* LBL is shown to be a strong agglutinating action against *E. coli*, it can be used as a ligand for sensing foodborne pathogens and is important in developing lectin-based biosensors.

Keywords: Bacterial agglutination, Lectin, Ligand, Biosensor

Introduction

Lectins are glycoproteins that are distinguished in accordance with strong selectivity in attaching carbohydrates including mannose, galactose, rhamnose, lactose, fucose, and N-acetyl glucosamine (Rubeena *et al.,* 2019). The most essential and beneficial discovery regarding lectins is that they can detect and bind carbohydrates precisely and reversibly. Lectin may adhere to sugar molecules found on the surfaces of bacterial cells, making them useful for detecting pathogenic bacteria in food. Their ability in differentiating cells has helped in the rapid recognition of pathogens (Raghu *et al.,* 2022) and has been explored as biological instruments for the scientific investigation of carbohydrate interactions as well as structure on the cell interface, and has been utilized including applied claims as distinguishing normal and cancerous cells (Sharon, 1993; Padma *et al.,* 1998) glycoconjugates separation (Yamamoto *et al.,* 1984), and binding of medications to improve their breakdown in the digestive system

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(Naisbett and Woodley, 1990). In addition, plant lectins have been employed as therapeutic agents as well as diagnostic and immunomodulatory tools in cell biology and immunology (Gondim *et al.,* 2017). Moreover, they might be employed to create sensor technology for the food industry, which validates the existence of pathogens and assures the integrity of industrialized goods and byproducts (Suzuki *et al.,* 2015; Selvaprakash and Yen, 2018).

Lectins are a component of many living things, including bacteria, plants, and mammals as well as viruses and bacteria (Raghu and Kumar, 2020). Plants are the primary foundation of lectins, which are present in a variety of parts including seeds, leaves, bark, roots, tubers, and fruits (Nareddy *et al.,* 2017). Although lectins are originated in all plant classes, their assemblies and activity differ depending on the plant from which they originate (Sharon, 1993; Padma *et al.,* 1998). Furthermore, the lectins found in legumes have multiple binding sites and can form dimers and tetramers. Legume lectins have different carbohydrate specificities, but their physicochemical characteristics are similar and typically, they are made up of two or four subunits, each of which has one location for binding carbohydrates (Sharon and Lis, 1990). Potential health advantages of bioactive proteins from plants, such as lectins, have drawn more attention over the past 20 years. Lectins are found in legumes, which may be an important food source for both humans and animals (Zhang *et al.,* 2010). *Phaseolus lunatus* L. is a species of plant from the legume family that is also known as the lima bean. Given the fact that lima beans are rich in protein and starch, they are not widely consumed due to their anti-nutritional factors (ANF) and toxins. Nonetheless, the health-promoting benefits of lima beans and their contents, such as hypoglycemic, anti-HIV, anticancer, antihypertensive, bile acid-binding, gastroprotective, cardiovascular disease defense, and antimicrobic characteristics, have already been documented (Lourembam *et al.,* 2020). According to Galbraith (1970) the presence of a true lectin, the Lima bean lectin (LBL), has been long known. Additionally, other lectins from different *P. lunatus* varieties were discovered and characterized (Aletor, 1987; Sparvoli and Bollini, 1998).

The main objective of this study was to isolate and characterize the activity of lectin from lima bean (*Phaseolus lunatus* L.) that are capable of bacterial agglutination.

Materials and methods

Collection and purification of samples

Lima bean samples were collected at Barangay Linglingay, Alicia, Isabela. Five hundred grams of lima beans were pulverized with liquid nitrogen and then

standardized with phosphate buffered saline (PBS) at pH of 7.2 in 10 mM concentration with agitation for an overnight at 4°C to isolate the lectins. The crude sample was then filtered and centrifuged to get a supernatant. The crude sample was purified using the aqueous two-phase system technique (ATPS). To make ATPS, 1 mL of crude sample solutions was mixed with 750 mg of ammonium sulphate, 0.9 g of polyethylene glycol 600 (PEG 600), and 400 mg of NaCl. The mixture should create 5 grams of total weight, and the pH of the system was adjusted at 7.5. Following ATPS separation, the lectin and proteins in the top phase were extracted and dialyzed against distilled water for 24 hours using a dialysis bag with a molecular cut-off of 12000 Da to eliminate salt and PEG 600. The lectin was further purified by different chromatographic techniques.

Chromatography

All chromatographic techniques were executed using ÄKTA™ start fast protein liquid chromatography (FPLC) (GE Healthcare, Sweden) with Unicorn 1.2 software as control system and Frac-30 fractionator (Cytiva, Sweden).

Anion exchange chromatography

Anion exchange chromatography was performed using diethyl aminoethyl (DEAE) Sephacel® (Sigma, USA). The column was packed at a flow rate of 3 mL/min and equilibrated with two column volumes of the binding buffer (20 mM PBS, pH 7.2) in 1 mL/min as its flow rate. The sample solution was then passed through the column at a flow rate of 1 mL/min and the resulting flow-through was collected. To elute the bound proteins, 1 M NaCl was passed through the column at a flow rate of 1 mL/min. The eluate was preserved and tested for hemagglutinating activity with the flow-through. Native and SDS-PAGE, as well as Coomassie blue staining, were performed on 10-microliter aliquots of the eluate and flow-through.

Gel filtration chromatography

Gel filtration chromatography was done using Sephadex™ 50-fine (Cytiva, Sweden). The column was packed at a flow rate of 3mL/min before being equilibrated with the elution buffer (20 mM PBS, pH 7.2) at 1mL/min. Forty milliliters of (DEAE) Sephacel® eluate were passed through the column at a flow rate of 1 mL/min. The fractions were collected and pooled and subjected to different characterization processes.

Hemagglutination assay

Agglutination assay was carried out using the protocol of Mojica and Merca (2004) in multi-well microtiter plates using human erythrocytes A, B, and O. A 50 uL volume of lectin solution was serially diluted in a suitable buffer for two folds. In the same buffer, the solution was added with 50 uL of 2% (v/v) erythrocyte suspension. The agglutination was examined visually after the plates were incubated at room temperature for one hour. The creation of a homogeneous coating on the well's surface suggested a positive result. A negative result, on the other hand, was signified by the appearance of a distinct button at the bottom of the well.

Polyacrylamide gel electrophoresis (SDS-PAGE and Native-PAGE)

All polyacrylamide gel electrophoresis steps were done following the methods of Laemmli (1970) using 10 % native or SDS polyacrylamide gel and 1X native (25 mM Tris, 192 mM glycine) or SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at 120 Voltz in a Mini-PROTEAN® Tetra Cell (Bio-Rad, USA) unless otherwise stated. 6X native or SDS loading dye was used in loading the samples in a loading dye to sample ratio of 3:10. Gel images were taken after Coomassie staining using Chemiluminescent Imager (Bio-Rad, USA).

Total protein concentration (Bradford Assay)

Total protein concentration was determined using the method described by Bradford (1976) with slight modification. About 30 µL of the purified sample was added to 3 mL of Coomassie blue reagent and was mixed thoroughly. Bovine serum albumin (BSA) (Sigma, USA) was used as a standard and was diluted to the following concentrations: 0, 50, 100, 200, 300,400, 500, 600, and 700 µg/mL. The absorbance of the mixture was read at 595 nm in the UV-vis spectrophotometer of Magenta Lab science.

Total carbohydrate determination

Phenol sulfuric method by Dubois *et al.* (1956) with slight modification was used to determine the carbohydrate content of the purified lectin. D-glucose was used as a standard. To each standard, blank, and sample, 50 uL of 80% (w/v) phenol solution was added. The solution was vortexed, and 2.0 milliliters of concentrated sulfuric acid was added to the stream then stood for 10 minutes at room temperature. The absorbance of the mixture was read at 490 nm in the UV-vis spectrophotometer of Magenta Lab science.

Hapten-inhibition assay

The sugars glucosamine, glucose, maltose, lactose, galactose, arabinose, mannose, fructose, xylose, raffinose, mannitol, and fucose were tested to see how they affected the agglutination process. Concentrations of 125mM, 250mM, 500mM, and 1000mM were used to determine the sugar specificity of the purified sample. The assay was done using the modified protocol of Occena *et al.* (2007). A 50 µL of the isolated lectin was placed in multi-well microtiter plates that contained 50 µL of the sugars mentioned above. Inhibition of agglutination was observed after adding 50 µL of 2% (v/v) erythrocyte suspension and the formation of a distinct button of red blood cells at the bottom of the well.

Bacterial agglutination assay

The bacterial agglutination assay was conducted to compare purified lectins based on their binding affinity to bacteria. The assay was done using the modified protocol of Nader *et al.* (2015). The procedure is divided into two parts: the preparation of cells, and the test procedure.

Preparation of cells

The collected cells from nutrient agar were washed three times with 1 mL of sterile 0.01 M PBS in pH 7.2. Washing was done by centrifugation at 2000 rpm for 10 minutes at 20 °C. After precipitation, the cells were suspended in 3 mL of PBS before being exposed to lectin.

Test procedure

A 50 uL aliquot of lectin solution was serially diluted twice in 0.01 M PBS before being mixed with 150 uL of bacterial culture in the same buffer. The plates were incubated for one hour at room temperature and visually checked for agglutination. A carpet of aggregated cellular material at the bottom of the tubes suggested a positive result, whereas a dot of cellular material in the center of the tube suggested a negative result. The suspension was then shaken twice at maximum speed in a vortex for 20 seconds each. One drop of the mixture was placed on a glass slide and covered with a glass slip. The agglutination was seen using a light microscope with a dark-filled illumination and classified as no agglutination (-) or positive agglutination (+). As a control, PBS was employed in the same manner as the lectin.

Results

Isolation and purification of lima bean lectin

The LBL was isolated and purified using a three-step process namely Aqueous two-phase system (ATPS), anion exchange chromatography (AEC) using DEAE-cellulose as resin, and gel filtration chromatography (GFC) using Sephadex™ G-50 fine as its final purification process. Table 1 showed the step-by-step purification of the LBL with its corresponding hemagglutination activity with blood types A, B, and O.

Table 1. Agglutination activity of LBL at different stages of purification

Sample	Protein Content (mg/mL)	Total Protein (mg)	Blood type	Titer	Agglutination Activity (ug ml^{-1})
Crude extract	3.0347	100.15	A	8388608	$3.62x10^{-4}$
			B	16384	0.19
			O	1024	2.96
ATPS	0.6105	15.26	A	8388608	$7.27x10^{-5}$
			B	16384	0.04
			O	16	38.16
AEC	0.3432	6.86	A	4194304	$8.18x10^{-5}$
			B	16384	0.02
			O	16	21.45
GFC	0.2612	3.13	A	4096	0.06
			B	8192	0.03
			O	8	32.65

Characterization of the purified lima bean lectin

Determination of molecular weight

The molecular weight of purified lima bean lectin was measured using Native and SDS-PAGE. The molecular weight of the native lectin was estimated to be 139 kDa, as demonstrated by a single protein band in Figure 1. Moreover, SDS-PAGE without β-mercaptoethanol revealed two bands, indicating partial denaturation of LBL. The first band is similar with Native-PAGE having 139 kDa and the second band is likely to have a molecular weight of 70 kDa.

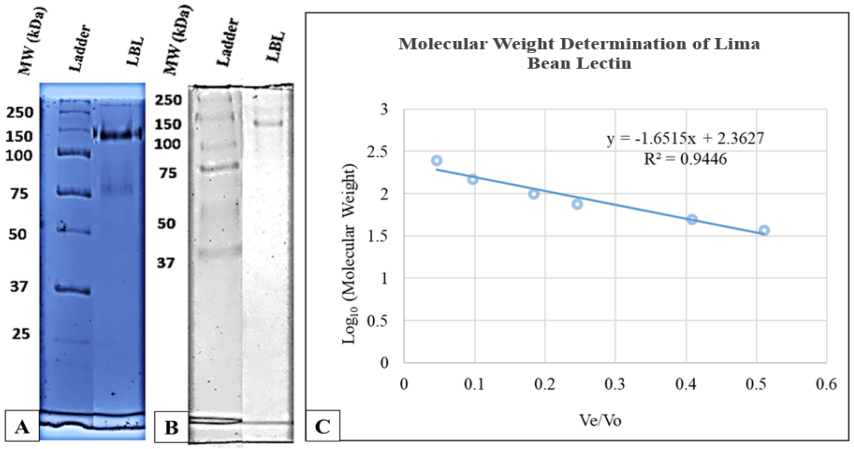

Figure 1. Molecular weight Determination of LBL. (a) SDS-PAGE with two subunits indicating the partial denaturation of LBL. (b) Native-PAGE with the single band with 139 kDa. (c) Standard curve for the molecular weight determination of LBL

Sugar-specificity

The concentration of test sugars increased from 125 mM up to 1000 mM. Result showed that at 125 mM of the twelve sugars tested inhibited the hemagglutinating activity of the LBL to human erythrocyte A, B, and O (Table 2). As the concentration reached 1000 mM, inhibition of hemagglutination activity was still observed. However, inhibition was evaluated merely with 12 sugars, therefore this may be sufficient to conclude that *P. lunatus* is sugar specific.

Table 2. Hapten inhibition assay of purified LBL

Sugar Sample	Concentration (mM)			
	125	250	500	1000
Arabinose	-	-	-	-
Xylose	-	-	-	-
Galactose	-	-	-	-
Glucose	-	-	-	-
Mannose	-	-	-	-
Fructose	-	-	-	-
Glucosamine	-	-	-	-
Mannitol	-	-	-	-
Lactose	-	-	-	-
Maltose	-	-	-	-
Sucrose	-	-	-	-
Raffinose	-	-	-	-

Legend: (+) Display of Hemagglutination Activity; (-) Absence of Hemagglutination Activity

Bacterial agglutinating activity

To test the binding affinity of LBL to different pathogenic bacteria, an agglutination assay was conducted against Gram-positive and Gram-negative bacteria such as *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Salmonella enterica*. After a careful microscopic observation, manifested by a carpet of aggregated cellular material encircled in red (Figure 2 and 3), it can be inferred that only the Gram-negative *Escherichia coli* and Gram-positive *Bacillus subtilis* exhibited positive agglutination as shown in Table 3. On the other hand, no agglutinating activity was observed against *Pseudomonas aeruginosa, Staphylococcus aureus, and Salmonella enterica.*

Table 3. Microbial agglutination assay of purified LBL

Test organisms	Results
Escherichia coli ATCC 8739	+
Salmonella enterica JCM 1651	-
Staphylococcus aureus ATCC 6538	-
Bacillus subtilis ATCC 6633	+
Pseudomonas aeruginosa ATCC 9027	-

Legend: (+) positive agglutination; (-) negative agglutination

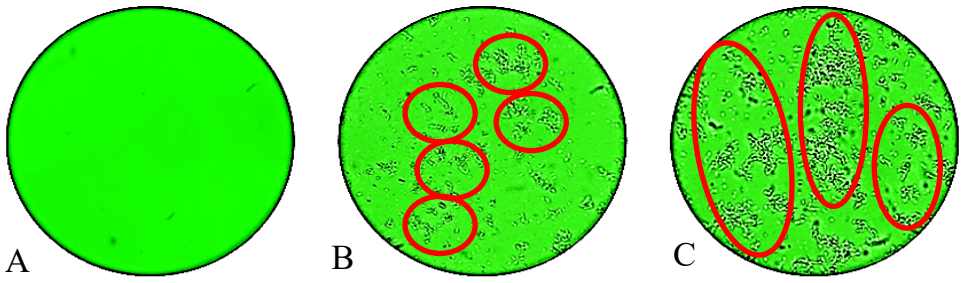

Figure 2. Microscopic image of the LBL bacterial agglutination using *B. subtilis*: (A) Negative control, (B) Pure LBL extract, (C) 2^1 dilutions of the pure LBL extract

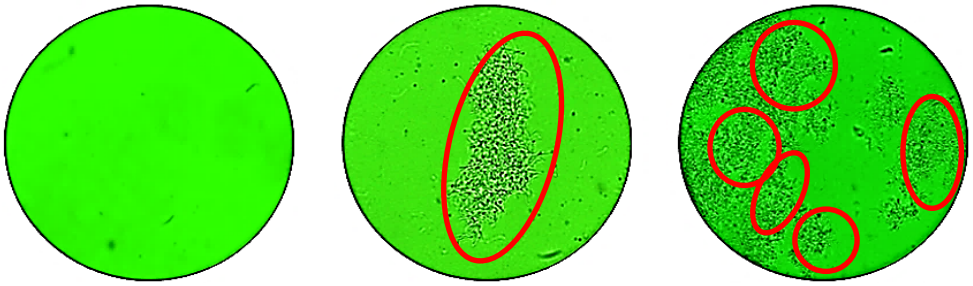

Figure 3. Microscopic image of the LBL bacterial agglutination using *E. coli*: (A) Negative control, (B) 2^1 dilutions of the pure LBL extract, (C) 2^2 dilutions of the pure LBL

Discussion

Lectins are a type of naturally occurring proteins/glycoproteins that precisely and exclusively attach to carbohydrate molecules on the cell exterior in a noncovalent connection (Michael and Smith, 1995). In the study of Yang *et al.* (2016), Concanavalin A is one of the legume lectins that has been chosen as a ligand. This harmless glycoprotein can identify and attach to exact sugars on the surfaces of cells without altering their natural structure.

The LBL protein composition from the crude sample decreases after the different purification methods such as ATPS, AEC, and GFC. The decreasing concentration of the protein content was expected and is due to the removal of the other unwanted proteins after each purification step. Additionally, it was observed that the titer value of LBL decreased but its agglutinating activity increased indicating that the pure LBL exerts greater activity towards hemagglutination assay as it becomes pure. The hemagglutination assay is the simplest technique for identifying lectins in biological sources, which separates lectin from the protein mixture. Lectin binds to erythrocytes during an agglutination reaction and creates numerous cross-bridges between them (Katoch and Tripathi, 2021). LBL activity was investigated using human blood types A, B, and O and the results suggest that LBL can be non-blood type-specific since it agglutinates human blood types A, B, and O. The result contrasts with the first reported lectin from *P. lunatus* which only agglutinates human blood type A and B but not blood type O (Galbraith and Goldstein, 1970). Moreover, their results also differ from the result of Galbraith (1970) in terms of specific activity since they observed the highest agglutination activity with blood type A while in this

study, it was observed with blood type B. Furthermore, LBL can be classified as a complete lectin resulting in its capacity to agglutinate red blood cells independently of metal ions or proteolytic enzymes. According to Singh and Bhari (2014), numerous lectins can adhere red blood cells from all human blood types at comparable concentrations. These lectins are known as panagglutinins due to their broad specificity. This agglutination property is commonly utilized to distinguish lectins from other proteins. Because of their enormous potential in the life sciences, lectins are becoming more and more significant. Lectins can bind a wide variety of sugars. No lectin, however, is specific to a single sugar (Goldstein *et al.,* 1977). The sugar molecules on the cell surface vary between human blood types. Blood type AB has the sugar determining factor for both A and B. Type A has N-acetyl-D-galactosamine, type B has D-galactose, and type O has L-fucose. The lectin's interaction with these sugar moieties results in agglutination. The lectin may not be blood type specific given that it has several binding sites where it could detect all the blood type determining factor (Aragones and Merca, 1998).

Lectins can attach to certain bacteria because their cell walls have a structure that matches the lectin's specific sugars. LBL is not a Gram-specific agglutinating protein since it agglutinates equally Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) bacteria. LBL exerts a strong affinity toward *E. coli*, a food-borne pathogenic bacteria. LBL binds to carbohydrate molecules on the surface of *E. coli* by interacting with mannose-specific lectins located on the bacterium's pili or fimbriae—hair-like appendages that facilitate attachment (Nizet *et al.,* 2017). In addition, *E. coli* exhibit lipopolysaccharide that contains galactose, glucose, N-acetylglucosamine, or colitose in its cell wall (Medearis *et al.,* 1968). Conversely, the cell wall of *B. subtilis* encompasses polysaccharides with galactose, glucose, and glucosamine as monosaccharide units (Streshinskaya *et al.,* 2011). The agglutination of these bacterial species could be due to the types of carbohydrates that are present in their cell wall. These saccharides could effectively inhibit the hemagglutination activity of LBL which means that LBL was specific to these types of saccharides, hence bacterial agglutination is observed. This activity of LBL can be employed in the development of biosensors. According to Mi *et al.* (2021), biosensors change biological lectin-polysaccharide linkages into measurable indicators that are easier to determine, analyze, and straightforward to use.

Similar with the study of Hendrickson *et al.* (2017), they created a lectin-based microplate examination using two of the most widespread bacterial pathogens, *E. coli,* and *S. aureus*, which investigated the attachment selectivity

of numerous plant lectins. According to the study, the specificity of the carbohydrate–lectin response is the most important aspect that indicates the usefulness of lectins as a diagnostic instrument (Dan *et al.,* 2016; Hendrickson and Zherdev, 2018). The availability of positive carbohydrate deposits in glycan particles dictates their specificity. The carbohydrate ligand structure on the cell surface thus serves a crucial function in selecting the specificity of the lectin-carbohydrate relationship (Brooks, 2017).

Overall, the purified LBL was found to be 139 kDa protein with subunit having a molecular weight of approximately 70 kDa. In agglutination and hapten-inhibition assays, it can be inferred that LBL is a non-blood type-specific lectin since it agglutinates human erythrocytes A, B, and O and possess carbohydrate specificity against 12 sugars that is glucose, N-acetyl-d-glucosamine, d-galactose, xylose, fructose, sucrose, d-maltose, mannose, mannitol, raffinose, arabinose, and lactose. The purified LBL solution was shown to have a total protein concentration of 0.261 mg/mL. Also, LBL revealed to be a glycoprotein possessing 13.82 ug of total carbohydrates or 0.053 µg of carbohydrates per milligram of protein. Furthermore, as the pure LBL exhibits significant agglutination against *E. Coli* and *B. subtilis*, it could potentially be utilized as a ligand for the recognition of foodborne diseases and is significant for the development of lectin-based biosensors.

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