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Purification of a novel chitin-binding lectin with antimicrobial and antibiofilm activities from a Bangladeshi cultivar of potato (*Solanum tuberosum*)

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A new chitin-binding lectin was purified from a Bangladeshi cultivar 'Deshi' of potato (*Solanum tuberosum* L.) through anion-exchange and affinity chromatographies using a chitin column. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) showed the molecular mass of the lectin as 20,000 Daltons. This molecular mass was almost half of the molecular masses of chitin-binding lectins derived from other potatoes. The lectin showed both bactericidal and growth-inhibiting activities against Gram-positive (*Listeria monocytogenes*) and Gram-negative (*Escherichia coli*, *Salmonella enteritidis* and *Shigella boydii*) pathogenic bacteria. It also showed antifungal activity against *Rhizopus* spp., *Penicillium* spp. and *Aspergillus niger*. Biofilm produced by the bacterium *Pseudomonas aeruginosa* was dose-dependently reduced by 5-20% in 24 h after administration of the lectin, which was attributed to the glycan-binding property of the lectin having affinity to GlcNAc polymers. It was the first observation that any potato lectin prevented biofilm formation by *P. aeruginosa* and, therefore, could have possible applications in clinical microbiology and biomedical science.

Keywords: *N*-Acetyl D-Glucosamine, Antibiofilm, Antimicrobial, Chitin, Lectin, *Solanum tuberosum*

Lectins are carbohydrate-binding proteins distributed among diverse organisms having complex roles in symbiosis or infections, innate immunity and antimicrobial activity. There are a variety of lectins recognizing oligomeric *N*-acetyl D-glucosamine (GlcNAc), especially chitin (polymer of GlcNAc) in fruit bodies and tubers of the plant family *Solanaceae*[1,2]. In case of potato (*Solanum tuberosum*), there are multiple varieties of chitin-binding lectins with the molecular mass of 45 to 65 kDa in different cultivars[3,4]. They have been specifically reported to be glycoproteins having glycan portions in a ratio of 50% of their molecular masses.

The potato originated in South America and diverse strains and cultivars have been bred according to different environments and cultures around the world. In Bangladesh, potato is regarded as one of the main foods and over thirty different cultivars are established, according to cooking habits with different physiological and morphological properties[5]. A cultivar named 'Deshi' is a representative variety in this country with relatively small size (2 to 4 cm in diameter) and bright red in color. The storage quality of potato might be closely related to the antimicrobial activity found in the tubers. Considering this background, it can be expected that potato lectins may function in the prevention of infection by microorganisms by binding with GlcNAc oligomers present in the bacterial peptidoglycan layer, as well as in the fungal cell wall[6,7]. Similarly, these lectins have also been predicted to prevent the formation of mucous-like components termed as 'biofilm' consisting of proteins and GlcNAc oligomers secreted by microorganisms that function to maintain the microbial ecology[8].

In this study, we report purification of a novel chitin-binding lectin from a domestic potato variety (Deshi) and investigated its antimicrobial property, as well as inhibitory effects against biofilm formation of *Pseudomonas aeruginosa* PAO1.

Materials and Methods

The tubers of potato (*Solanum tuberosum* L. *cv*. 'Deshi') were purchased from the local market

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Abbreviations: DEAE, diethylaminoethyl; GlcNAc, *N*-acetyl D-glucosamine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; StL-20, 20 kDa chitin-binding lectin from *Solanum tuberosum.*

in Rajshahi, Bangladesh and stored in a refrigerator at 4°C.

Chitin and DEAE-cellulose were procured from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Automated microtiter plate reader was purchased from Mikura Ltd. (West Sussex, UK). All other chemicals and reagents used in this study were of analytical grade.

Purification of lectin

The skins of potatoes (300 g) were peeled off and the potatoes were sliced and homogenized using a blender with the addition of 10 mM Tris-HCl (pH 8.2) containing 50 mM NaCl. After centrifugation at 20,000 × *g* for 15 min, the supernatant was collected and dialyzed against the same buffer at 4°C for 6 h. Hemagglutination assay was performed in 96-well U-bottomed plates[9]. 20 l of a 2-fold dilution of the crude potato extract in 150 mM NaCl containing 10 mM Tris-HCl, pH 8.2 (TBS) was mixed with 20 l of a 2% suspension (with TBS; v/v) of mice erythrocytes. The plate was incubated at room temperature for 30 min and the formation of a sheet (agglutination-positive) or dot (agglutination-negative) was observed and scored as the hemagglutination titer. This crude supernatant was applied on to a DEAE-cellulose column (2 × 25 cm) and eluted by a linear gradient of 0 to 400 mM NaCl containing 10 mM Tris-HCl (pH 8.2).

The fractions (2.5 mL each) having hemagglutination activity were subjected on to a chitin column (2 × 25 cm) previously equilibrated with TBS and eluted by 0.5 M acetic acid[3,4]. The pH of eluted fractions was neutralized by the addition of an aliquot of 1 M Tris-HCl buffer (pH 8.2) to each tube and then dialyzed overnight. The molecular mass and hemagglutination activity of purified lectin were determined by SDS-PAGE of 15% separating gel under reducing conditions[10] and the hemagglutination assay was performed as described previously[9].

Antibacterial assay

Escherichia coli O157:H18, *Salmonella enteritidis* ATCC 13076, *Listeria monocytogenes* ATCC 35152 and *Shigella boydii* ATCC 9905 were grown at 37ºC overnight in liquid nutrient medium. Each bacterial strain was collected as pellets by centrifuging at 4000 × *g* for 3 min, washed with 20 mM Tris-HCl buffer saline (pH 7.8) and re-suspended in the same buffer with a turbidity of 1.0 at optical density (OD) at 640 nm (OD_{640}). Antibacterial activity of purified lectin was estimated by the agar disc diffusion method[11] using sterile petri dishes containing 30 mL of nutrient agar. Pathogenic bacteria were seeded separately on to the surface of plates, followed by the placement of standard paper discs on the agar surface of plates. An aliquot of potato lectin (50 µg in 100 µl) was added on to the discs and bacterial cells were allowed to grow at 30ºC for 12 h. A transparent ring around the paper discs signified the antibacterial activity.

Assay of bacterial growth inhibition

E. coli O157:H18, *S. enteritidis* ATCC 13076, *L. monocytogenes* ATCC 35152 and *S. boydii* ATCC 9905 were grown for 18 h at 37ºC in nutrient broth and absorbance was adjusted to 0.3-0.35 at OD_{630} with liquid nutrient medium. 50 µl of each bacterial suspension was mixed with a serial dilution of the protein samples to a final volume of 100 µl in a 96-well microtiter plate. Negative control wells contained broth only. The plate was agitated at 28ºC and the reading was taken after 8 h at OD_{630} using an automated microtiter plate reader. The percentage of bacterial growth inhibition was determined according to the formula:

$$\% \text{ Inhibition} = \text{Absorbance of (control - test)} / \text{Absorbance of control} \times 100$$

Antifungal assay

Antifungal activity assay of the lectin was investigated by the agar disc diffusion method against fungal strains — *Rhizopus* spp., *Penicilium* spp. and *Aspergillus niger*. Sterile petri dishes were used containing 30 mL of potato dextrose agar. Fungal mycelia were placed on solid potato dextrose agar and sterile filter paper discs were put on the plate. The discs were soaked with 100 µl (containing 50 g) of purified lectin. Incubation of petri dishes was carried out at 30°C until the development of mycelial growth. Crescent-like transparent zones were formed around the discs containing the lectin.

Anti-biofilm activity of lectin against *Pseudomonas aeruginosa*

Pseudomonas aeruginosa PAO1 were grown overnight at 37°C in nutrient broth. The colonies were transferred into test tubes and centrifuged at 4000 × *g* for 3 min. The turbidity of bacterial cell suspensions was adjusted to OD_{640} of 1.0. Then, 50 µl of bacterial suspension was mixed with a serial dilution of purified lectin to a final volume of 100 µl in a 96-well microtitre plate and incubated in a humid environment for 48 h at

37°C. 20 µl of 0.1% (w/v) crystal violet solution (filtered through a 0.45 µm filter paper) was added in each well for the detection of biofilm and stained for 10 min at room temperature. The solution containing bacteria was carefully removed using a micropipette, so that only the biofilm remained on the plate. The wells were washed three-times successively with phosphate buffered saline (pH 7.5) to remove any free dye. After drying, the biofilm for 15 min, 150 µl of 95% ethanol was added into each well to release the crystal violet which was used to stain the biofilm (at room temperature for 10 min). The extracted dye was transferred to another 96-well titer plate and OD values were recorded by an automated microtiter plate reader at 570 nm[12]. The percentage of reduction in OD indicated inhibited biofilm production by the addition of lectin compared to the control and was determined according to the formula:

% of Reduction of OD = Absorbance of (control - test)/ Absorbance of control × 100

Statistical analysis

The experimental results were presented as mean ± standard error (SE). Differences in means were evaluated by two-tailed Student's t-test, with P values < 0.05 considered to be statistically significant.

Results

Purification of lectin

The crude supernatant extracted from the Deshi cultivar of potato showed strong hemagglutinating activity against mice erythrocytes. The activity was specifically inhibited by the addition of chitotriose, however, not inhibited by the GlcNAc in the microtiter plate assay (data not shown), which is a common property of other potato lectins[4]. From anion-exchange chromatography of the supernatant applied on to the DEAE-cellulose column, four peaks were eluted from the column by linear gradient of NaCl concentration (0-400 mM) (Fig. 1A). Since the first peak had significant hemagglutinating activity, those fractions (no. 15-25, Fig. 1A) were collected together and applied on to the chitin column. After washing the chitin column with TBS extensively, a single peak was eluted from the column by the addition of 500 mM acetic acid (Fig. 1B). Eluted fractions (no. 17-32, Fig. 1B) were collected, neutralized by the aliquot of 1 M Tris-HCl (pH 8.2) and dialyzed against TBS. SDS-PAGE showed that the 20 kDa chitin-binding lectin of *S. tuberosum* L. *cv.* Deshi (StL-20) consisted of a major 20 kDa band with two faint 22 and 17 kDa bands (Fig. 2) under reducing conditions. About 16 mg StL-20 was purified from 300 g of Deshi potatoes and the recovery of the lectin was found to be 33% (w/w) (Table 1).

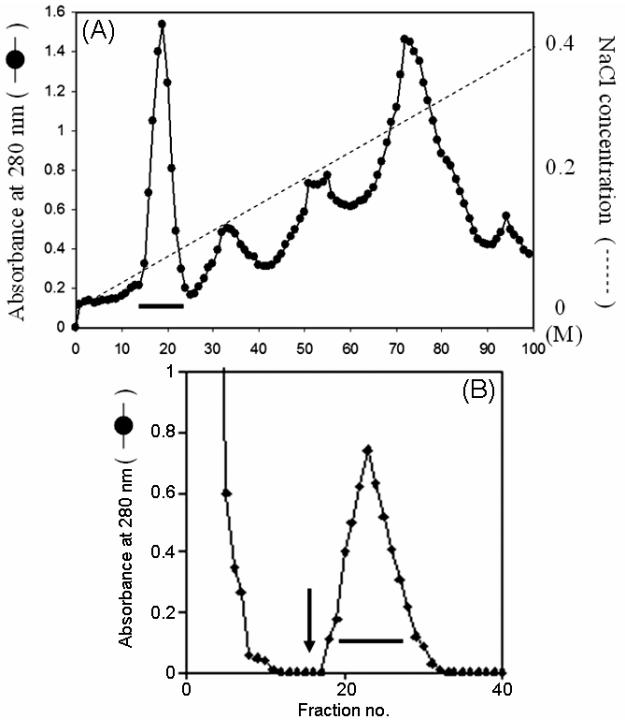

Fig. 1—Purification of StL-20 [(A): crude supernatant from Deshi potato was applied on to DEAE-cellulose column (2 × 25 cm) and eluted by a linear gradient of 0 to 400 mM NaCl containing 10 mM Tris-HCl (pH 8.2). The first peak (with the bar) indicates the fractions containing hemagglutinating activity due to the presence of 20 kDa lectin; and (B): Fractions were collected and subjected on to a chitin column (2 × 25 cm) and washed with TBS. The lectin was eluted by 0.5 M acetic acid (indicated by the arrow)]

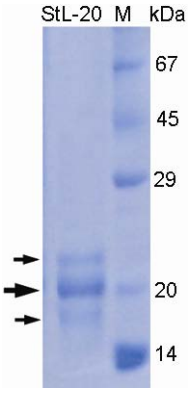

Fig. 2—Molecular mass of StL-20 by SDS-PAGE [Standard protein markers (M): bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and lysozyme (14 kDa). Purified lectin (StL-20): Arrows indicate major (20 kDa) and minor (22 kDa and 17 kDa) proteins]

Table 1—Purification of StL-20 from *Solanum tuberosum*

Purification steps	Titer (HU)	Volume (mL)	Total activity[a]	Protein conc. (mg/mL)	Specific activity[b]	Purification ratio (fold)[c]	Recovery of activity[d] (%)
Crude extract	256	300	76,800	2.5	0.34	1	100
Ion-exchange chromatography	512	66	33,792	0.8	9.70	29	44
Affinity chromatography	1024	25	25, 600	0.65	16.30	48	33

[a]Total activity calculated as titer × volume
[b]Specific activity calculated as titer/mg of protein
[c]Purification ratio calculated by comparing the value of specific activity on the crude extract *vs.* purified lectin
[d]Recovery of activity calculated by comparing the value of total activity on the crude extract *vs.* purified lectin

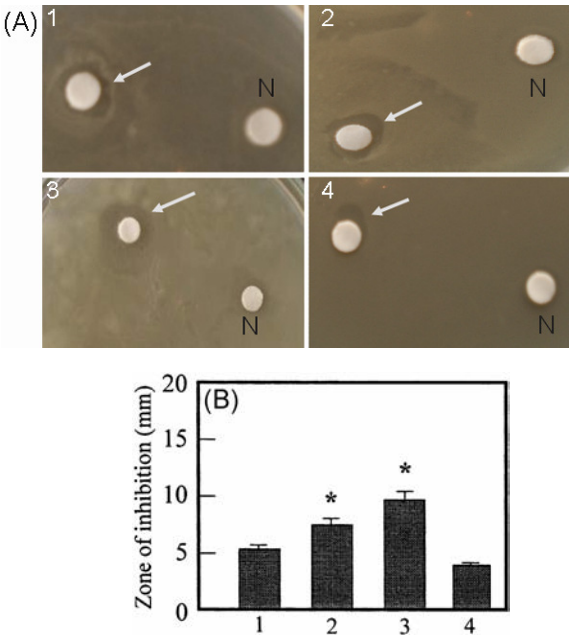

Fig. 3—Antibacterial activity of StL-20 [(A): *E. coli* O157:H18 (1), *S. enteritidis* ATCC 13076 (2), *L. monocytogenes* ATCC 35152 (3) and *S. boydii* ATCC 9905 (4) were plated and 50 g/mL of StL-20 was applied on the discs. Arrows indicate transparent zones of killed bacteria by StL-20; and (B): The zones of inhibition of bacteria by StL-20. Numbers correspond to the bacteria shown in A. Error bars: SE calculated from three independent experiments. The negative control (N) is not graphically represented.*, P <0.05 was considered statistically significant]

Antibacterial activity of StL-20

StL-20 was found to have notable antibacterial activity against *E. coli* O157:H18, *L. monocytogenes* ATCC 35152, *S. enteritidis* ATCC 13076 and *S.boydii* ATCC 9905 (Fig. 3). The gram-positive bacteria *L. monocytogenes* ATCC 35152 was significantly sensitive (Fig. 3B, column 3) to the lectin, compared to the gram-negative *S. boydii* ATCC 9905 (Fig. 3B, column 4).

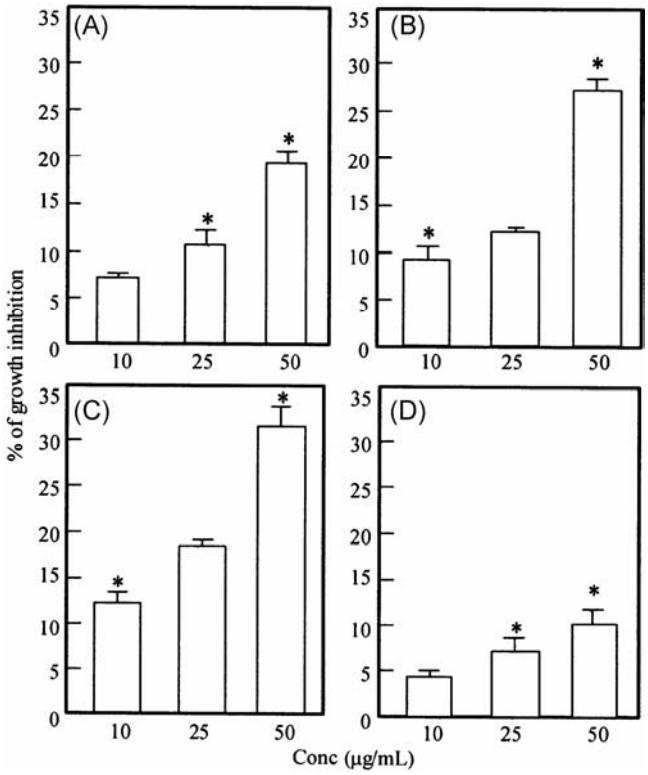

Fig. 4—Bacterial growth inhibition by StL-20 [*E. coli* O157:H18 (A), *S. enteritidis* ATCC 13076 (B), *L. monocytogenes* ATCC 35152 (C) and *S. boydii* ATCC 9905 (D) were cultured in the 96 well-plate with 10, 25 and 50 g/mL of StL-20 for 8 h. Inhibition of the bacterial growth by the addition of StL-20 was measured at OD_{630}. Error bars: SE calculated from three independent experiments. The negative control is not graphically represented. *, P <0.05 was considered statistically significant]

StL-20 inhibited the growth of bacteria

Microtiter plate culture of the bacteria in the presence of lectin indicated that StL-20 dose-dependently inhibited the growth of all tested bacteria (Fig. 4). The growth of *L. monocytogenes* ATCC 35152 was significantly decreased to 36 % by applying 50 µg/mL of StL-20 (Fig. 4C).

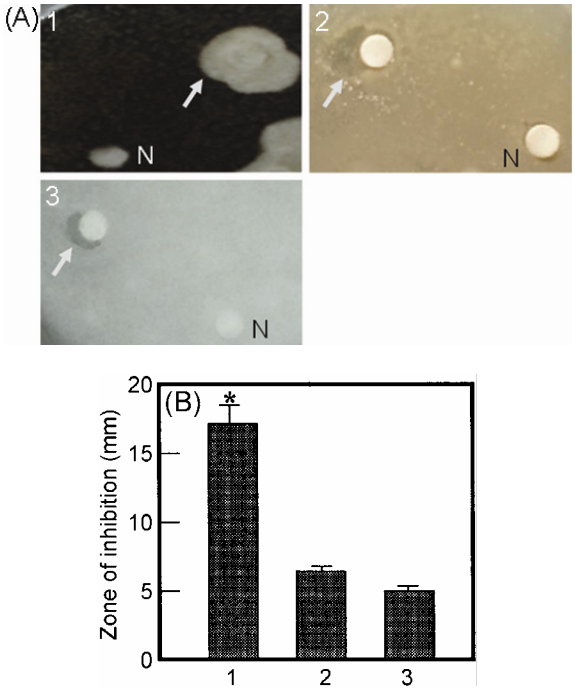

Fig. 5—Antifungal activity of StL-20 [A: *Aspergillus niger* (1), *Penicilium* spp (2) and *Rhizopus* spp (3). 50 g/mL of StL-20 was applied on the discs. Arrows indicate transparent zones of killed fungi by StL-20; and B: The zones of inhibition of fungi by StL-20. Numbers correspond to the fungi shown in A. Error bars: SE calculated from three independent experiments. The negative control (N) is not graphically represented.*, P <0.05 was considered statistically significant]

S. boydii ATCC 9905 was the least susceptible against the lectin (Fig. 4D), showing that this tendency was analogous with the result of bactericidal activity by this lectin (Fig. 3).

Antifungal activity of StL-20

StL-20 exerted antifungal activity against *Rhizopus* spp., *Penicillium* spp. and *A. niger* (Fig. 5A). *A. niger* was strongly susceptible to StL-20 (Fig. 5B, column 1) comparing with other fungi (Fig. 5B, column 2 and 3).

Anti-biofilm activity of StL-20 against *P.aeruginosa* PAO1

StL-20 inhibited the production of biofilm by *P. aeruginosa* PAO1, as revealed by the assay for the quantification of biofilm formation (Fig. 6). Administration of StL-20 reduced the formation of biofilm from 5 to 20% according to the concentrations. The inhibitory effect steadily increased at concentrations 2.5 to 15 μg/mL and became almost constant over 20 μg/mL.

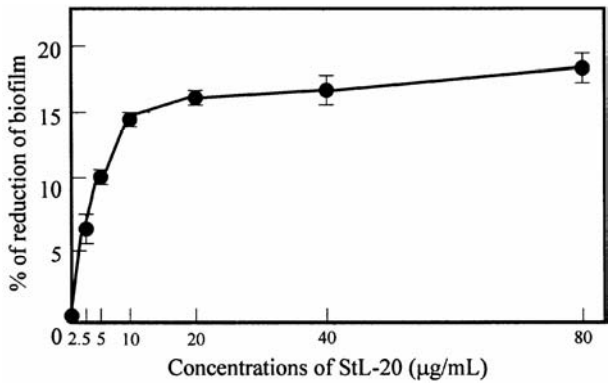

Fig. 6—Prevention of biofilm formation by StL-20 [*P. aeruginosa* PAO1 was cultured in the presence of different concentrations of StL-20 for 12 h in a 96-well culture plate. Formation of biofilm and its reduction in the presence of lectin was detected by measuring the color developed by crystal violet staining at OD_{570}. Error bars: SE calculated from three independent experiments]

Discussion

Purification of StL-20 provided useful knowledge regarding the biochemical diversity of chitin-binding lectins in potatoes[3,4,13,14]. In these lectins, L-arabinose (a pentose) has been found to be the main component of glycans through the O-linkage of hydroxyproline-rich domains[15]. The smear-like minor bands (22 and 17 kDa) around the major 20 kDa band in present study might have been derived from different levels of glycosylation, though it needs further confirmation. However, the classical potato lectin comprises of two nearly identical chitin-binding domains, consisting of 90 amino acids interfused with a highly glycosylated extensin-like domain of 60 kDa[16]. In our study, StL-20 might become less glycosylated around the shorter extensin-like domain. The elucidation of primary structure of StL-20 might provide valuable structural evidence for plant lectins. Besides, the glycan-binding profiles of different chitin-binding lectins in potato tubers can elucidate the roles played by the each molecule.

Plants are more prone to microbial infection compared to animals because they do not have well-developed immune systems and cannot move. To continue the production of offspring from one generation to the next, the reproductive organs like fruit bodies and tubers are developed to store a number of self-protective molecules against the invading microbes. In the *Solanaceae* family, lectins play this role by binding with peptidoglycans and other cell wall components of microorganisms

that contain the GlcNAc polymer. The presence of different chitin-binding lectins in potato tubers provides the resistance against diverse microorganisms having different oligosaccharide structures. Previously, it is reported that an 18 kDa lectin in a sessile animal, Japanese black sponge could also bind chitotriose and core GlcNAc of *N*-linked glycoproteins[17]. It would be interesting to study any correlation between the bactericidal StL-20 and the 18 kDa chitotriose-binding lectin in the Japanese black sponge, which is known to have a symbiotic relationship with bacteria.

StL-20 showed both bactericidal and fungicidal activities, but sensitivity against each strain was different; *L. monocytogenes* ATCC 35152 and *Rhizopus* spp. were comparatively more sensitive to the lectin than the other tested species. The presence of a single outer peptidoglycan layer in the cell walls of Gram-positive bacteria like *L. monocytogenes* ATCC 35152 increases the vulnerabiltiy to many synthetic and natural antibiotics, compared to the multilayered and complex cell walls of Gram-negative bacteria[18]. It might be fascinating to find out, if this difference in bactericidal activity was a mechanism of killing that occurred through a lectin-glycan interaction, though it is still unclear. It could be investigated by examining the relationship between the amount of GlcNAc in microorganisms and the sensitivity of lectins. In case of antifungal activity, perhaps this lectin exerted lethal effects, leading to the disruption of the fungal cell wall like other chitin-binding plant lectins[19,20]. Similar to the lectins, chitinases also play vital roles to prevent the growth of microorganisms in potatoes, indicating the complexity of the regulation of anti-microbial activities of potatoes[21].

P. aeruginosa PAO1 is a well-known bacterial species that can form biofilm to protect itself from natural or synthetic chemicals and antibiotics[22]. StL-20 moderately (5-20%) inhibited the formation of biofilm. As lectins are divalent molecules, StL-20 might have binding sites for saccharides, proteins and/or other major biofilm components and through this process disturbed the polymerization of those components.

It is evident that exquisite recognition molecules like lectins are present in potatoes that can bind with GlcNAc polymers, one of the essential components of pathogenic microorganisms since the primary stage of their evolution[23]. These recognition molecules emerged spontaneously through the continuous process of infections and their protections. Discovery of StL-20 can help to hypothesize that presence of plural number of lectins in potato provides more effective defense capability against diverse invading microbes for their survival and reproduction.

Additionally, these types of antimicrobial lectins can be used in medicine and clinical diagnostics[24], thus can also be regarded as important from a public health perspective. Nowadays, medical device colonization constitutes a global threat. Biofilms are difficult to remove from medical devices like catheters, as well as from a number of pharmaceutical appliances[24,25]. Previously, it is reported that a number of plant lectins tested could reduce the development of dental caries by inhibiting the biofilm formation of *Streptococcus mutans*, though none of those lectins have inhibited the bacterial growth and multiplication[26]. Therefore, the inhibitory effect of StL-20 against biofilm formation may play a promising role in different biomedical and clinical applications.

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