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Purification, Characterization and Bioefficacy of Legume Lectins against Mustard Aphid

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ABSTRACT

Background: Lectins are carbohydrate binding proteins which perform diverse roles in plants. One important role is in plant defense. These proteins hold great potential as entomotoxic proteins as a part of integrated pest management.

Methods: Lectins were purified and characterized from seeds of two legumes, *Glycine max*-Soybean and *Lens culinaris*-Lentil, employing ammonium sulfate fractionation and ion exchange chromatography. Bioefficacy of the purified lectins was evaluated against mustard aphid.

Result: Lectins isolated from seeds of soybean (*Glycine max* agglutinin GMA-I, II) and lentil (*Lens culinaris* agglutinin LCA-I) were purified upto 9.30 (GMA-I), 4.60 (GMA-II) and 8.70 (LCA-I) fold, respectively. Lectin characterization revealed that soybean agglutinin and lentil agglutinin were specific towards D-Galactose and D-mannose, respectively. Insect bioassay was carried out with five different concentrations (10, 20, 30, 40, 50 µg/ml) of purified lectins of soybean and lentil against mustard aphid. The lethal concentration LC_{50} value for GMA-I was obtained as 32.1 µg/ml with a 95% confidential interval of 18.2 to 40.5 µg/ml and that of LCA-I was 19.1 µg/ml with a 95% confidential interval of 9.3 to 26.8 µg/ml. Lentil lectin (LCA-I) with lower LC_{50} value, was found to be the potential candidate for integrated pest management.

Key words: Antibiosis, Characterization, Haemagglutination activity, Lectins, Mustard aphid, Purification.

INTRODUCTION

Lectins are carbohydrate-binding proteins which bind to glycoproteins, glycolipids and also polysaccharides. They recognize specific carbohydrate structures and agglutinate a variety of animal cells by binding to their cell-surface glycoproteins and glycolipids (Van Damme *et al.*, 1998; Bharathi, 2017). Lectins are highly diverse in structure, molecular weight, composition, and number of sugar binding sites present per molecule (Laija *et al.*, 2010). They are widely distributed in nature and can be found in plants, animals and microorganisms (Jawade *et al.*, 2016) with most abundance in plants, especially in legume seeds, where they constitute 15% of the total proteins (Loris *et al.*, 1998; Sagar and Dhall, 2018).

Lectins play an important role in nitrogen fixation, plant defense and stress physiology, symbiotic interactions between the plants and microorganisms, carbohydrate metabolism and packaging of storage proteins (Ayesha and Rao, 2020; Thakur *et al.*, 2013). Lectins are highly resistant to proteolysis, can bind to insect proteins mainly in the gut, thus retain inside the insect body (Lagarda-Diaz *et al.*, 2017). The anti-insect activity of plant lectins against a broad range of insect species has been identified (Fitches *et al.*, 2010). Thus, lectins could act as one of the promising agents against insect pests under integrated pest management strategies. Several insect-resistant transgenics have been developed in economically-important crops like cotton, maize, rice and potato, which primarily carry genes of bacterial origin encoding *Bacillus thuringiensis* toxins (Hussain *et al.,* 2008; Wang *et al.,* 2005). Now other sources of potential insecticidal gene products are also being studied, mainly from plant defense proteins such as lectins or protease inhibitors or both as fusion gene (Singh *et al.*, 2006).

Mustard aphid (*Lipaphis erysimi*) is a major pest of oilseed crops. It is a phloem-sap sucking pest that can cause up to 75% loss in crop yield (Kumar *et al.*, 2011). Parasitic feeding in *Brassica* oilseeds leads to yellowing and curling of leaves, followed by wilting and stunting of plant parts, that eventually results in retarded growth, poor seed formation and low oil content. The aphids are currently being controlled with the indiscriminate use of insecticidal sprays. The focus has recently moved towards developing the alternatives for example, entomotoxic proteins so as to curtail down the usage of chemical pesticides (Jaber *et al.*, 2010). Lectins as entomotoxic proteins could offer a promising strategy that needs to be explored against insect pests (Paul and Das, 2020; Sathyapriya *et al.*, 2012).

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There are a few scientific reports on purification of lectins isolated from soybean and lentil, but the information about the insecticidal effect of purified lectins against mustard aphid is still lacking. Hence, the main objective of the present study was to isolate and purify lectins from the seeds of Soybean variety SL 525 and Lentil variety LL 931, respectively and assess their efficacy against mustard aphid (*Lipaphis erysimi*).

MATERIALS AND METHODS

Plant material

Seeds of pulses (*Glycine max*- Soybean variety SL 525 and *Lens culinaris*- Lentil variety LL 931) were procured from Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana. Experiments were conducted during 2012-13 at Department of Biochemistry, Punjab Agricultural University, Ludhiana.

Protein purification

For isolation, the seedflour (20 g) was defatted with petroleum ether (60°-80°C). The defatted seedmeal (10g) treated with 0.9% sodium chloride solution (100 ml), was shaken for 2 hours and kept overnight at 4°C. After centrifugation at 10,000 rpm (4°C), the supernatant was subjected to $(NH_4)_2SO_4$ fractionation and dialyzed against normal saline solution (Bajaj *et al*., 2001). The dialysed ammonium sulfate fraction (2.5 ml) was loaded on treated ion exchange resin Toyopearl DEAE-650 M column (13 cm × 1.0 cm) equilibrated with 0.01 M PBS (pH 7.4) and eluted the fractions (2 ml each) with stepwise NaCl gradient, 0-500 mM (Bala, 1998; Oliveira *et al*., 2008). The haemagglutination activity of the preparations was determined following serial dilution technique (Liener and Hill, 1953) and the protein content was also estimated (Lowry *et al*., 1951). The same purification protocol was followed for both the legume samples.

Assay for haemagglutination activity

A serial two-fold dilutions of the lectin solution in microtiter U plates were mixed with 50 µl of 2% suspension of red blood cells in 0.9% normal saline and were allowed to stand for two hours at 37°C. Agglutination and the clumping of cells was then observed. A tube containing 50 µl of saline and 50 µl of 2% RBC suspension served as a negative control. One haemagglutination unit (HU) was assigned to the tube with maximum dilution showing haemagglutination. Lectin activity was expressed as HU/ml. Specific activity was expressed as HU/mg protein.

Sodium dodecyl sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of lectins

The molecular mass of purified lectin preparation was studied by SDS-PAGE using the standardized protocol of Walker (1996) with 10% resolving gel and 5% stacking gel composition. Protein bands were coomassie brilliant blue stained to detect proteins after electrophoretic separation (Merril *et al*., 1981).

Temperature, pH, metal ion stability and sugar specificity of lectins

The samples were incubated at different temperatures viz. 40°C, 50°C, 60°C, 70°C, 80°C and 90°C for 30 min to find out the thermostability of lectins (Bala, 1998). Lectin stability at different pH was studied using PBS with a pH range of 5.0-9.0 (Bala, 1998; Oliveira *et al*., 2008). The metal ion requirement for lectin activity was examined by demetalizing the sample and then treating with different metal ions (Kawagishi *et al*., 1990). Sugar specificity of lectins was estimated by determining the inhibition of agglutination with specific sugars. Negative agglutination indicated the specificity for that sugar (Bala *et al*., 2010).

Artificial diet bioassay

Liquid artificial diet mixed with different concentrations (0- 50 µg/ml) of purified lectin were prepared and introduced between the two layers of parafilm forming a diet sachet (Fig 1). Liquid diet consisted of leaf tissue extract of mustard plant and 15% sucrose (Wille and Hartman, 2008). The diet was changed every 24 hours. The insects were reared in the incubator at 22°C at a photoperiod of 16 hours light/8 hours dark. The percent mortality of the aphid was recorded after every 24 hours for a total period of 96 hours.

Aphid mortality

Considering the natural death of the insect, the percentage of insect mortality was determined using Abbott's formula:

$$\% \text{ Mortality} = \frac{X - Y}{X} \times 100$$

Where,

X = Percentage of survivability in the control where no toxin is present.

Y = Percentage of survivability in treated sample.

The data was subjected to Standard Probit Analysis to find out the values of LC_{50} (median lethal concentration). Percentage of mortality values were further converted to probability unit (probit) with the help of computerized program POLO (Russell *et al*., 1977). A linear regression

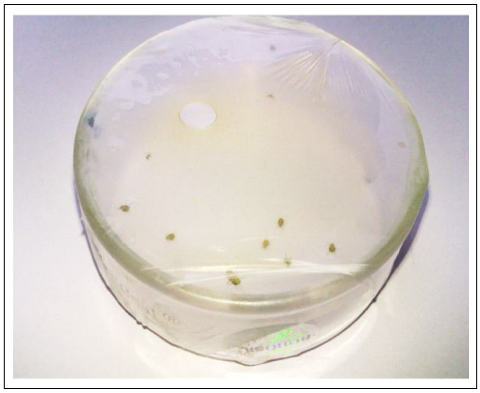

Fig 1: Diet sachet for studying efficacy of purified lectins against mustard aphid (*L. erysimi*).

was obtained by plotting probit values vs. $\log_{10}$ of doses to get the LC_{50} values.

RESULTS AND DISCUSSION

Isolation, purification and characterization

Lectins were extracted and purified from seeds of soybean (*Glycine max*) and lentil (*Lens culinaris*). The purification chart of purified lectins represented in Table 1 and Fig 2. The haemagglutination activity in the elution graph was plotted by considering maximum agglutination activity as 100% (Suseelan *et al.*, 1997). In case of *Glycine max*, the dialyzed protein sample fractionated into two peaks, F1 (fraction 11-20) and F2 (fractions 40-50), on ion exchange chromatography using Toyopearl DEAE 650 M column (Fig 2). The haemagglutination active proteins separated into two isoforms corresponding to respective protein peaks. The first isoform designated as GMA-I (*Glycine max* agglutinin-I) eluted at 100 mM NaCl concentration. The second isoform eluted at 200 mM NaCl concentration and was designated as GMA-II (*Glycine max* agglutinin-II). The recovery of GMA-I was 32.1% with a purification fold of 9.3 and that of GMA-II was 9.4% with a purification fold of 4.6. In *Lens culinaris*, the dialyzed ammonium sulfate fraction when subjected to ion exchange chromatography on Toyopearl 650 M column separated into two protein peaks *viz.* F1 (fractions 14-20) and F2 (fractions 33-42). The haemagglutination activity corresponded with F1 protein peak (fraction 11-21). The peak corresponding to lectin activity eluted at 100 mM NaCl concentration and was designated as LCA-I (*Lens culinaris* agglutinin-I). LCA-I was purified to 8.7-fold with a yield of 27.1%.

The homogeneity of purified lectins was checked on SDS-PAGE. Purified *Glycine max* agglutinin (GMA-I) gave a single band of approx 11 kDa on SDS-PAGE while *Lens culinaris* agglutinin (LCA-I) showed two bands of approx. 14 and 22 kDa respectively (Fig 3). A study conducted by Lin *et al.* (2008) reported that yellow soybean lectin was a tetramer of 30 kDa, while black soybean lectin a dimer of 25 kDa.

Stability studies and sugar specificity of purified lectins

Lectins purified from both the sources were stable upto 40°C with complete inactivation at 70ºC in case of GMA- I while *Lens culinaris* lectin lost haemagglutination activity completely at 80°C (Fig 4). The pH sensitivity profile of the lectins is shown in (Fig 5). *Glycine max* agglutinin (GMA-I) was stable in the pH range of 7.0 to 8.5. pH optimum for GMA-I and LCA-I was found out to be 7.5-8.0 and 7.0-7.5, respectively, corresponding to maximum haemagglutination activity in their respective pH range.

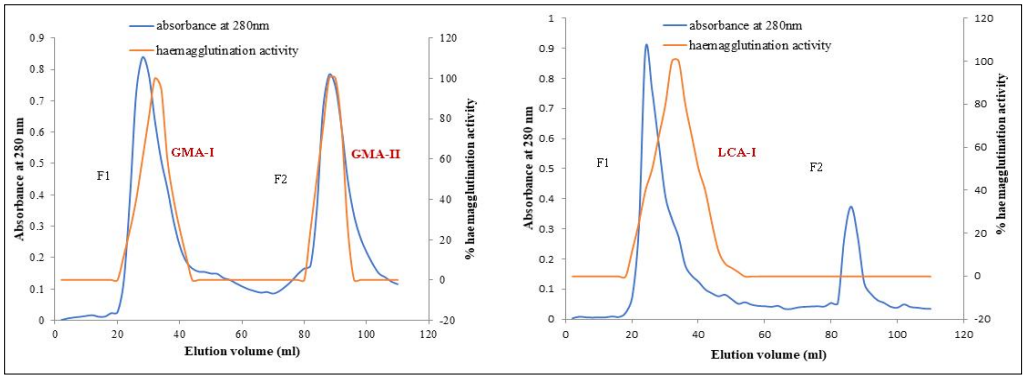

Fig 2: Ion exchange chromatography of purified lectins on Toyopearl resin DEAE-650 M using NaCl stepwise gradient (0-500 mM).

Table 1: Purification chart of isolated lectins.

Legume	Sample	Total protein (mg)	Total HU†	Sp. Act. (HU/mg protein)	Purification fold	Recovery of aggl. act. (%)
Glycine	Crude extract	5180.00	7,16,800.00	138.40	1.00	100
max	0-80% $(NH_4)_2SO_4$ fraction	840.00	3,84,000.00	457.10	3.30	53.6
	Ion exchange chromatography Peak1 (GMA-I-100 mM NaCl)	180.00	2,30,400.00	1280.00	9.30	32.1
	Ion exchange chromatography Peak2 (GMA-II-200 mM NaCl)	105.00	67,200.00	640.00	4.60	9.4
Lens	Crude extract	5200.00	3,20,000.00	61.50	1.00	100
culinaris	0-80% $(NH_4)_2SO_4$ fraction	407.00	1,18,400.00	290.20	4.70	37.0
	Ion exchange chromatography (LCA-I - 100 mM NaCl)	162.80	86,826.00	533.30	8.70	27.1

†HU- Haemagglutination Unit.

The incubation of *Glycine max* and *Lens culinaris* lectin with 10 mM EDTA abolished the haemagglutination activity which was later restored completely with the addition of divalent cations *viz.* 1 mM $MnCl_2$ in *Glycine max* lectins and 1 mM $MgCl_2$, 1 mM $MnCl_2$ in *Lens culinaris* lectins (Table 2). These results are in agreement with the findings of Rao *et al*. (1998) who reported that soybean agglutinin (SBA) contained a single carbohydrate binding site and required Ca^{2+} and Mn^{2+} ions for haemagglutination activity. Similarly, Bhattacharyya *et al*. (1985) also reported the metalloprotein nature of lentil lectin (LcH) that requires the metal ions (Ca^{2+} and Mn^{2+}) for its saccharide binding activity.

Sugar specificity of lectins was evaluated by determining the inhibition of agglutination by different sugars. Minimum inhibitory concentration (MIC) is the lowest concentration of sugar capable of complete inhibition of agglutination. *Glycine max* agglutinin showed specificity towards D-Galactose and N-Acetyl D-galactosamine. Both these sugars were effective for inhibiting the agglutination of rabbit erythrocytes at concentration of 0.01 M (Table 3). Bashir *et al*. (2010) reported the carbohydrate specificity of purified

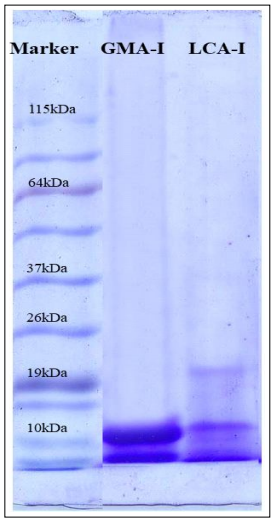

Fig 3: Sodium dodecyl sulfate - polyacrylamide gel electrophoresis of purified lectins.

Table 2: Effect of metal ions on haemagglutination activities (HU/ml) of purified lectins.

Experiment	*Glycine max* agglutinin (GMA-I)	*Lens culinaris* agglutinin (LCA-I)
Normal saline control	1280	640
Saline containing 10 mM EDTA	-	-
Saline containing 1 mM $MgCl_2$	-	640
Saline containing 1 mM $MnCl_2$	1280	640
Saline containing 1 mM $BaCl_2$	-	-

Table 3: Inhibition of lectin-mediated haemagglutination by different sugars.

Sugar tested	D-Mannose	D-Galactose	D-Fructose	D-Glucose	L-Rhamnose	L-Arabinose	N-Acetyl D-galactosa Mine	N-Acetyl D-glucosa Mine	Sucrose	Maltose
Glycine max agglutinin (GMA-I)	+	-	+	+	+	+	-	+	+	+
Lens culinaris agglutinin (LCA-I)	-	+	+	-	+	+	+	+	-	+

"+" Haemagglutination; "-" Haemagglutination inhibition.
‡Maximum concentration of sugars tested was 0.1 M.

soybean lectin towards N-acetyl galactoasmine, galactose and other carbohydrates containing galactopyranosyl residue. In case of *Lens culinaris* agglutinin, the agglutination was readily inhibited by D-mannose at 0.01 M; D-glucose and sucrose at 0.02 M concentration.

Bioefficacy against mustard aphid (*Lipaphis erysimi* kalt.)

Effect of liquid artificial diet mixed with different concentrations (0-50 µg/ml) of purified lectin was studied against mustard aphid. Mortality of aphids was monitored after 24 hours interval upto 96 hours. The data of corrected % mortality at 48 hours is presented in Table 4, Fig 6. The results revealed that mortality increased with increase in lectin concentration. The per cent mortality at different concentrations ranged from 0 to 81.3 for both the purified lectins, GMA-I and LCA-I respectively. The LC_{50} value of the purified lectin against *L.erysimi* was calculated by Probit analysis with a 95% confidence interval and are presented in Table 5. The LC_{50} values obtained were 32.1 and 19.1 µg/ml for GMA-I and LCA-I respectively. It is evident that lower the value of LC_{50}, the more toxic the lectin is. Thus, mannose-

Table 4: Effect of purified lectins on the mortality of mustard aphid (calculated as percentage corrected mortality at 48 hours).

Conc. of lectin (µg/ml)	% Corrected mortality at 48 hours	
	Glycine max agglutinin (GMA-I)	*Lens culinaris* agglutinin (LCA-I)
0	0	0
10	9.4	34.4
20	31.3	46.9
30	43.8	59.4
40	53.1	78.1
50	81.3	81.3

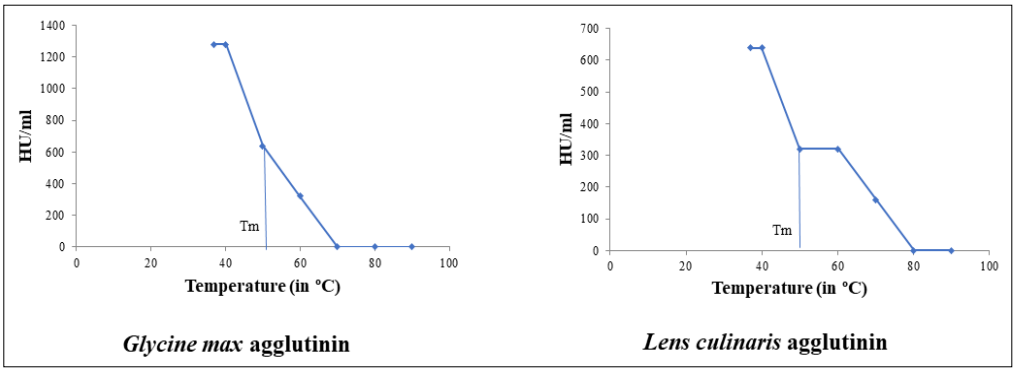

Fig 4: Effect of temperature on haemagglutination activity of purified lectins.

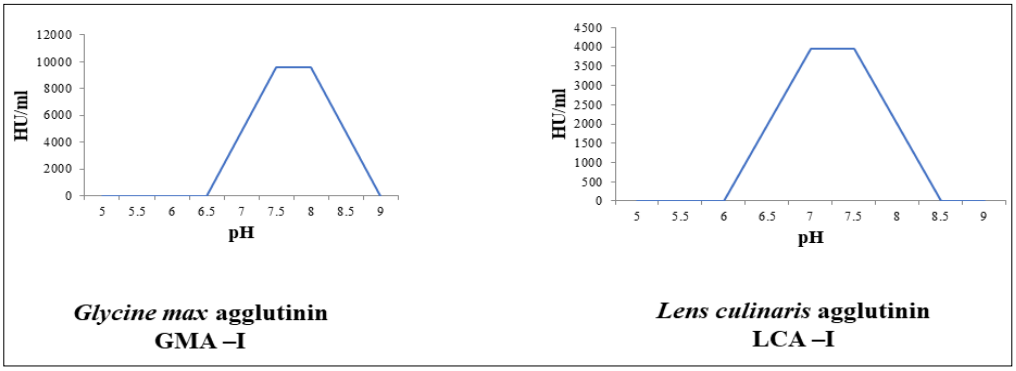

Fig 5: Effect of pH on haemagglutination activity of purified lectins.

Table 5: Dose mortality response of purified lectins against *L. erysimi.*

Lectin	χ^2 value	d.f	Heterogeneity	Slope	LC_{50}	95% Fiducial limits
Glycine max agglutinin (GMA-I)	2.44	3	0.82	3.12±1.0	32.1	18.2 to 40.5
Lens culinaris agglutinin (LCA-I)	1.31	3	0.44	1.97±0.5	19.1	9.3 to 26.8

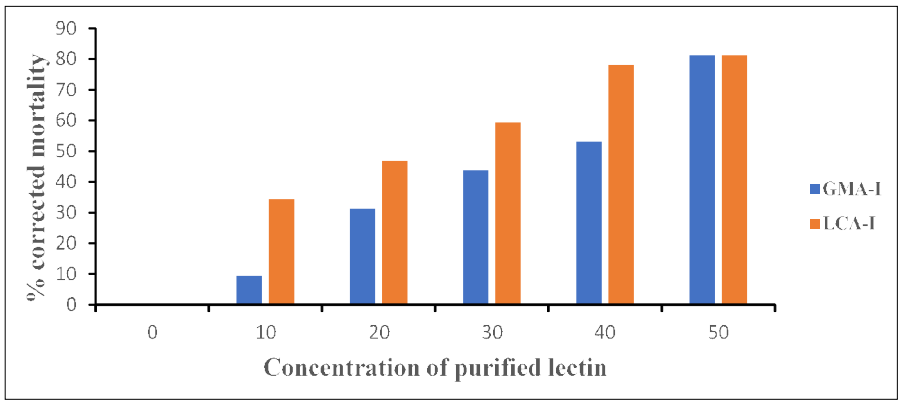

Fig 6: Bioefficacy of purified lectins w.r.t. adult mustard aphid (*L. erysimi*).

specific LCA-I is more effective against mustard aphid as compared to galactose-specific GMA-I. Our findings are consistent with the results of other workers who reported that lectins with mannose binding specificity were most effective against hemipteran insects (Hussain *et al.,* 2008; Majumder *et al.,* 2004; Van Damme 2008; Zapata *et al*., 2016). A lucrative solution to protect crop plants from sap-sucking insects would be the production of transgenic plants expressing lectin genes compared to routine chemical insecticides used to date.

Further, there are some reports which shows that genetic engineering of crop plants based on lectin gene confers wide resistance against whitefly, aphids, lepidopterans and hemipteran insects (Dias *et al*., 2015; Dutta *et al.,* 2005; Boddupally *et al*., 2018).

CONCLUSION

The lectins from seeds of two important legumes viz. *Glycine max* and *Lens culinaris* have been purified to homogeneity. Sugar specificity for *Glycine max* agglutinin and *Lens culinaris* agglutinin was found to be D-Galactose and D-Mannose respectively. Lectin from lentil seeds showed higher mortality rate in aphids in artificial diet bioassay. Based on our results it could be proposed that the lectin gene from lentil could be a potential candidate for the development of transgenic plants which may help to reduce the losses caused by sap-sucking insects in oilseed brassicas.

Conflict of interest

This is Dr. Deeksha, on behalf of all the authors of the article, hereby state that there is no conflict of interest with respect to any financial or non financial matters.

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