

***In vitro* callus induction for determination of lectin activity in pea (*Pisum sativum* L.) variety (AP-1)**

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Abstract

From 8-10 days old seedlings of *Pisum sativum* L. (variety AP-1), various parts root, cotyledon as well as leaf were separated to use as explants. Root and cotyledonary explants did not show any response on MS (Murashige & Skoog) media supplemented with 2, 4-D at a concentration of 5 ppm. However, leaf explants showed callus formation on this media. The callus so formed was subcultured on MS media supplemented with 2, 4-D, IAA, and kinetin. After eight weeks, callus (green and friable) was harvested and used for extraction of lectin protein, known for a wide variety of biological activities in plants, animals and microbes. Seeds were also used to extract lectin protein. Seed extract showed haemagglutination activity of 800 HU/ml, where as callus extract showed 100HU/ml. In seed extract, specific haemagglutination activity was 4 times more than callus extract. Moreover, lectin from seed extract was found to be more thermostable than that of callus extract. In pH range of 6.0-8.0, no loss of lectin activity was observed in seed as well as callus extract. Above and below this pH range, lectin activity showed reduction. Among the various tested sugars, seed as well as callus extract showed specificity for fructose, glucose, maltose, mannose, N-acetyl glucosamine and sucrose.

Keywords: Callus, haemagglutination, lectins, pea, *Pisum sativum* L., proteins

Introduction

Lectins, the carbohydrate binding proteins or glycoproteins of non immune origin, capable of agglutinating and precipitating polysaccharides or glycoconjugates, are widely distributed in nature. These glycoproteins seem to be ubiquitously present among plant, animal and microbial cells. In plant kingdom, majority of these are present in leguminous plants and within the plant itself, lectins are found mainly in the endosperm or cotyledonary tissue of seed (DIAZ & al. [1]), but may also occur to some extent in leaves, stems and root (PUSZTAI & al. [2]), embryo (HOWARD & al. [3]), mature embryonic axis (MELGAREJO & PEREZ [4]) and callus (BHATTACHARYA & al. [5], D'SILVA & al. [6], D'SILVA & PODDER [7], GUPTA & SRIVASTAVA [8], SILVA & al. [9]).

Lectins possess multiple binding sites for combining with specific saccharide branches of lipid and protein molecules present at cell's outer surface and cause agglutination and clumping of the later. A wide variety of cells agglutinated by lectins include red blood cells, lymphocytes, fibroblasts, spermatozoa, yeast, bacterial, and fungal cells. Lectins play an important role in symbiotic interactions between the plants and microorganisms (PUSZTAI

[10]), plant defense and stress physiology, carbohydrate metabolism and packaging of storage proteins (ETZLER [11], KIJNE & al. [12], SHARON & LIS [13], SHUKLA & al. [14]). Lectins are also known to exhibit a wide variety of biological activities in animals (SONI & al. [15]). Lectins have been tested for their toxicity and may be useful as anticancer drugs (OPPENHEIMER & al. [16]) and anti HIV agent (NAEEM & al. [17]).

Lectins from leguminous seeds are among the most extensively studied plant lectins. Lectins found in legume seeds constitute a family of related proteins sharing structural and amino acid sequence homologies (SHARON & LIS [18]). Typically, these lectins are glycoproteins consisting of subunits with molecular masses in a range of 25 to 35 KD arranged as dimers or tetramers. These are particularly abundant in legume seeds, accounting as much as 10 % of the total seed protein (ETZLER [11]). The seed lectins accumulate in protein storage vacuoles of cotyledons and are degraded during seed germination as well as maturation of the seedlings (PUSZTAI [10]). Pea seeds as well as seedling parts i.e. root, shoot etc. have already been reported to contain lectins (DIAZ & al. [1], VAN DRIESSCHE & al. [19], BAJAJ & SONI [20]). In general, vegetative lectins are present at much lower levels than those of seed and also have not been characterized as well. Although some vegetative lectins possess strong homologies with seed lectins, others display unusual carbohydrate-binding characteristics and an inability to agglutinate erythrocytes (PUSZTAI [10], ETZLER [11]).

Plant tissue and cell cultures could be an alternative continuous source of lectins and could also be a useful and important tool to study their regulation and biosynthesis. Although *in vitro* production of lectin from different sources have been reported (BHATTACHARYA & al. [5], D'SILVA & al. [6], D'SILVA & PODDER [7], GUPTA & SRIVASTAVA [8], SILVA & al. [9]) and callus using different explants has been raised using different hormonal combinations in grass pea (*Lathyrus sativus L*) (OCHATT & al. [21]) and pea (*Pisum sativum L.*) (SHARMA & KAUSHAL [22], SIDHU & DAVIES [23]). But there are a few/ no reports regarding the studies on lectins in callus cultures of pea plants. So present study has been undertaken to establish tissue cultures of pea (*Pisum sativum L.*) variety (AP-1) and screening of these for presence of lectins along with seeds. The interest in the present study is based on the fact that lectins have been employed in various biotechnology applications and callus cultures could represent a convenient system to obtain and purify considerable amounts of lectins.

Material and Methods

Seed sample:

The seeds of pea (*Pisum sativum L.*) variety AP-1 were obtained from Agriculture Research Station, Beechwal, Rajasthan Agriculture University, Bikaner.

Rabbit blood:

Rabbit Blood used for making RBC suspension required for haemagglutination study was procured from National Research Centre on Camel (NRCC), Bikaner, Rajasthan, India.

Explant preparation

Seeds were first washed thoroughly under flowing tap water, followed by several washings with solution of liquid detergent and sterile distilled water. Seeds were then treated with 0.1 % HgCl₂ for 2 to 3 minutes followed by treatment with bevisin and then rinsed 3 to 4 times with sterile distilled water. Paper bridges were prepared with filter paper strips taken in test tubes with their ends soaked in distilled water and autoclaved for 15 to 20 minutes. Surface sterilized seeds were aseptically inoculated on the paper bridges. From 8-10 days old

seedlings, roots, leaves and cotyledons were excised aseptically and used as explants for inoculation on MS medium.

Culture media and conditions

The explants were transferred to MS media (MURASHIGE & SKOOG [24]) containing 3% (w/v) sucrose and 0.8% (w/v) agar. MS medium was supplemented with 5 ppm 2,4-D. pH of media was adjusted to 5.8 prior to autoclaving at 121°C and 1.05 kg/cm square (15 psi) pressure for 15–20 minutes. After inoculation, culture vessels were labeled and kept in culture chamber. The culture chamber had aseptic uniform conditions of temperature 26±2°C, 55±5% relative humidity (RH) and diffused light (300 lux).

Harvesting of callus tissues

The callus thus obtained was maintained by frequent sub-culturing after 4-6 weeks on fresh MS medium supplemented with 2, 4-D (1.5 ppm), IAA (0.5 ppm) and kinetin (0.5 ppm). The tissues samples so grown were harvested regularly after 8 weeks for further biochemical analysis.

Biochemical Evaluation of Callus and Pea seeds

Extraction of lectins and determination of haemagglutination activity along with protein content

The lectins were extracted from the pea seeds and callus tissues using normal saline (0.9 % sodium chloride). The crude extract obtained was used for determination of haemagglutination activity and protein content. The haemagglutination activity of lectin extract was assayed by serial dilution technique of LIENER & HILL [25], whereas protein was estimated by the method of LOWRY & al. [26].

Thermostability of pea lectins

In order to determine the thermostability, the lectin extracts were incubated at different temperatures i.e. 40, 50, 60, 70 and 80°C for 30 minutes. The extracts were brought to room temperature and activity was determined by serial dilution technique using rabbit erythrocytes.

Lectin stability under different pH conditions

To study the stability of lectins in media having different pH, phosphate buffer saline (PBS) with pH range of 5.0-9.0 (pH tolerated by rabbit erythrocytes) was prepared. The haemagglutination assay was carried out by mixing 0.1 ml of each extract in 0.9 ml of PBS with pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. The agglutination assay was run by using PBS of the respective pH for the two fold serial dilution as well as for control.

Sugar specificity

Sugar specificity of lectins was determined by finding the inhibition of agglutination activity by different sugars. For this purpose, 0.5 ml of the last dilution preparation showing positive agglutination and 0.1 ml of sugar solution of known molarities were mixed. After incubation for 2 h at room temperature, 0.4 ml of 2% rabbit erythrocytes suspension was mixed with each mixture and observed for haemagglutination. Negative agglutination indicated the specificity for that sugar. Different sugars tested were arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, melibiose, N-acetyl galactosamine, N-acetyl glucosamine, ribose and sucrose.

Statistical analysis

Statistical analysis was performed using Excel 2007 and data was expressed as mean ±Standard Deviation of three replicates.

Results and discussion

Among various seedling parts used as explants on MS media supplemented with 2, 4-D (5ppm), root and cotyledons explants did not show any response whereas the leaf explants showed hypertrophy after inoculation. After 8-10 days of hypertrophy, hyperplasia was

observed. Callus was observed along the midrib and margins of leaf. Complete callusing of explant was observed after 25-30 days of inoculation. The callus so obtained was greenish white in color.

This callus was sub-cultured on MS media supplemented with 2, 4-D (1.5ppm), IAA (0.5ppm) and kinetin (0.5ppm). After 8 weeks, green and friable tissues were harvested. SHARMA & KAUSHAL [22] observed callus induction in pea on MS medium containing 2, 4-D (2mg/l) when used leaf, epicotyls and root as explants. They also reported more callus formation from leaf explant for the various hormonal combinations tested by them. The callus produced from leaf explants in the present study was friable in nature and similar observation has also been reported by SHARMA & al. [27].

Lectins were extracted from the seeds and callus tissues of pea (*Pisum sativum* L.) variety (AP-1) using normal saline. The crude extract from seed showed haemagglutination activity of 800 HU/ml. The protein content was found to be 9.49 (mg/ml) and the specific haemagglutination activity of seed extract was 84.30 (Table1). In case of pea, lectin has already been reported in mature seeds by BAJAJ & SONI [20]. The perusal of data in Table 1 revealed that haemagglutination activity in callus tissues was also detected but it was low (100 HU/ml) as compared to seed extract activity.

Table 1. Haemagglutination activity, protein content and specific haemagglutination activity of seed and callus extract of *Pisum sativum* L. variety AP-1

Sample	Haemagglutination activity HU/ml	Protein mg/ml	Specific activity HU/mg protein
Seed extract	800	9.49 ±0.11	84.30 ±0.98
Callus extract	100	4.42 ±0.03	22.59 ±0.16

Each value is mean ±SD of three determinations.

Protein content was 4.42 (mg/ml) and the specific haemagglutination activity was found to be 22.59 HU/mg protein. The results revealed that seed extract showed 4 times more specific haemagglutination activity than the callus extract. Whereas, SILVA & al. [9] reported that the specific haemagglutinating activity from crude callus extract (2.4 HU/mg) was comparable to that obtained from seeds (3.2 HU/mg). Similarly, GUPTA & SRIVASTAVA [8] reported much higher haemagglutination activity in callus of *Zizyphus mauritiana* than its seeds. But in the present study, pea callus was found to be having less haemagglutination activity as compared to seeds. BHATTACHARYA & al. [5] also reported very less haemagglutinating activity of lectins during *in vitro* lectin production from *Abrus* cultures.

Thermostability test revealed that lectin activity in seed and callus extracts was present up to 70° C and 50° C, respectively. HOWARD & SAGE [28] showed that *Lens culinaris* lectin was stable up to 65° C. In our results, lectin from callus was found to be more thermolabile as compared to seed lectin as shown in Table 2.

Table 2. Thermostability of lectins extracted from seed and callus extract of Pea (*Pisum sativum* L) Variety AP-1

Exposure temperature (°C)	Haemagglutination activity (HU/ml)			
	Seed extract		Callus extract	
	HU/ml	% activity	HU/ml	% activity
37	800	100*	100	100*
40	800	100	100	100
50	512	64	64	64
60	256	32	ND	ND
70	128	16	ND	ND
80	ND	ND	ND	ND

Each value is mean ±SD of three determinations.

NDNot detected

*Activity of preparation incubated at 37 °C was taken as 100 %

Data from Table 3 revealed that there was no loss of lectin activity in seed as well as callus extract at pH range of 6.0-8.0, whereas at pH below 6.0 and above 8.0 there was reduction in activity. The pH sensitivity of *Zizyphus mauritiana* and *Abrus spp.* callus lectin also lies in the pH range 6.0 and 7.6 (GUPTA & SRIVASTAVA [8], SILVA & al. [9]).

Table 3. Stability of lectins at different pH media

pH	Haemagglutination activity (HU/ml)			
	Seed extract		Callus extract	
	HU/ml	% activity	HU/ml	% activity
5.0	512	64	64	64
5.5	512	64	64	64
6.0	800	100	100	100
6.5	800	100	100	100
7.0	800	100*	100	100*
7.5	800	100	100	100
8.0	800	100	100	100
8.5	512	64	64	64
9.0	512	64	64	64

Each value is mean \pm SD of three determinations.

*Activity at pH 7.0 was taken as 100%

Sugars which are specific for pea seed and callus lectins and which inhibited lectin mediated haemagglutination are listed in Table 4.

Table 4. Inhibition of pea lectin mediated haemagglutination by different sugars

Sugars tested *	Haemagglutination activity	
	Seed extract	callus extract
Arabinose	+	+
Cellobiose	+	+
Fructose	-	-
Galactose	+	+
Glucose	-	-
Lactose	+	+
Maltose	-	-
Mannose	-	-
Melibiose	+	+
N-acetylgalactosamine	+	+
N-acetylglucosamine	-	-
Ribose	+	+
Sucrose	-	-

Each value is mean \pm SD of three determinations.

*Maximum concentration of sugars tested was 0.1M

+ haemagglutination - haemagglutination inhibition

It is evident from results that fructose, glucose, maltose, mannose, N-acetyl glucosamine and sucrose inhibited lectin activity of seed as well as callus extract showing they are glucose/mannose specific. Similar kind of results has been observed by other workers in other varieties and different parts of pea (BAJAJ & SONI [20],). Similarly, PUSZTAI [10] and ETZLER [11] had also reported that some vegetable lectins possess strong homologies with seed lectins.

Conclusions

From the present study it is concluded that pea callus raised from leaf explants is friable in nature. The cotyledon and root explants did not give rise to callus formation under

our tested conditions. Lectin activity was detected from pea callus although activity was far less than that of pea seeds. It was found to be thermolabile as compared to pea seed lectin but it performed similar to seed lectin with different sugars and under different pH media.

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