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Isolation and characterization of a lectin with antifungal activity from Egyptian *Pisum sativum* seeds

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Abstract

A plant lectin isolated in its pure state from the Egyptian seeds of *Pisum sativum* (PSL) produced two bands in SDS–PAGE (5.53 and 19.3 kDa; i.e. α and β chain) but one peak by gel filtration chromatography on Sephadex G-100, corresponding to 50 kDa, i.e., a dimeric structure of two monomers, each consisting of one α and one β subunit. PSL is a glycoprotein bound with glucose (2 mol/mol of protein) and stabilized by 2 atoms of each of Ca^{2+} and Mn^{2+} per molecule of protein. It highly agglutinated human, rabbit and rat erythrocytes but weakly agglutinated chicken erythrocytes, while no agglutination occurred with sheep erythrocytes. Hemagglutination was markedly affected by acidic pH, but was heat stable below 60 °C for 30 min. Among the various tested sugars, PSL agglutination was most inhibited by mannose. PSL is rich in hydroxyl amino acids while totally lacking sulfur amino acids. PSL inhibited the growth of *Aspergillus flavus, Trichoderma viride* and *Fusarium oxysporum*.

Keywords: *Pisum sativum*; *Leguminoseae*; Purification; Characterization; Mannose binding lectin; Antifungal; Hemagglutination activity

1. Introduction

Lectins are naturally occurring glycoproteins that bind carbohydrate residues selectively and non-covalently (Van Damme, Van Damme, Prumans, Barre, & Rouge, 1998). Lectins can be found in all kingdoms of life ranging from viruses through bacteria and plants to animals (Loris, 2002). Simple lectins consist of a small number of subunits, not necessarily identical, of molecular weight usually below 40 kDa. This class comprises practically all known plant lectins in addition to the galectins, a family of galactose specific animal lectins (Lis & Sharon, 1998). Legume lectins represent the largest and most thoroughly studied family of the simple lectins. The members of this protein family consist of two or four subunits (or protomers), either identical or slightly different, each with a single, small carbohydrate-combining site with the same specificity. Most legume lectins contain one ion each of tightly attached calcium and manganese per subunit, which are required for carbohydrate binding (Sharon & Lis, 2002). Mannose specific lectins are widely distributed in higher plants and are believed to play a role in the recognition of high mannose glycans of foreign microorganisms or plant predators (Barre, Bourne, Van Damme, Peamans, & Rouge, 2001). Mannose (or glucose) binding lectins form seeds of the family leguminosae including pea (*Pisum sativum*), lentil (*Lens culinaris*), fava bean (*Vicia faba*), common vetch (*Vicia cracca*) and the forage legume sainfoin (*Onobrychis viciiffolia*) have been isolated and well-characterized (Goldstein & Hayes, 1978). Pea lectin of a molecular weight 49 kDa was reported to exist as a dimer, where each monomer built up from two β sheets of seven (curved front face) and six (flat back face) anti parallel strands, respectively, interconnected by turns and loops. A third smaller β-sheet made of five short strands, also referred to as the S-sheet, helps to keep together the front and back sheets. The structures resemble flattened bell-shaped domes containing a shallow pocket at their apex, which forms the

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carbohydrate-binding site. The bottom of the pocket contains binding sites for bivalent metal ions (Barre et al., 2001; Rudiger & Gabius, 2001). In this report, we describe the purification and characterization of a lectin present in Egyptian *P. sativum* seeds cultivated under warm climatic conditions and report on its antifungal activity. The lectin carbohydrate binding specificity, molecular structure, and biochemical properties were investigated and compared to other reports on plant lectins.

2. Materials and methods

2.1. Lectin source

Garden pea (*Pisum sativum*, Lincoln variety) seeds were obtained from Legume Research Institute, Agricultural Research Centre, Ministry of Agricultural, Giza, Egypt.

2.2. Blood samples

Human blood cells (A, AB, B and O) of healthy doners obtained by intravenous withdrawal using heparinized syringes were kindly offered by the blood bank of General Zagazig Hospital. Rabbit, rat, and sheep blood cells were obtained from the animal house of the Faculty of Veterinary Medicine, Zagazig University.

2.3. Fungi

Fusarium oxysporum, Aspergillus flavus and *Trichoderma viride* were obtained from Prof. Tahany Abdel Rahman, Micro Analytical Center, Faculty of Science, Cairo University.

2.4. Chemicals

Sephadex G-100 was purchased from Pharmacia (Uppsala, Sweden). Reagents for electrophoresis were from Bio-Rad laboratories (Richmond, CA, USA). Molecular mass markers for SDS–PAGE were purchased from Sigma (St. Louis, MO, USA). Sugars (all of D-configuration) were obtained form El-Naser Pharmaceutical Chemicals Company, Abu Zaabal, Egypt. All other reagents were either of analytical grade or of the highest quality available.

2.5. Protein determination

The method described by Bradford (1976) was used with bovine serum albumin (BSA) as standard. Absorbance was measured at 280 nm to determine the protein content obtained during the purification process and to monitor elution profiles during chromatography.

2.6. Hemagglutination and sugar specificity tests

Serial twofold dilutions of purified pea lectin (10 mg/ml) in Tris buffered saline (50 μl) were incubated with 50 μl of erythrocyte suspension in V-shaped microtiter plates and the agglutination was scored after 1 h at room temperature. The hemagglutination unit (HU) was expressed as the reciprocal of the highest lectin dilution showing detectable visible erythrocyte agglutination and the specific activity was calculated as HU/mg protein. Hemagglutination activity was assayed against rabbit, rat, chicken, sheep and human ABO erythrocytes (2–4% erythrocyte suspension). Carbohydrate binding specificity of the lectin was assessed by the ability of sugars to inhibit agglutination, measured by incubating 50 μl of serial twofold dilutions of a sugar solution (0.1 M initial concentration) with an equal volume of lectin. After 30 min, 50 μl of a 2–4% suspension of rabbit erythrocytes were added, and the hemagglutination was scored after 1 h. The lowest sugar concentration giving full inhibition of agglutination was determined. The sugars tested were D-mannose, D-glucose, D-sucrose, D-fructose, and D-galactose.

2.7. Purification of the lectin

The lectin was purified from plant materials according to Rüdiger protocol (Rudiger, 1993) as follows: Ten grams of dry seeds were soaked in water at 4 °C overnight, before homogenization in 100 ml of 0.05 M Tris–HCl buffer, pH 8.0 containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.02% NaN_3 first with a house hold mixer for 30 s, then with an ultraturrax for an additional 30 s, and stirred for 2 h at 4 °C. The suspension was filtered through nylon tissue and centrifuged for 20 min at 23,500*g*. Storage proteins were precipitated by slowly adding 1 M acetic acid to stirred solution until pH 5.0 was reached. After an additional hour of stirring at 4 °C, the suspension was recentrifuged (20 min at 23,500*g*) and the supernatant was readjusted to pH 8.0 by adding a suitable aliquot of 1 M NaOH. The latter solution (the prepurified extract) was dialyzed exhaustively against the same buffer.

2.8. Affinity chromatography

Sephadex G-100 was equilibrated with Tris buffer pH 8.0 and transferred to 2.8 × 40 cm column. The prepurified extract was applied to the column at flow rate of 60 ml/h. The column was washed with the buffer at the same speed until the A_{280} fell down to <0.05. The buffer was exchanged by the eluting buffer (0.25 M Glucose, and 0.02% NaN_3) to desorbs the lectin from the column. The fractions containing lectin were combined on the basis of A_{280}, dialyzed against water, frozen and lyophilized.

2.9. Gel electrophoresis and molecular mass determination

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed in a discontinues system with 12.5% separating and 5% stacking gels according to the method of Laemmli and Favre (1973). The samples were boiled for 5 min with 2% SDS in the presence and absence of

β-mercaptoethanol before electrophoresis. Staining the post electrophoresis gel was carried out using Coomassie brilliant blue R-250 for 30 min. The molecular mass standards (Sigma) used included, bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa) and aprotinin (6.5 kDa).

2.10. Gel filtration

The apparent molecular mass of the purified lectin was estimated using Sephadex G-100 column (1.1 × 60 cm) chromatographic analysis (Hagel, 1994). The column was equilibrated with 20 mM Tris–HCl buffer (pH 8.0) containing 150 mM NaCl, 100 mM D-glucose and calibrated with proteins of known molecular masses, i.e.; bovine serum albumin (66 kDa), ovalbumin (45 kDa), and cytochrome C (12.5 kDa). The lyophilized lectin (5 mg) was dissolved in 1 ml of the equilibrating buffer, and centrifuged at 23.500*g*, 4 °C for 20 min. The resulting supernatant was filtered and applied to the column at 60-ml/h-flow rate. The elute absorbance was monitored at 280 nm and compared to the standard mass markers to deduce the molecular weight of lectin.

2.11. pH-dependence of agglutination activity

The effect of pH on lectin hemagglutinating activity was determined by incubating lectin samples at various pHs (1–12) at room temperature (~25 °C) for 1 h. The residual hemagglutinating activity was assayed after adjusting the mixture to pH 7.0. At least five replicates were done for each test to confirm the results.

2.12. Thermal inactivation

The effect of temperature on lectin hemagglutinating activity was determined by incubating lectin samples (pH 7) at various temperatures (30, 35, 40, 45, 50, 55, 60, 65, 70, 75, and 80 °C) at room temperature (~25 °C) for 30 min. The residual hemagglutinating activity was assayed after adjusting the temperature to 4 °C. At least five replicates were done for each test to confirm the results.

2.13. Effect of EDTA, Ca^{2+} and Mn^{2+}

The purified lectin (1 mg/ml) was incubated for 10 h with 50 mM EDTA with continuous shaking. The lectin sample was dialyzed exhaustively against 150 mM NaCl, and the hemagglutinating activity was assessed before and after addition of 50 mM Ca^{2+} and 50 mM Mn^{2+} ions.

2.14. Amino acid analysis

The amino acid composition of experimental samples were determined using HPLC-Pico-Tag method according to Millipore Cooperative (Cohen, Meys, & Travin, 1989). A sample corresponding to 100 mg protein was weighed into 25 × 150 mm hydrolyzed tube, an aliquot of 7.5 ml of 6 N HCl was added, purged with nitrogen for 60 s and the tube was capped immediately. The tube was placed in a 110 °C oven for 24 h, removed and allowed to cool. The contents of the tube were quantitatively transferred to a 25 ml volumetric flask and completed to volume with HPLC grade water. About 1 ml of the solution was filtered through a 0.45 μm Millipore membrane filter. The amino acid derivatization was performed on an aliquot of fifty micro liters of the filtered sample in 6 × 50 mm tubes placed into a drying vial and dried in a Freeze-dryer workstation for 10–15 min. An aliquot (30 μl) of redried solution (consisting of a mixture of 200 μl methanol, 200 μl of 0.2 N sodium acetate and 100 μl triethylamine) was added to the sample tubes and redried again in the workstation. An aliquot (30 μl) of the freshly prepared derivatization reagent [performed by mixing 350 μl methanol, 50 μl (PTC) phenylthiocyanate] was added to the tube contents and allowed to react for 20 min, before drying in the workstation for 15 min. Thirty microliters methanol was added and redried again. Finally, 250 μl of sample diluents (Waters, USA) were added to the dried tube, vortexed and transferred to injection vials. The standard amino acid (Sigma, USA) solution was treated similarly. Liquid chromatography analysis was conducted on Spectra Physics Analytical P4000 Multisolvent Delivery System, USA with Spectra Physics P2000 variable wavelength detector (adjusted to 254 nm) and Spectra Focus optical scanning detector, using stainless steel Pico-Tag amino acids column (150 × 3.9 mm) at a flow rate of: 1 ml/min. The solvent (A) was sodium acetate trihydrate pH 4.6 and solvent (B) Acetonitrile 60%. The amino acids were quantified by comparing the peak area of the unknown sample with those corresponding to standard amino acids using the Spectra Physics Data System Program.

2.15. Carbohydrate analysis

Lectin sample (100 mg) was hydrolyzed by combining with 12.5 ml of 4% sulfuric acid and boiling for 60 min. The hydrolyzed lectin solution was neutralized by the addition of 2 g of barium carbonate to each sample and insoluble barium salts were removed by filtration. The clear supernatant was lyophilized and dissolved in 20 μl water/acetonitrile (8:92 v/v) before injection on a Perkin–Elmer Series 10 HPLC injection and pump unit. The samples were passed at a pressure of 1000 p.s.i at a flow rate of 0.5 $cm^3\ min^{-1}$ through a 25 cm × 4.6 mm SS Amino (sugar) column (Phase separation Ltd) at ambient temperature (22 °C). The eluting buffer was 70/30 acetonitrile/water mixture (v/v), the column eluate was successively mixed with reagent A (1% decolorized 2-cyanoacetamide) and reagent B (0.5 M borate) at 0.5 ml/min by the use of Teflon Y-shaped joint. The mixture was passed through an open tubular Teflon reaction coil (10 cm × 0.5 mm

i.d.) held at 100 °C in a thermostatted bath containing glycerol (80 s contact time) followed by an air – cooled Teflon cooling coil (1 m × 0.5 mm i.d). The absorbance of the eluant was determined at 280 nm (Chaplin, 1994).

2.16. Metal analysis

The presence of Ca^{2+} and Mn^{2+} was determined by a Unicom SP 9 atomic absorption spectrophotometric. Absorption was measured at 422.7 and 279.5 nm using 10 and 12 mA analytical line lamp for Ca^{2+} and Mn^{2+}, respectively, and calculated as mole metal per mole protein.

2.17. Assay of antifungal activity

Antifungal activity was performed on sterile Petri plates (100 × 15 mm) containing 10 ml Dox's agar (Water 1000 ml, $NaNO_2$ 3.0 g, K_2HPO_4 1.0 g, $MgSO_4 \cdot 7\,H_2O$ 0.5 g, $FeSO_4 \cdot 7\,H_2O$, 0.01 g, Sucrose 30.0 g, Agar 15.0 g) sterilized at 15 psi and 120 °C for 20 min. Sterile paper disks, 1 cm in diameter, were placed at the surface of heavily seeded medium with the tested organism. A 10 µl aliquot of the purified pea lectin in 0.05 M Tris–HCl buffer pH 8.0 containing 0.15 M NaCl was added to a disk. Petri dish was incubated at 25–27 °C for 5 day-period, at the end of which the diameter of the clear zone of inhibition surrounding the sample was taken as a measure of the inhibitory power of the sample against the particular test organism (Muanza, Kim, Euler, & Williams, 1994; Grayer & Harbone, 1994; Irob, Moo-Young, & Anderson, 1996).

3. Results

After saline extraction, acetic acid precipitation and dialysis, the crude protein preparation from *P. sativum* seeds was chromatographed on Sephadex G-100 to yield two adsorbed fractions (Fig. 1), designated as A (β-subunit) and B (α-subunit), respectively, in accordance with Driessche, Foriers, Stroberg, and Kanarek (1976). The purification procedure of lectin from seed extract is summarized in Table 1. The lectin from *P. sativum* seeds (PSL) showed variable hemagglutinating activities when tested against chicken, rat, rabbit and human (ABO system) erythrocytes (Table 2), referring to relatively higher potency against rabbit erythrocytes. The carbohydrate binding specificity of the lectin was studied by incubating the lectin with various sugars in the hemagglutination assay of the tested carbohydrates (Table 3). D-Mannose and D-glucose were the most potent inhibitors ($IC_{50} = 5$ mM and 7 mM, respectively) of hemagglutinating activity. Two main protein bands with M_r of 5.53 kDa (subunit α) and 19.3 kDa (subunit β) were obtained in SDS–PAGE either under normal or reducing conditions (Fig. 2a). The apparent molecular mass of the lectin determined by gel filtration on Sephadex G-100 was approximately 50 kDa (Fig. 2b). Amino acid analysis, expressed in moles of amino acid per mole protein (Table 4), revealed the absence of sulphur amino acids and affluent levels of hydroxyl amino acids. The (PSL) hemagglutinating activity remarkably decreased after metal removal by prolonged dialysis against 50 mM EDTA, followed by dialysis against 0.15 M NaCl; however, when Ca^{2+} and Mn^{2+} (50 mM) were readded to the assay medium, the PSL activity was fully restored. Quantitative minerals analysis of purified Pea lectin (Table 5) showed 4 atoms of Ca^{2+} and Mn^{2+} per lectin molecule. So the PSL was Ca^{2+} and Mn^{2+} dependent metalloprotein. PSL was heat stable below 60 °C for 30 min, where it maintained 100% of its activity. Increasing temperature above 60 °C dropped hemagglutinating activity gradually to 90, 60 and then 20% when the temperature was increased to 65, 70 and 75 °C, respectively, until complete inactivation when heating at 80 °C for less than 15 min (Fig. 3b). Hemagglutination was markedly affected by acidic pH, while it maintained 100% of its activity at a pH range 6–8. Decreasing the pH to 5 and 4 lead to decreasing the activity by 30% and 70%, respectively, and the agglutination was completely abolished when lowering the pH below 4 (Fig. 3a). On the other side, increasing the pH above 8, has gradually decreased hemagglutinating activity by 10%, 20%, 60% and 80% at the pH values 9, 10, 11 and 12, respectively. The determination of carbohydrate content by HPLC indicates that PSL is a glycoprotein bound with 2 mol of glucose per lectin molecule. Traces of mannose were also detected (Table 6). Growth inhibition of *F. oxysporum, A. flavus*, and *T. viride* was noticed in the presence of PSL at several serial dilution (Fig. 4) where the original concentration was (100 mg/ml). PSL inhibited the growth of *A. flavus*, *T. viride* and *F. oxysporum* with LIC of 0.1, 0.01 and 0.01 mg/ml, respectively.

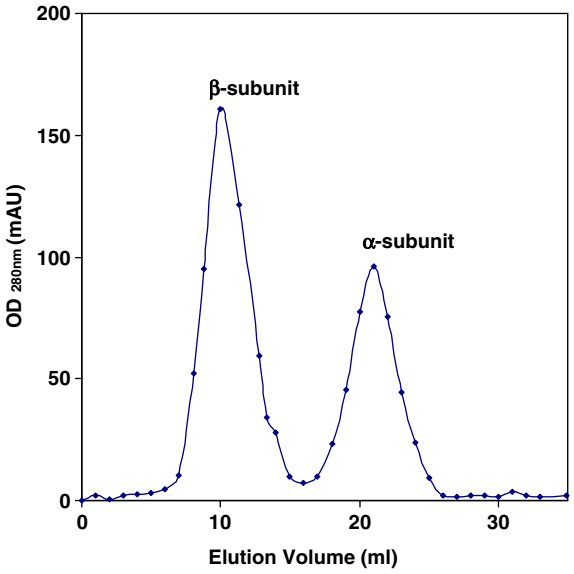

Fig. 1. Affinity chromatogram of pea lectin on Sephadex G-100 column (2.8 × 40 cm) equilibrated with 50 mM Tris–HCl buffer (pH 8.0) containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.02% NaN_3. Lectin was eluted with 250 mM glucose at a flow rate of 60 ml/h.

Table 1
Purification of the lectin from *Pisum sativum* seeds

Stage	Volume (ml)	Protein (mg/ml)[a]	Total protein (mg)	Total lectin (titer[b] × ml)	Specific activity[c] (HU/mg)	Purification fold[d]	Yield (%)
Crude extract	100	42	4200	6400	1.5	1	100
pH 5.0 supernatant	21	28	588	5376	9	6	84
Purified lectin[e]	5	3.2	16	5120	320	213	80

[a] Crude protein extract from 10 g of cotyledons.
[b] Minimal concentration of protein able to cause visible agglutination of a 2–4% suspension of rabbit erythrocytes.
[c] Specific activity is defined as the hemagglutination unit (HU) divided by the protein concentration (mg/ml) of the assay solution. Rabbit erythrocytes were used for the assay.
[d] Purification index was calculated as the ratio between the minimal concentration of the crude extract able to cause visible agglutination of the rabbit erythrocytes and that of the protein fraction obtained at each purification step.
[e] The lectin was obtained by affinity chromatography on Sephadex G-100.

Table 2
Hemagglutinating activity of *Pisum sativum* lectin (PSL) against human and animal erythrocytes

	Erythrocyte source	Agglutination (titer[a])
Human		
	Type A	2^{10}
	Type B	2^{10}
	Type AB	2^{10}
	Type O	2^{11}
Rabbit		2^{12}
Rat		2^{10}
Chicken		2^{6}

[a] Titer is defined as the reciprocal of the end point dilution causing detectable agglutination of erythrocytes. The initial amount of PSL used in these assays was 500 μg and diluted by 1/2 for the subsequent serial dilutions.

Table 3
Carbohydrate inhibition of the hemagglutinating activity of *Pisum sativum* lectin (PSL)

Sugar	IC_{50}[a] (mM)
D-Mannose	5
D-Glucose	7
D-Sucrose	7.5
D-Fructose	14.5
D-Galactose	24

[a] IC_{50}: concentration required to inhibit hemagglutinating activity by 50%. The amount of PSL used in these assays was 500 μg and diluted by 1/2 for the subsequent serial dilutions.

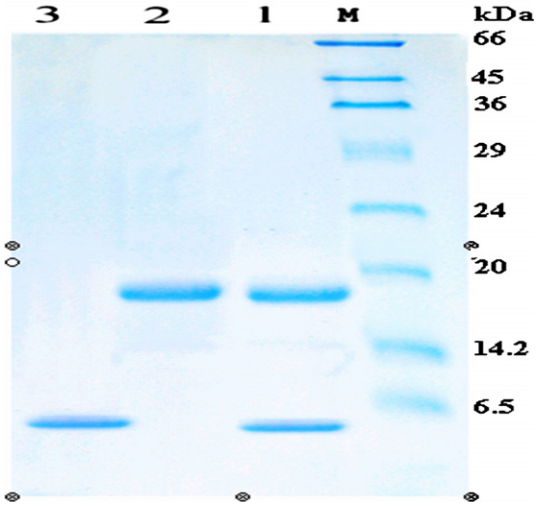

Fig. 2a. SDS–PAGE analysis of PSL. Lane M, molecular weight standards; Lane 1, whole lectin; lane 2, β subunit; lane 3 α subunit.

4. Discussion

The aim of the present study was to isolate a lectin from Egyptian *P. sativum* seeds and investigate its antifungal activity. The lectin could be successfully purified in a single step by affinity chromatography on Sephadex G-100. Loading of prepurified extract on an affinity column followed by washing out the unbound proteins then eluting the bound lectin with 250 mM glucose (Fig. 1) led to increments in the specific activity up to 320 titer/mg corresponding to 80% yield. The final obtained lectin yield was ca. 160 mg per 100 g dry seed weight. This value is exactly the same as obtained by Rudiger (1993). SDS-electrophoretic patterns of PSL in the presence and absence of β-mercaptoethanol manifested two bands of 5.54 and 19.3 kDa corresponding to α and β chains, non-covalently bound (Fig. 2a). Hence, the bands seen in SDS–PAGE most likely represented the subunits of a dimeric lectin; a common occurrence in plant lectins (Van Damme et al., 1998). Each two subunits are bound together to produce one monomer of a molecular weight of ca. 24.8 kDa. Molecular estimation of PSL by gel filtration on Sephadex G-100 in the presence of 100 mM D-glucose produced one major peak corresponding to a molecular mass around 50 kDa (Fig. 2b) referring to the dimeric structure of that lectin. PSL showed no specificity in its ability to hemagglutinate human (A, B, AB and O) erythrocytes and indiscriminately agglutinate rabbit, rat and chicken. However, hemagglutinating activity against chicken erythrocyte was comparatively the lowest one (Table 2). This difference in the agglutination activity may be due to the nature of the glycoproteins protruding on the cell surface, which are weakly or not totally recognized by the lectin (Oliveria

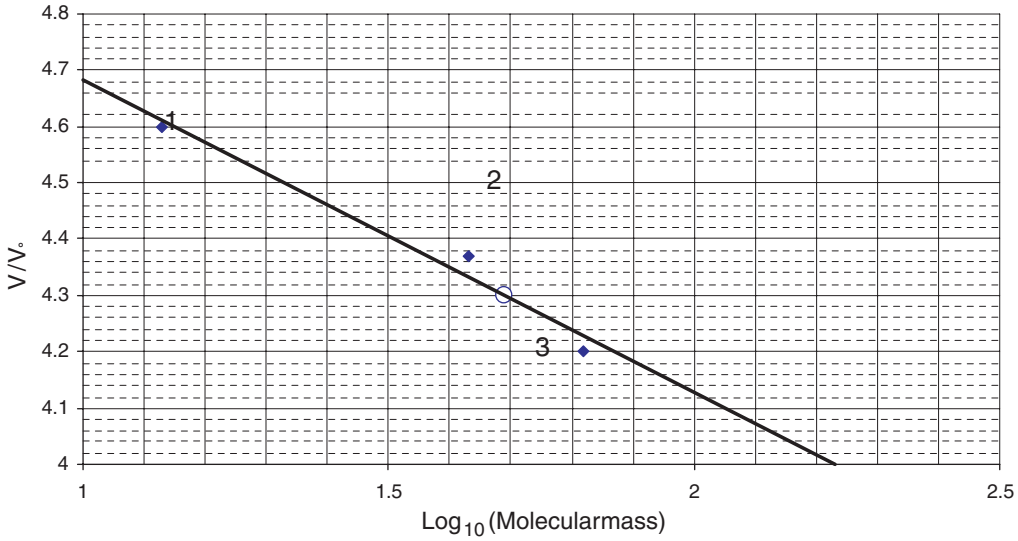

Fig. 2b. Molecular mass estimation of pea lectin by gel filtration affinity. Purified pea lectin and standards were loaded to Sephadex G-100 (Pharmacia) with 50 mM Tris–HCl (pH 8.0) containing 150 mM NaCl and 100 mM glucose. Standard proteins are: (1) Cytochrome C (12.51 kDa); (2) ovalbumin (43 kDa) and (3) Bovine serum albumin (66 kDa).

Table 4
Amino acid composition of α and β subunits of *Pisum sativum* lectin (PSL)

Amino acids (AA)	Mole AA/mol of protein	
	α Subunit	β Subunit
Aspartic	6	19
Glutamic	9	14
Serine	20	24
Glycine	5	12
Histidine	4	5
Arginine	2	8
Threonine	14	32
Alanine	8	18
Proline	2	12
Tyrosine	5	14
Valine	11	18
Methionine	ND	ND
Cystine	ND	ND
Isoleucine	2	16
Leucine	7	16
Phenyl alanine	4	22
Lysine	2	18

Table 5
Mineral analysis of PSL by atomic absorption

Metal	Lamp current (mA)	Wavelength (nm)	Concentration (%)	Gram atom/mol of protein
Calcium	10	422.7	0.16	2
Manganese	12	279.5	0.22	2

et al., 2002). The agglutinating activity of PSL was inhibited by mono and disaccharides, with D-mannose being the most potent ($IC_{50} = 5$ mM) while D-galactose was the weakest one ($IC_{50} = 24$ mM) referring to the highly selectively of PSL for mannose. Although both glucose and galactose are epimers for mannose (Lis & Sharon, 1998), they were different in their inhibitory actions on the hemagglutinating activity. It seems that the conformation of hydroxyl group is very decisive on this activity especially that of hydroxyl groups on C2 and C4 which is highly determinant for this activity. More precisely, the conformation of hydroxyl group on C2 is most determinant for the hemagglutinating activity since it is different in both Glucose and Galactose from that of mannose. The hydroxyl conformation on C4 comes in the next order since it is only the galactose, which has different conformation while glucose has the same conformation as mannose. This comparison explains why glucose was next to mannose in this inhibitory profile while galactose showed the least inhibition. Globally, the inhibition of hemagglutination activity by sugars was similar to that observed with *L. culinars* D-mannose and D-glucose binding lectins (Goldstein & Portez, 1986). The amino acid composition of the purified lectin, generally characterized by the absence of sulphur containing amino acids and high levels of hydroxyl amino acids, is in accordance with the results of Marik, Entlicher, and Kocourek (1974). The hemagglutinating activity of the lectin was inhibited by 50 mM EDTA, indicating that the lectin required divalent cations for its optimal activity. A similar behavior has been reported for several lectins such as the lectin of *Erythrina speciosa* seeds (Konozy et al., 2003), which showed decreased activity after prolonged dialysis against 50 mM EDTA. However, the activity of *Parkia javanica* lectin was not influenced by EDTA (Utarabhand & Akkayamount, 1995). Probably due to different protein structure that does not need such divalent cations for its activity. PSL was a metalloprotein having 4 atoms each of Ca^{2+} and Mn^{2+}/mol protein, providing evidence that purified pea contains one ion each of tightly attached calcium and manganese per subunit (or protomer). This

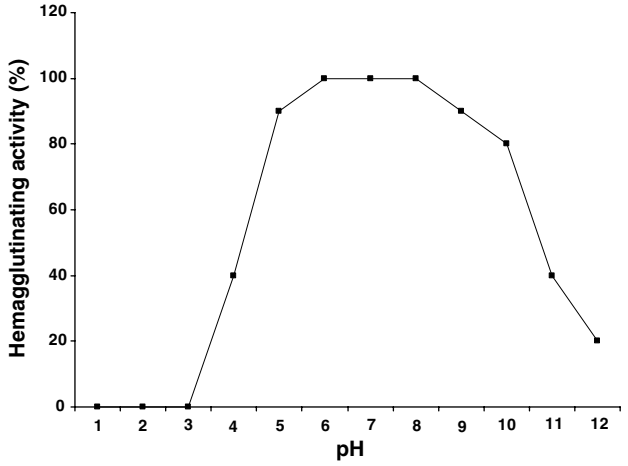

Fig. 3a. Effect of pH on the agglutinating activity of Pea Lectin towards human erythrocyte suspension (Type A). Each point on the line represents the average of five replicates. Full activity (100%) corresponded to a titer of 2^{11}.

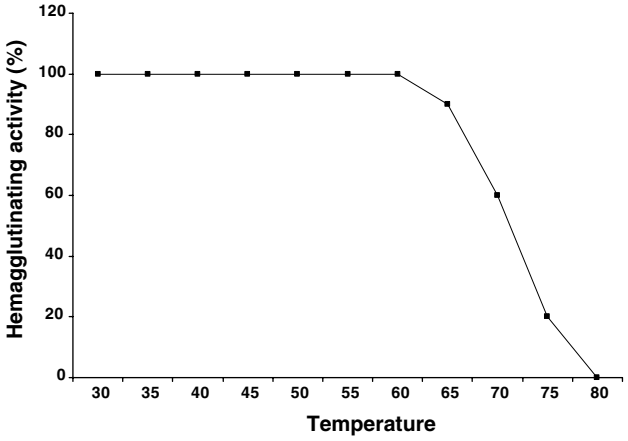

Fig. 3b. Effect of temperature on the agglutinating activity of pea lectin towards human erythrocyte suspension (Type A). Each point on the line represents the average of five replicates. Full activity (100%) corresponded to a titer of 2^{11}.

Table 6
Carbohydrate analysis of PSL by HPLC

Peak No.	Retention time (Min)	Bound sugar	Concentration (%)	Mole of sugar (mol of protein)$^{-1}$
1	7.68	Mannose	0.08	0.23
2	8.94	Glucose	0.74	2.06

finding is similar to that reported by Rini, Hardam, Einspahr, Suddath, and Carver (1993). Thermal denaturation results of PSL showed that the lectin remained significantly stable below 60 °C for 30 min without losing its hemagglutinating activity. Above 60 °C lectin activity was gradually lost until attaining complete loss of activity when heating at 80 °C for less than 15 min (Fig. 3b). The loss of hemagglutinating activity with increasing temperature is evidently

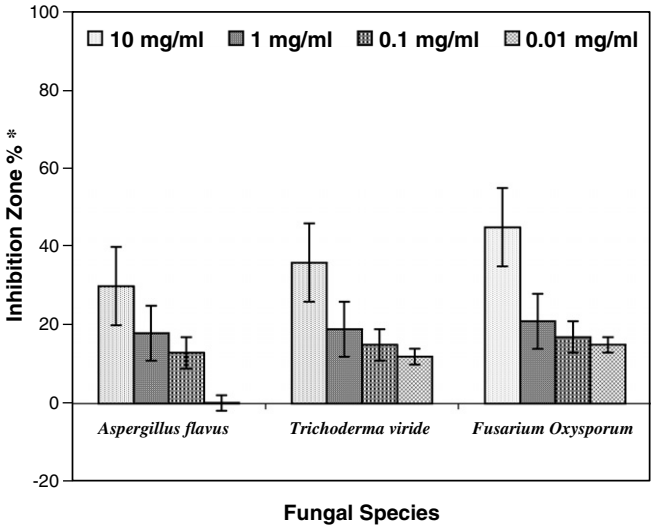

Fig. 4. Antifungal activity of *Pisum sativum* lectin (PSL)* Inhibition zone divided by the total plate zone (90 mm).

due to heat-induced denaturation of the lectin. This denaturation may expectedly weaken the interaction between lectin and the carbohydrate ligand (Schwarz, Puri, Bhat, & Surolia, 1993) leading consequently to attenuated agglutination activity. The pH profile showed that PSL was most stable at pH 5.0–9.0 (Fig. 3a). These characteristics were similar to those of the lectin from *Annona muricata* seeds (Damico et al., 2003). The complete abolishment of lectin hemagglutinating activity at pH lower than 4 is apparently due to the dissociation of lectin into its α and β subunits at the acidic conditions (Schwarz et al., 1993). It was also confirmed that pea lectin secondary and tertiary structures of PSL shows little change at acidic conditions until reaching pH 2.4, where further pH reductions led to successive loss of the ordered structures since this protein has 27 acidic amino acid residues (Naseem & Khan, 2005). The maximum activity is evidently associated with neutral pH where lectin exists as a dimer (Loris, Hamelryck, Bouckaert, & Wyns, 1998; Srinivas et al., 2001). This relationship indicates the importance of dimerisation for lectin hemagglutinating activity. The relatively lower reduction of activity, at the basic pH values may be due to some degree of base-induced denaturation. However, the acidic condition is more influential on the activity due to the dissociation of lectin subunits. In parallel, the acidic conditions lead to leaching out the divalent cations (e.g. Ca^{2+} and Mn^{2+}), which are essential for the stability of lectin structure and activity. The observed antifungal activity of PSL against *A. flavus*, *T. viride* and *F. oxysporum* agrees with the results obtained from other plant legume lectins (Yan, Jiang, Yang, Deng, & Han, 2005; Ciopraga, Gozia, Tudor, Brezuica, & Doyle, 1999; Ye, Ng, Tsang, & Wang, 2001; Peumans & Van Damme, 1995). This activity was concluded to be related to the lectin carbohydrate binding property (Damico et al., 2003), that might endow lectin molecules with binding activity towards certain carbohydrate

components in the fungal cell wall affecting its activity and viability as most lectins recognize either *N*-acetylneuraminic acid, *N*-acetylglucosamine, *N*-acetylgalactosamine, galactose, mannose, or fucose in accordance with the conclusion of Lis and Sharon (1998). Alternatively, it was stated that antifungal activity of some proteins or peptides was associated with chitin binding property and the active proteins should have a specific amino acid sequence and a cysteine/glycine rich chitin binding domain (Grenier, Potvin, Trudel, & Asselin, 1999). This chitin binding property might simulate the carbohydrate binding property as chitin is composed of modified glucose subunits (*N*-acetyl glucose amine) which can be equally recognized by lectin as glucose. Chitin binding might lead to the disruption of the fungal cell wall that increase toxicity (Yun et al., 1998; Chrispeels & Raikhel, 1991), since chitin, which is a major component of the fungal wall, is a polymer of *N*-acetylglucosamine (Ye, Ng, Tsang, & Wang, 2001). Although hydroxyl group on C-2 of sugar molecule is essential for lectin specificity, exists as bound to an acetamide group in case of chitin, this does not preclude the binding interaction between the carbohydrate and lectin. It was previously established that most lectins that bind glucose interact equally with *N*-acetylglucosamine and even the acetamide group may enhance the binding activity (Lis & Sharon, 1998). In parallel many species of lectins (e.g. red kidney beans and wheat germ lectin), which specifically bind the sugar *N*-acetylglucosamine, its oligomers, and chitin were proved to possess antifungal activity (Pusztai, 1993).

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