Glycoconj J (2009) 26:535–545
DOI 10.1007/s10719-008-9205-x

The unique enzymatic function of field bean (*Dolichos lablab*) D-galactose specific lectin: a polyphenol oxidase

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Received: 1 September 2008 / Revised: 10 October 2008 / Accepted: 10 October 2008 / Published online: 31 October 2008

Abstract The polyphenol oxidase (PPO) of field bean (*Dolichos lablab*) is a tetramer made up of two subunits of mass 29,000 and 31,000 Da. The amino acid sequence of the tryptic peptides showed approximately 90% sequence identity to the D-galactose specific legume lectins. The haemagglutinating activity of a pure and homogenous preparation of PPO measured using human erythrocytes was 1261 HAU mg^{-1} protein and was inhibited by D-galactose. Purification by galactose-sepharose chromatography also indicated that the PPO and haemagglutinating activities were associated with a single protein. Crude extracts of other legumes did not exhibit PPO activity, yet cross reacted with anti-PPO antibodies. This dual function protein with PPO and haemagglutinating activity is unique to field bean. The two activities are independent of each other occurring at different loci on the protein. These observations further evidence and strengthen the assumption that galactose specific legume lectins have enzymatic function. Both PPO and lectins are proteins that play a vital role in the defense mechanism of plants. The complementarity of these two simultaneous and independent powerful defense mechanisms exhibited by a single protein renders it a candidate gene for the development of inbuilt plant protection.

Keywords Catechol oxidase · Haemagglutination · Galactose sepharose · Legume lectin · *Dolichos biflorus*

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Abbreviations

MBTH	3-methyl-2-benzothiazolinone hydrozone
PPO	polyphenol oxidase
ibCO	*Ipomea batatas* catechol oxidase
EDTA	ethylene diamine tetra acetic acid
TBS	Tris buffered saline
TPCK	Tosyl phenyl alanine chloromethyl ketone
TBC	tertiary butyl catechol
PVPP	polyvinylpolypyrrolidone

Introduction

Polyphenol oxidase (1, 2 benzene:oxygen oxidoreductase; EC 1.10.3.1, PPO) is a widely distributed enzyme in plants and animals. It is a bifunctional copper containing enzyme, which in the presence of molecular oxygen catalyses the hydroxylation of monophenols to *o*-diphenols (monophenolase, cresolase activity) and further oxidizes the *o*-diphenols to *o*-quinones (diphenolase, catecholase activity) [1]. The generated, unstable highly reactive *o*-quinones subsequently react with themselves, amino acids or proteins evolving into brown, black or red heterogeneous polymers often referred to as melanins. The PPO mediated browning of fruits and vegetables' following damage to the organism suggests a role in plant defense against insects and pathogens [2]. The PPOs have been implicated in chloroplast oxygen scavenging [3] and biosynthesis of water-soluble betalanins [4]. The effect of PPO against herbivory is proposed to reside in the propensity of PPO-generated *o*-quinones to covalently modify and cross-link the dietary protein [5].

A detailed knowledge of the structure of plant PPOs is required to elucidate the diverse functions of this enzyme.

The first three-dimensional structure of a PPO (catechol oxidase) available to date is that from *Ipomea batatas* [6]. The purification and characterization of a PPO from field bean (*Dolichos lablab*) seed, which exists as a single isoform was reported by us [7]. The single isoform of field bean seed PPO, a catecholase renders it suitable for both primary structure determination and three-dimensional analysis. Efforts to obtain the full-length cDNA encoding field bean PPO were initiated to better understand and correlate at the molecular level the structure activity relationship of PPO. The cloning strategies using degenerate primers targeting the NH_2-terminal sequence of field bean PPO and highly conserved Cu^{2+} binding regions of plant PPOs did not yield suitable cDNA. Therefore, internal peptides of the purified PPO were generated by TPCK-trypsin cleavage and sequenced. A BLAST search of the peptides sequences showed >90% sequence identity to the D-galactose specific legume lectins. Recently, Latha *et al.*, [8] reported the purification of a D-galactose specific lectin by affinity chromatography from a different *Dolichos lablab* cultivar, the Indian lablab bean. The NH_2-terminal sequence, native molecular weight and subunit architecture of Indian lablab were identical to that reported for the field bean seed PPO [7]. This prompted us to evaluate the haemagglutinating properties of the purified field bean PPO. In this paper, we report that the purified PPO displayed associated haemagglutinating activity against human erythrocytes and is a D-galactose specific lectin. The PPO and haemagglutinating activities are associated with a single protein referred to as 'PPO-haemagglutinin'. Using inhibitors specific to either PPO or haemagglutinating activity we demonstrate that these two activities are independent of each other and occur at different loci of the protein. In addition, we show the dual lectin-PPO activity among legumes is unique to field bean and not present in other legumes. This observation adds further evidence and strengthens the assumption made by Hankins *et al*., [9] that the galactose specific legume lectins have enzymatic function.

Materials and methods

Materials

Field bean (*Dolichos lablab* var lignosus) seeds were procured from the local market. Catechol, *t*-butylcatechol (TBC) D-galactose, *N*-acetyl-D-galactosamine, D-galactosamine, D-lactose, D-glucose, D-rhamnose, D-arabinose, D-fructose, L-fucose, 3-*O*-methyl D-glucopyranose, tropolone, diethyldithiocarbamate, 2-mercaptobenzimidazole, 2-mercaptobenzothiazole, EDTA, 3-methyl-2-benzothiazolinone hydrazone (MBTH), TPCK-trypsin, Sepharose 6B, DEAE-sepharose, polyvinyldiflouride membrane (PVDF; 0.45 μm), poly(vinylpolypyrrolidone) (PVPP), goat anti-rabbit IgG HRP conjugate, SDS-PAGE molecular weight markers were obtained from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals used were of the highest purity available.

Purification of PPO

Field bean PPO was isolated and purified as reported [7] with minor modifications. Defatted field bean powder (10 g) was extracted for 16 h at 4°C with 0.1 M Tris-HCl buffer (pH 7.0, 50 mL) containing 2% (*w*/*v*) PVPP and 1.2% (*w*/*v*) NaCl. The extract was centrifuged at 15,000 rpm for 45 min. To the supernatant, solid $(NH_4)_2SO_4$ (226 g L^{-1}) was added to obtain 40% saturation at 4°C. The precipitated protein was removed by centrifugation at 15,000 rpm for 45 min at 4°C and discarded. The supernatant was brought to 80% saturation by adding solid $(NH_4)_2SO_4$ (258 g L^{-1}) and allowed to stand overnight at 4°C. The precipitate was dissolved in 0.01 M Tris-HCl buffer (pH 8.2), containing 1.2% NaCl (*w*/*v*) and dialyzed against the same buffer (3×500 mL) and loaded onto a DEAE-Sepharose column (15×4 cm) equilibrated with the same buffer. The column was washed with the same buffer. The PPO was recovered as the unbound protein fraction in the buffer wash (Fig. 1). The active fractions were pooled as shown and subjected to 80% $(NH_4)_2SO_4$ precipitation. The 80% precipitate was loaded onto a HiLoad 16/60 Superdex-200 prep grade column attached to an AKTA FPLC system, equilibrated in 0.1 M Tris-HCl (pH 7.0) containing 1.2% NaCl (*w*/*v*). The PPO

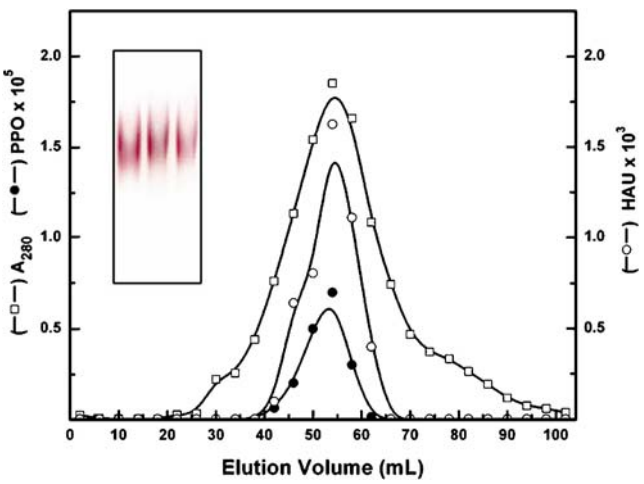

Fig. 1 DEAE-Sepharose chromatography profile of unbound protein fraction. Protein (—□—), PPO (—●—) and HAU (—○—)). The *inset* shows Native-PAGE of fractions collected across the peak and stained for PPO activity with catechol and MBTH. From left to right the lanes correspond to V_e of 50, 54 and 58 mL respectively

active fractions were pooled (Fig. 2) and stored at 4°C for further studies.

Lectin purification by galactose affinity chromatography

A 40–80% $(NH_4)_2SO_4$ fractionation was carried out as described above. The precipitate was dissolved in Tris buffered saline (TBS, 25 mM Tris-HCl buffer pH 7.4 containing 1.2% NaCl). The galactose-sepharose affinity matrix was prepared according to the method described by Sivakumar and Rao [10], wherein mannose was replaced with D-galactose. The D-galactose-sepharose-6B-column (13×3 cm) was pre-equilibrated with TBS containing 1.5 M $(NH_4)_2SO_4$. The 40–80% $(NH_4)_2SO_4$ fraction was applied to the packed D-galactose-sepharose column and washed with the same buffer to remove unbound proteins. The bound protein was eluted with TBS. The fractions exhibiting haemagglutinating activity were pooled as shown (Fig. 3) and stored at 4°C until used.

Polyphenol oxidase assay

Field bean PPO was assayed spectrophotometrically at 25±2°C using a Shimadzu UV-Visible spectrophotometer (Model 1601) at 400 nm using TBC (ε_{400}=1150 M^{-1} cm^{-1}). The assay mixture consisted of 0.9 mL of 0.05 M sodium acetate buffer (pH 4.5), 0.1 mL of 0.04 M TBC and 10–100 μg of enzyme. The quinone formed was measured at 400 nm. One unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of *tert*-butylquinone per minute under the assay conditions. For the inhibition, studies PPO was pre-incubated with the inhibitors/sugars for 10 min and residual PPO activity measured.

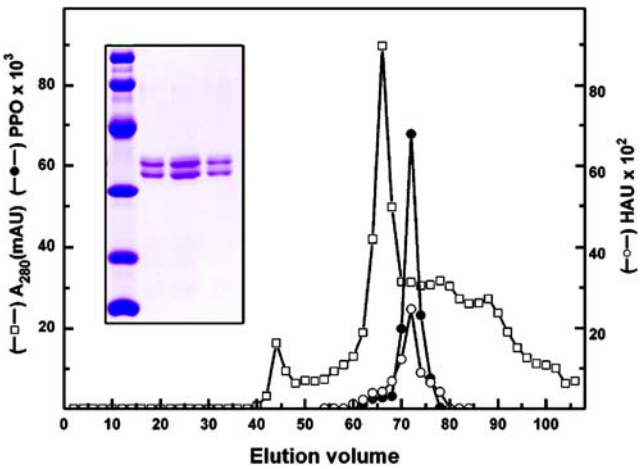

Fig. 2 Size exclusion chromatography (Superdex 200) elution profile of PPO and haemagglutinin activities. Protein (—□—), PPO (—●—) and HAU (—○—). Inset shows SDS-PAGE (12.5% T, 2.7% C) of fractions collected across the peak with molecular weight markers 97,400 Da; phosphorylase, 66,000 Da; ovalbumin, 43,000 Da; albumin, 29,000 Da; carbonic anhydrase, 20,100 Da; soy bean trypsin inhibitor, 14,300 Da; lysozyme. From *left to right* the *lanes* correspond to V_e of 70, 72 and 74 mL respectively

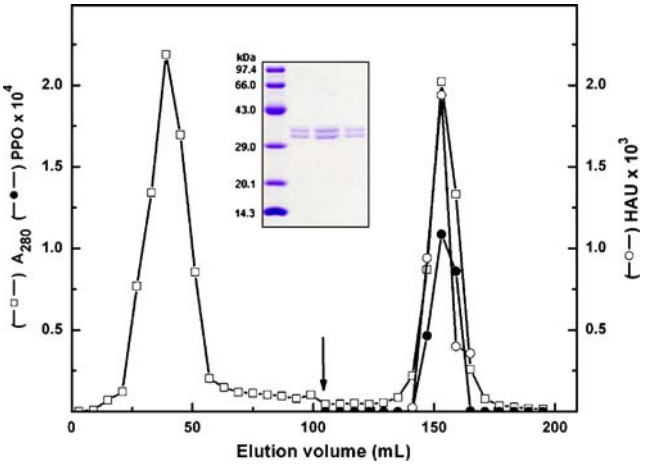

Fig. 3 Galactose-Sepharose elution profile of PPO and haemagglutinin activities. Protein (—□—), PPO (—●—) and HAU (—○—). *Arrow* shows the start of elution with TBS. The *inset* shows SDS-PAGE (12.5% T, 2.7% C) of 100 μL samples collected across the peak. Molecular weight markers 97,400 Da; phosphorylase, 66,000 Da; ovalbumin, 43,000 Da; albumin, 29,000 Da; carbonic anhydrase, 20,100 Da; soy bean trypsin inhibitor, 14,300 Da; lysozyme. From *left to right* the *lanes* correspond to V_e of 148, 152 and 156 mL respectively

Haemagglutination assay

Haemagglutinating activity was measured using 2% tryptinized human erythrocytes suspension in phosphate buffered saline (PBS). Two fold serial dilutions of 200 μL of lectin (10–50 μg) in PBS (pH 7.4) were incubated with 200 μL of 2% suspension of tryptinized human erythrocytes in a microtiter plate for 60 min at 37°C and titer of visible agglutination noted. One haemagglutination unit (HAU) is defined as the lowest concentration of lectin that causes visible erythrocyte agglutination. During purification, the haemagglutinating activity was measured in the presence of D-glucose (0.5 M final concentration) to differentiate the galactose and glucose specific agglutination. The sugar inhibition assays were carried out using stock solutions of the D-sugars dissolved in 0.9% NaCl. To each well 0.1–500 mM of sugar solution and 4 HAU of lectin in a total volume of 200 μL was added and pre-incubated for 1 h at 25±2°C. Later 200 μL of a 2% suspension of tryptinized human erythrocytes was added and incubated for 1 h at 37°C. The lowest concentration of sugar that inhibited agglutination was taken as the minimum inhibitory concentration (MIC). Similar inhibition of agglutination in the presence of PPO inhibitors was carried out.

Protein and carbohydrate estimation

Protein concentration was determined by the dye binding method of Bradford [11]. BSA was used as the standard. Total neutral sugar was determined colorimetrically by the phenol/sulfuric acid method [12] using glucose as the standard.

Polyacrylamide gel electrophoresis (PAGE)

Native-PAGE (7.5% T, 2.7% C) was performed as described by Zhang and Flurkey [13]. Duplicate samples were run simultaneously for protein and enzyme staining. The gels were stained for protein with Coomassie Brilliant Blue R-250. PPO activity was visualized by incubating the gel in 0.05 M sodium acetate buffer (pH 4.0) containing 0.05 M catechol and 0.002 M MBTH. PPO is detected as a pink to red band against a transparent background. SDS-PAGE (12.5% T, 2.7% C) of the column fractions was carried out according to the method of Laemmli [14] at pH 8.8.

Determination of pI

Isoelectric focusing was performed on pre-cast PAG gels (Amersham Biosciences, USA; pH range 3–10) following the manufacturer's instructions. The pH gradients were determined from the results of simultaneous runs performed with the wide range isoelectric protein calibration kit (Amersham Biosciences USA).

Electro blotting of PPO

Protein was transferred to either PVDF or nitrocellulose by a semi-dry procedure [15, 16] using 10 mM CAPS (pH 11.0) containing 10% methanol (*v*/*v*) and 0.1% SDS (*w*/*v*). The membrane was stained in 0.5% (*w*/*v*) coomassie blue R-250 in 50% methanol and de-stained with 50% methanol until the protein bands were visible. The membranes were washed and bands excised for protein sequencing. Alternatively, the transferred proteins were probed with antibodies.

Partial identification of NH_2-terminus

The protein was subjected to SDS-PAGE and transferred to a PVDF membrane by the semidry procedure described above. Protein bands corresponding to the two subunits (29,000 and 31,000) were excised individually and subjected to Edman degradation using the standard protocols on a 477A pulsed gas phase protein sequencer (Applied Biosystems, USA) according to the manufacturer's instructions. Alternatively, the RP-HPLC purified peptides obtained by TPCK-trypsin cleavage were prepared for sequencing using the Perkin-Elmer Prosorb sample cartridges. A β-lactoglobulin standard was used to validate the performance of the instrument.

TPCK-trypsin cleavage

The purified PPO was cleaved at R–X and K–X bond by digestion with TPCK-trypsin at 2:100 (*w*/*w*) enzyme to protein ratio using the following protocol: PPO (5 mg) was dissolved in 50 μL of 0.4 M ammonium bicarbonate containing 8 M urea. The pH was adjusted to between 7.5 and 8.5. Five microliter of 45 mM dithiothreitol was added and incubated at 50°C for 15 min. After cooling to 25±2°C, 5 μL of 100 mM iodoacetamide was added and incubated at 25±2°C for 15 min followed by the addition of water (140 μL). TPCK-trypsin (2% *w*/*w*) was added and incubated at 37°C for 24 h. The sample was concentrated to dryness and re-dissolved in 0.5 mL of 0.1% TFA. The peptides were purified by RP-HPLC [15]. Individual peptides collected over several runs were subjected to Edman degradation as described above.

Erythrocytes haemagglutinin overlay assay

The erythrocyte haemagglutination overlay assay was performed as described by Souza *et al*. [17]. The PPO-haemagglutinin was separated by native PAGE (7.5% T, 2.7% C) and transferred to nitrocellulose using a semidry system operating at 0.8 mA cm^2 for 2 h. The membrane was incubated in TBS containing 1% Triton X-100 for 1 h at 25±2°C. The membrane was washed three times with TBS followed by incubation in TBS containing 1% BSA (TBS-BSA) for 1 h at 25±2°C. The membrane was finally incubated with a human erythrocyte suspension (2% in TBS-BSA) for 1 h at 25±2°C with gentle shaking. The membrane was fixed for 10 min in TBS containing 3% formalin. The lectin band was visible by the bound erythrocytes.

To detect PPO, the membrane with the bound erythrocytes was washed once with TBS to remove formalin and subsequently incubated with TBS for 30 min at 25±2°C. The membrane was then stained for PPO activity using catechol and MBTH as described under PPO assay. PPO appears as a pink band.

Immunodetection of PPO

The legume seed crude extracts were transferred by dot blot onto a nitrocellulose membrane. The membrane was washed with immunoblot buffer (5% skimmed milk powder in PBS, pH 7.0) four times for ~2 h. The washed membrane was incubated overnight (16–18 h) in immunoblot buffer

containing primary antibodies raised against PPO (1:1,000 dilution). After repeated washes in the same buffer, the membrane was incubated with the secondary antibody; horseradish peroxidase (HRP) conjugated goat anti-rabbit immunoglobulin for 2 h at 25±2°C. After four washes in immunoblot buffer and finally in substrate buffer (50 mM sodium citrate, pH 5.5), the HRP activity was detected with a mixture of 3% H_2O_2 and diaminobenzidine in substrate buffer.

Metal ion analysis

The contents of copper and manganese in extensively dialyzed and lyophilized protein samples were measured by atomic absorption spectrometry. Purified PPO-haemagglutinin (10 mg) was refluxed in 2 mL of nitric acid for 2 h and cooled. The volume was adjusted to 10 mL using high purity water exhibiting an electrical resistance of 18-megaohm cm^{-1} (Millipore Corp.). The copper atomic absorption standards (Inorganic Ventures Inc.) and manganese standard (E. Merck Germany) were diluted appropriately. An AA6701F Atomic absorption flame spectrophotometer (Shimadzu Inc Japan) with the following set parameters: 324.8 nm for copper and 279.5 nm for manganese with a 0.2 nm slit width and air–acetylene flame with a 10 cm slot burner was used. The absorption was recorded. Quantity of Cu^{2+} and Mn^{2+} present was determined from the calibration curves of peak height versus standard concentration. Experimental values were corrected for background levels of copper and manganese in the controls without protein.

Results and discussion

Although the first PPO, a mushroom tyrosinase was discovered in 1856, the first three-dimensional structure of a plant PPO, a sweet potato catechol oxidase (ibCO) was reported in 1998 [6]. PPO has been isolated and purified from a variety of plant sources. Pigment contamination and the occurrence of multiple forms have frequently hampered its characterization. During the purification of the glucose-mannose specific lectin from field bean, severe browning of the crude extracts was observed [18]. The extracts showed high PPO activity, when assayed with catechol. Consequently, a single isoform of PPO was purified to homogeneity and characterized [7]. Like many other PPOs, field bean PPO required catalytic quantities of diphenols to activate the monophenolase activity [19]. In keeping with other plant PPOs, the field bean PPO also existed in a latent form. Field bean PPO was activated manyfold by the anionic detergent SDS and on exposure to acid pH [20]. Subtle differences in the structure of diphenols, which render them as either inhibitors or substrates of PPO, was explained by molecular docking using the reported 3-D structure of ibCO [21].

Internal peptide sequence of purified PPO

To investigate the structure-activity relationships peptides were generated by TPCK-trypsin digestion and purified by RP-HPLC. The sequences of the major peptides obtained by Edman degradation are listed (Table 1). A BLAST search of these peptide sequences revealed a high degree of sequence identity to a group of D-galactose-specific legume lectins. Only the lectins showing >70% identity are listed. The generated peptides show a higher sequence identity to the *Phaseolus sp*. Maximum identity (93%) was observed with a group of uncharacterized legume lectins from *Phaseolus filiformus and Phaseolus parvulus.* The list includes the well studied DBL and DB58 of *Dolichos biflorus* [22].

The purified PPO is a lectin

Compelling evidence as detailed by the sequence identity and a recent report of a D-galactose specific lectin [8] with an NH_2-terminal sequence and subunit architecture identical to the purified PPO (7) led to the question 'Is the field bean PPO a galactose-specific lectin?' An answer to this was achieved by purifying the field bean PPO (Table 2) and the D-galactose specific lectin (Table 3) independently and measuring both the activities in the two preparations. PPO eluted as a single unbound protein peak in the DEAE-sepharose column wash (Fig. 1). The haemagglutinating activity is also coincident with this unbound fraction. A native-PAGE profile of 50 μL fractions across the DEAE-sepharose peak reveals a single staining region for PPO (Fig. 1 inset), the intensity of which co-incides with the A_{280} and haemagglutinating activity peak. The active fractions were pooled (Fig. 1), concentrated and subjected to size exclusion chromatography on a Superdex-200 column. As revealed the PPO and haemagglutinating activities are coincident in the same fractions on the descending shoulder of a major protein peak (Fig. 2). SDS-PAGE of 100 μL portions of the three peak fractions (Fig. 2, inset) show the presence of two very similar sized polypeptides of Mr ~29,000 and 31,000 Da. The intensity of the protein stain is coincident with the peak haemagglutinating and PPO activity. The specific activity of PPO and haemagglutination increased ~26 fold. The ratio of PPO to haemagglutinating activity was nearly constant during purification (Table 2) indicative of the same protein exhibiting two independent activities.

The 40–80% $(NH_4)_2SO_4$ precipitate of a crude extract of field bean powder was dissolved in TBS and applied to a

Table 1 Sequence of peptides obtained from TPCK-trypsin cleavage and their sequence identity with legume lectins

Sequence of peptides	Identity (%)	Name and accession number
NAWDPETYHIGIDVNSK	85–90	*Sophora japonica* (bark, AAB51458)
		Sophora japonica (seed, AAB51441)
		Phaseolus leptostaachyus (CAH60215)
		Phaseolus maculates (CAH60256)
		Phaseolus filiformis (CAH60216)
		Vigna unguiculata (CAF18557)
		Phaseolus oligospermus (CAH60173)
		Phaseolus microcarpus (CAH60170)
		Vigna linearis var.latifolia (CAD 43280)
FSIGRAFYTTPIRVWDK	75-80	*Phaseolus oligospermus* (CAH 60172)
		Phaseolus vulgaris (CAD 29133)
		Phaseolus coccineus (CAD27654
		Phaseolus acutifolius (AAA82181)
ATVADGLAFALVPVGAQPR	90–95	*Phaseolus maculates* (CAH60256)
		Phaseolus filiformis (CAH60216)
		Vigna unguiculata (CAF 18557)
		Phaseolus oligospermus (CAH60173)
		Phaseolus microcarpus (CAH60170
		Phaseolus costaricensis (CAH 60989)
		Phaseolus parvulus (CAH 60255)
		Phaseolus augusti (CAH 59200)
		Phaseolus vulgaris (CAD 28674)
		Phaseolusigna linearis (CAD43280)
	80	*Dolichos biflorus* (DBL, DB 58)

galactose sepharose CL-6B column, equilibrated with TBS containing 1.5 M $(NH_4)_2SO_4$. The bound protein was eluted with TBS minus $(NH_4)_2SO_4$. The haemagglutinating activity eluted as a single symmetrical peak (Fig. 3), which also exhibited PPO activity. SDS-PAGE of 100 μL portions of the peak activity fractions show the presence of two very similar sized polypeptides of Mr ~29,000 and 31,000 Da identical to that obtained earlier (Fig. 2, inset). The intensity of the protein staining is commensurate with both the peak haemagglutinating and PPO activity. The haemagglutinating activity was 1,240 HAU mg^{-1}.To exclude the possibility of any hydrophobic interactions with the purification matrix the 40–80% $(NH_4)_2SO_4$ precipitate was applied to a Sepharose CL-6B column equilibrated in TBS. The PPO and haemagglutinating activities were recovered in identical unbound wash fractions (results not shown). This observation further strengthens the galactose binding property of the protein. The purification by galactose–sepharose affinity chromatography summarized in Table 2 shows the ratio of PPO to haemagglutinating activity is constant.

As observed the haemagglutinating and PPO activity co-purify irrespective of the method used (Figs. 1, 2, 3). The specific activity 3.27×10^4 U mg^{-1} obtained for PPO (Table 2), is comparable to that of 3.24×10^4 U mg^{-1} obtained by galactose affinity purification (Table 3). The haemagglutinating activities obtained by the two methods

Table 2 Conventional purification of field bean PPO [7]

Step	Total protein (mg)	Total activity ($U\times10^4$)		Specific activity (U/mg)	Fold purification	Yield (%)	PPO(U) /HAU
Crude extract	474.6	PPO	60.5	1275.8	1	100	26.9
		HAU	2.25	47.4	1	100	
$(NH_4)_2SO4$ precipitation (40–80%)	102.8	PPO	33.3	3239.2	2.54	55.0	26.0
		HAU	1.28	124.5	2.63	56.9	
DEAE-sepharose chromatography	7.14	PPO	14.32	20058.8	15.72	23.7	25.6
		HAU	0.56	784.0	16.54	25.7	
Superdex-200 chromatography	3.0	PPO	9.8	32666.7	25.61	16.2	25.9
		HAU	0.38	1261	26.60	16.9	

These are the results of a typical purification starting from 10 g of defatted field bean powder. These values are reproducable in three separate purifications. HAU was measured in presence of 0.5 M D-glucose

Table 3 Purification of D-galactose specific lectin of field bean

Step	Total protein (mg)	Total activity ($U \times 10^4$)		Specific activity (U/mg)	Fold purification	Yield (%)	PPO(U) /HAU
Crude extract	474.6	PPO	60.5	1275.8	1	100	26.9
		HAU	2.25	47.4	1	100	
$(NH_4)_2SO4$ precipitation (40–80%)	102.8	PPO	33.3	3239.2	2.54	55.0	26.0
		HAU	1.28	124.5	2.63	56.9	
D-galactose-sepharose affinity chromatography	4.64	PPO	15.1	32488.1	25.5	25.0	26.2
		HAU	0.58	1240	26.2	25.7	

These are the results of a typical purification starting from 10 g of defatted field bean powder. These values are reproducible in three separate purifications. HAU was measured in presence of 0.5 M D-glucose

are also concurrent. As little as 0.39 µg of the protein is sufficient to cause agglutination. The agglutinating activity titer is very similar to other galactose specific lectins. The ratio of the HAU to PPO activity is nearly constant between the steps of purification and between the two purification methods (Tables 2 and 3). These results advocate that the two activities are associated with a single protein species hereafter called PPO-haemagglutinin.

An erythrocyte overlay assay was performed to conclusively advocate that the PPO and haemagglutinating activity are associated with one protein. The purified PPO (Table 2) after native-PAGE was electro-blotted onto a nitrocellulose membrane and incubated with human A^+ erythrocytes. Erythrocyte binding was clearly discernable (Fig. 4a). Further, to demonstrate that this protein is associated with PPO activity the membrane was washed to remove formalin and then stained with catechol and MBTH. The appearance of a pink PPO activity band coincident with the previous erythrocyte binding region (Fig. 4b) undoubtedly confirms that the two activities occur on the same protein. A similar assay was used by Souza *et al*., [17] to show the absence of erythrocyte binding in the presence of D-galactose for a latex lectin of *Synadenium carinatum*. These results clearly advocate that the field bean PPO is a lectin.

Molecular mass and NH_2-terminal sequence

The SDS-PAGE profile of the protein from the final purification step of both isolation methods are identical (Figs. 2 and 3 Inset). Previously, SDS-PAGE (10% T, 2–7% C) of field bean PPO showed it to be a tetramer of 30,000± 1,500 Da [7]. Increasing the total acrylamide concentration to 12.5% in this study resolved the PPO in two subunits of almost similar size. The extrapolated molecular weight for the two subunits was 29,000± 2,000 Da and 31,000±4,000 Da. These two subunits were electroblotted and subjected to NH_2-terminal sequence by Edman degradation. The NH_2-terminal sequence analysis led to an unambiguous identification of residues 1–20 for both subunits. The NH_2-terminal sequences of the first 20 residues were identical for both the subunits. The NH_2-terminal sequence was compared and aligned with other legume lectin NH_2-terminal se-

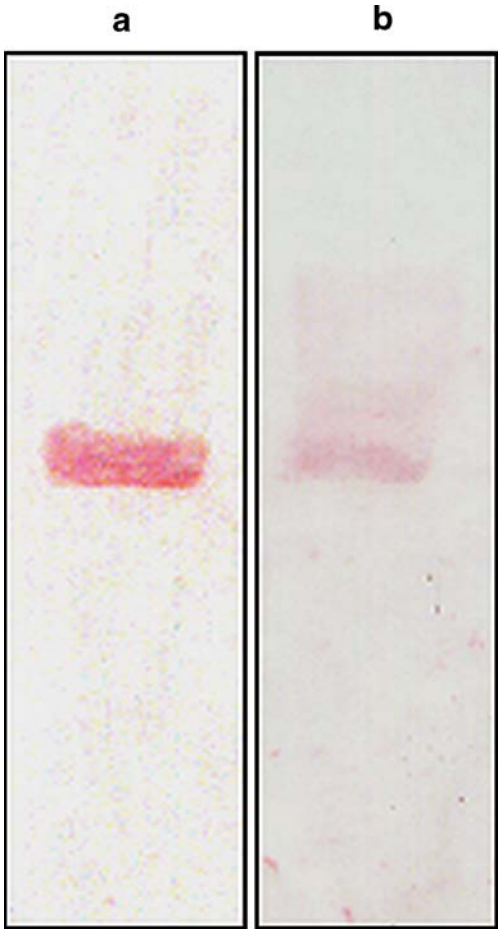

Fig. 4 Erythrocytes overlay assay to detect haemagglutination: *Lane a*; Native PAGE (7.5% T, 2.7% C) in 0.1% SDS of PPO-haemagglutinin transferred to nitrocellulose membrane followed by incubation with human A^+ erythrocytes. *Lane b*; the same membrane washed to remove formalin and stained for PPO activity with catechol and MBTH

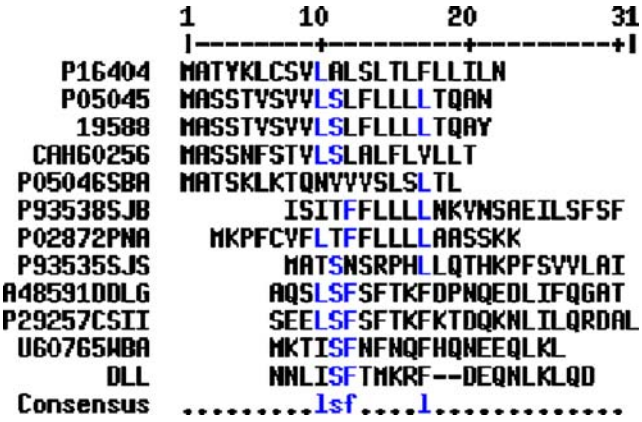

Fig. 5 Multiple alignment of the NH_2-terminal sequences of legume lectins: *P16404*; *Erythrina corollodendron*, *P05045*; *Dolichos biflorus* seed lectin, *19588*; *Dolichos biflorus* (DB58), *CAH60256*; *Phaseolus maculatus*, *P05046SBA*; soybean agglutinin (*Glycine max*), *P93538SJB*; *Sophora japonica* (bark lectin), *P02872PNA*; *Arachis hypogea, P93535SJS*; *Sophora japonica* (seed lectin,), *A48591DDLG*: *Dolichos lablab* (glu/man lectin), *P29257CSII*; *Cytisus scoparius*, *U60765WBA*; *Psophocarpus tetragonolobus*, *DLL*; *Dolichos lablab* (PPO-haemagglutinin this study)

quences (Fig. 5). This comparison showed that the PPO-haemagglutinin has significant sequence identity to other legume lectins. In particular, the consensus sequence I–S–F for legume lectins was present in the sequence of both the subunits. A phylogenetic tree constructed based on the NH_2-terminal sequence shows that the PPO-haemagglutinin clusters with the other D-galactose specific lectins (figure not shown).

The exact molecular weight of the two subunits as determined by MALDI-TOF was 29,808.492 and 31,087.042. The mass spectrum data is consistent with the mass obtained by SDS-PAGE (Fig. 3 Inset). The two subunits differ by a mass of 1,279 Da, which could come from a deletion of 10 or 11 residues. These results coupled with the identical NH_2-terminal sequences of the two subunits suggest that the smaller subunit is a truncated form of the large subunit. These results are similar to that reported for DBL, where the mass difference between the subunits is due to a C-terminal truncation of 10 amino acid residues [23]. The glycoprotein nature of the PPO-haemagglutinin was indicated by the positive phenol-sulfuric acid test as well as glycoprotein PAS staining (results not shown). The estimated content of neutral sugar was 8%, which correlates with the general range (3–12%) for carbohydrate content in lectins [24]. Isoelectric focusing of the purified protein on a precast Ampholine PAG plate showed a pI of 9.3 for the protein. The cDNA sequence (786 bp) of the PPO-haemagglutinin coding for 262 amino acid residues (unpublished results) has since been submitted to Gene Bank EF204527.

Blood group specificity of PPO-haemagglutinin

The haemagglutinating ability of the PPO-haemagglutinin with different erythrocyte types (human, rabbit, rat and sheep) revealed some interesting observations. The protein showed strong haemagglutinating activity when tested against native human blood groups (A, AB, B and O) with no specific group specificity. However, it was distinctly characterized by its slight preference towards the A group (Table 4). The galactose/*N*-acetylgalactosamine specific lectin from the seeds of hairy vetch (*Vicia villosa* Roth) and horsegram, DBL preferentially agglutinate blood group A_1 and have wide applications in blood typing [25, 26]. The sensitivity was enhanced following trypsination of the erythrocytes. The specific activity of 3424 HAU mg^{-1} toward rat erythrocytes was unusual. Preferential agglutination of rabbit erythrocytes over human is however common to most lectins [27]. The PPO-haemagglutinin did not exhibit any agglutination against sheep erythrocytes.

Sugar specificity of PPO-haemagglutinin

The carbohydrate binding specificity of the PPO-haemagglutinin was evaluated by sugar inhibition assays. Several sugars (all sugars of D configuration) were examined for their ability to inhibit the agglutination reaction. Among the sugars tested for the inhibition, galactose and its derivatives galactosamine, *N*-acetyl galactosamine and lactose inhibit the agglutination of human erythrocytes. The minimum inhibitory concentrations of these sugars indicate that it is a D-galactose specific lectin (Table 5). An eight-fold higher concentration of galactosamine was required to inhibit agglutination as compared to D-galactose and *N*-acetylgalactosamine. These minimum inhibitory concentrations are similar to that reported for galactose/*N*-acetyl galactosamine specific lectins [28]. The higher binding capacity of *N*-acetyl galactosamine as compared to galactosamine indicates a preference for the acetylated form.

Table 4 Agglutination of erythrocytes by purified PPO-haemagglutinin

Erythrocytes	HAU mg^{-1}[a]
Human A	1,261
Human B	628
Human AB	1,261
Human O	312
Rabbit	628
Rat	3,424
Sheep	0

[a] Average values of four independent agglutination assays

Table 5 Sugar inhibition of agglutination by PPO-haemagglutinin

Sugars	MIC (mM)[a]
D-galactose	3.9
N-acetyl-D-galactosamine	3.9
D-galactosamine	31.0
D-lactose	500.0
D-glucose	NA
D-rhamnose	NA
D-arabinose	NA
D-fructose	NA
L-fucose	NA
3-*O*-methyl D-glucopyranose	NA

All the sugars used were of D configuration except L-fucose
MIC: minimum inhibitory concentration of agglutination, *NA* no agglutination observed even at 1 M
[a] Average values of four independent agglutination assays

PPO-haemagglutinin is unique to field bean (*Dolichos lablab*)

Crude extracts of horsegram (*Dolichos biflorus*), soya bean (*Glycine max*), kidney bean (*Phaseolus vulgaris*) and Faba bean (*Vicia faba*) seeds were prepared as described above for field bean. Crude extracts equivalent to 45 μg protein were immobilized on a nitrocellulose membrane and assayed for PPO using catechol and MBTH. PPO activity is observed only in the purified protein and field bean extract (Fig. 6A Lanes 2 and 3). Therefore, the presence of a PPO-haemagglutinin is unique only to field bean. Horsegram seed extract does not show any PPO activity either in the gel staining or when assayed with TBC (results not shown), although the two lectins are similar. The faba bean extract shows a trace of PPO activity when assayed with TBC. The well-characterized galactose specific lectin DBL of horsegram seed exhibits an intrinsic lipoxygenase activity far higher than any of the known sources [29]. In contrast, the PPO-haemagglutinin does not exhibit lipoxygenase activity. Antibodies against the purified field bean PPO [7] were available with us. The crude extracts were evaluated for their immunological cross-reactivity with the anti-PPO. The extracts of horsegram, soybean, kidney bean and faba bean reveal cross reactivity (Fig. 6B). Incidentally, a more intense cross reactivity is noticeable with the horse gram extract. Commercially available purified galactose specific lectins from *Dolichos biflorus* (DBL), G*lycine max* (SBA), and *Phaseolus vulgaris* (PHA-M) also showed very strong cross reactivity (results not shown). The cross reactivity of the PPO-haemagglutinin with DBL, SBA and PHA, reveal that these lectins have similar antigenic determinants and are structurally similar, yet the PPO activity is unique to field bean.

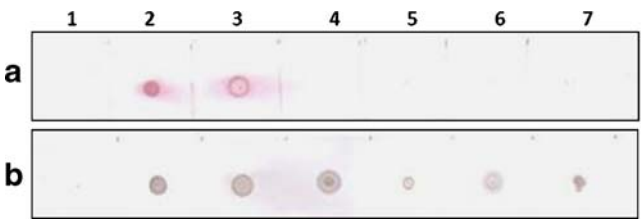

Fig. 6 **A** Dot blot PPO assay of crude legume seed extracts and **B** Immunodetection of PPO by dot blot. *Lane 1*; Bovine serum albumin, *Lane 2*; purified PPO-haemagglutinin, *Lane 3*; Field bean (*Dolichos lablab*), *Lane 4*; Horsegram (*Dolichos biflorus*), *Lane 5*; Soyabean (*Glycine max*), *Lane 6*; Kidney bean (*Phaseolus vulgaris*) and *Lane 7*; Faba bean (*Vicia faba*)

PPO and haemagglutinating activity occur at different loci

The catalytic PPO and haemagglutinating activities are independent of each other. The presence of tropolone a potent competitive inhibitor of PPO (K_i=0.57±0.02 mM) did not alter the agglutinating activity at the concentrations studied (2–25 mM). A very low concentration of tropolone (0.8 mM) is sufficient to bring about 50% inhibition of PPO. At concentrations as high as 10 mM the presence of galactose and *N*-acetylgalactosamine that inhibit agglutination had no effect on the PPO mediated oxidation of TBC.

Table 6 Effect of D-sugars and PPO inhibitors on haemagglutinating and PPO activity

Inhibitor	Concentration (mM)[a]	Relative activity (%)	
		PPO	HAU
Tropolone	0.8[b]	50	100
Diethyldithiocarbamate	0.009[b]	50	100
	0.100	0	100
2-Mercaptobenzimidazole	0.029[b]	50	100
	0.100	0	100
2-Mercaptobenzothiazole	0.001[b]	50	100
	0.10	0	100
EDTA	0.20	98	0
D-galactose	3.9	99	0
	100	98	0
N-acetyl-D-galactosamine	3.9	97	0
	100	97	0
D-Galactosamine	31	99	0
	100	96	0
Lactose	500	98	0

MIC minimum inhibitory concentration of agglutination
[a] Average values of three independent experiments
[b] IC_{50}: Concentration required to bring about 50% inhibition of PPO

Carbohydrate binding activity of legume lectins depends on the presence of metal ions mainly Ca^{2+}. In contrast, PPO activity depends on the presence of Cu^{2+} at the active site. The presence of Cu^{2+} required for PPO activity is evident from the potent inhibition of TBC oxidation by the Cu^{2+} chelators: tropolone, 2-mercaptobenzimidazole, 2-mercaptobenzothiazole and diethyldithiocarbamate (Table 6). Metal analysis showed the presence of 2 mol copper mol^{-1} protein. The Mn^{2+} content was 0.40 mol mol^{-1} protein. The D-galactose binding lectin of *Limulus polyphemus* exhibiting hemocyanin activity contains Cu^{2+} and is inhibited by these chelators [30]. In the presence of these Cu^{2+} chelators, haemagglutinating activity remains unaltered. The haemagglutinating activity was completely abolished in the presence of 0.2 mM EDTA indicating the requirement of Ca^{2+} (Table 6). However, there was no loss in PPO activity at an EDTA concentration as high as 10 mM (data not shown). These results are suggestive of independent sites on the protein for the PPO and haemagglutinating activities.

Conclusions

The purified PPO-haemagglutinin is therefore a unique dual functional protein that exhibits PPO and haemagglutinating activity. Both in mung bean and soybean seeds it has been conclusively demonstrated that the α-galactosidase and haemagglutinin activities are associated with a single species [31, 32]. The soybean α-galactosidase-haemagglutinin can be reversibly converted by pH changes from its tetrameric form, which shows enzyme and lectin activity to the monomeric form, which displays only enzymic activity. In its monomeric form, it is enzymatically active and displays different pH optima and carbohydrate specificity. The PPO-haemagglutinin also undergoes conformational changes, when exposed to acid pH with no change in the quaternary structure. However, a large change in the hydrodynamic radius with a change in PPO pH optima occurs [20]. Changes in the agglutinating power could not be studied with these forms of PPO-haemagglutinin as RBC agglutination occurs in the presence of SDS alone.

The molecular weight, subunit architecture and NH_2-terminal sequence of the D-galactose specific PPO-haemagglutinin indicate that it is distinct from the glucose/mannose specific lectin of the same seed. These results support the previous conclusions of Hankins *et al.* [9] that at least two distinct non-homologous classes of proteins with haemagglutinating activity exist in legume seeds. The galactose specific haemagglutinin of field bean is associated with exceptionally high levels of PPO activity (Tables 2 and 3). Using TBC and catechol as substrates it is evident that the PPO activity is unique only to this legume. The premise that the sugar binding and PPO activity exist on different loci is reflected by the inhibition studies (Table 6). Parisi *et al.* [33] also report an additional cysteine-peptidase of the well known mannose specific lectin from garlic (*Allium sativum*) bulbs.

Observing that the galactose specific lectins of *Vigna radiata*, soybean, *Phaseolus vulgaris* and mung bean exhibit an associated enzymic function, Hankins and Shannon [34] opined that most if not all galactose specific legume lectins have enzymatic function. Both the field bean PPO-haemagglutinin and horsegram DBL-lipoxygenase [29] are galactose specific lectins, which aptly justify this assumption. All these observations coupled lead to an important conclusion that a distinct class of multifunctional functional proteins termed 'enzymic lectins' do exist in legume seeds. All the reported enzyme activities associated with the lectin also play an important role in plant defense against predators as inferred from toxicity to insects, and inhibition of fungal pathogenesis. PPO is a direct plant defense enzyme that cross-links proteins and decreases the nutritive value of an insect wounded plant. Plant lectins are capable of interfering with the functioning of pathogens/predators through interactions on the surface or in the digestive tract. We conclude that this powerful dual defense mechanism of the PPO-haemagglutinin renders it a candidate gene for the development of inbuilt plant protection. The physiological implications of the presence of two different orders of plant defense exhibited by a single molecule need further investigation.

Acknowledgements The authors wish to thank Dr V. Prakash, Director CFTRI, Mysore for his keen interest in this work. We wish to thank Dr A. G. Appu Rao, Head, Department of Protein Chemistry and Technology, CFTRI, Mysore for the useful discussions. We acknowledge the technical help of Dr Asha Martin, Scientist, Food Safety and Analytical Quality Control, CFTRI and Mr K Anbalagan, Central Instrument Facility and Services, CFTRI. SRK and DHR are recipients of a CSIR Senior Research Fellowship, New Delhi. This study was carried under the CSIR networking project: NWP 009.

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