RESEARCH ARTICLE

Seeds of Amazonian Fabaceae as a source of new lectins

Andreia V. Fernandes[1], Márcio V. Ramos[2], José Francisco C. Gonçalves[1]*, Paulo A. C. Maranhão[1], Larissa R. Chevreuil[1] and Luiz Augusto G. Souza[1]

[1] Laboratory of Plant Physiology and Biochemistry, National Institute for Research and Innovation in the Amazon (MCTI-INPA), Manaus, Amazonas, Brazil

[2] Federal University of Ceará, Biochemistry and Molecular Biology Department, Fortaleza, Ceará, Brazil

*Corresponding author: Fone/Fax +55-92-3643-1938; jfc@inpa.gov.br

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ABSTRACT

Seeds from fifty native Amazonian Fabaceae species (representing subfamilies Caesalpinioideae, Mimosoideae and Faboideae) were screened for the presence of new lectins. Their crude protein extracts were assayed for hemagglutinating activity (HA). The protein fractions of *Anadenanthera peregrina*, *Dimorphandra caudata*, *Ormosia lignivalvis* and *Swartzia laevicarpa* exhibited HA, and this activity was inhibited by galactose or lactose but not by glucose or mannose. The crude extract of *S. laevicarpa* exhibited HA activity only after ion exchange chromatography, and its lectin was further purified by affinity chromatography on immobilized lactose. Despite the large number of lectins that have been reported in leguminous plants, this is the first description of lectins in the genera *Anadenanthera, Dimorphandra* and *Ormosia*. The study of lectins from these genera and from *Swartzia* will contribute to the understanding of the evolutionary relationships of legume lectins in terms of their protein processing properties and structures.

Key words: Evolutionary relationships; plant lectins; protein-carbohydrate interactions; *Swartzia*; tropical Fabaceae

INTRODUCTION

Bioprospecting from wild plant diversity (i.e., natural bioresources) in search of useful medicinal, agricultural and other non-wood forest products can help solve some of humanity's pressing challenges. This effort can also help address evolutionary questions that have not yet been answered. Plant lectins found in the seeds of Amazonian leguminous species are a particularly intriguing target for bioprospecting. Lectins are constitutive proteins that possess non-catalytic carbohydrate-binding sites and are non-immune origin (Van Damme et al., 1998). Lectins are widespread in nature, and a detailed definition based on their structural features has been proposed to classify these proteins (Mo et al., 2000; Pinto et al., 2009; Ito et al., 2011). Lectins have been reported in bacteria, viruses, fungi, animals and plants, but most known lectins are found in legume seeds (Nasi et al., 2009).

Because lectins can bind glycoconjugates regardless of their origin, they have become an important tool in a wide variety of studies, including cell-cell recognition and cell activation (Debray et al., 2009; North et al., 2011). They also provide a unique model to study protein-carbohydrate interactions at the atomic level (Oliveira et al., 2008; Debray et al., 2009). These potential uses have motivated the continued search for new lectins and for seeds to be prioritized for screening. Although more than a century has passed since the first lectin was reported, and some

screening studies periodically reveal novel lectins, many potential lectin sources remain to be examined. Throughout the history of lectin research, legume seeds have been screened for lectin activity. Almost all of our current knowledge regarding the structure, specificity and affinity of lectin-carbohydrate interactions has been achieved by studying legume lectins (Loris et al., 1998). Furthermore, structural data for legume lectins have helped to establish the evolutionary relationships among leguminous taxa (Rougé et al., 1987; Rougé and Varloot, 1990; Barre et al., 1994). The amino acid sequences and post-translational processing of lectins from the more derived legume tribes, such as Parkiae and Phaseoleae, have proven useful in supporting the chemotaxonomy of these groups (Gallego del Sol et al., 2005; Moreno et al., 2008). However, numerous legume taxa, especially in the more primitive groups, such as Sophoreae and Swartziae (Subfamily Faboideae), have been poorly studied. The study of lectins of the genera *Swartzia*, classified in the tribe Swartzieae, it will contribute to the understanding of the evolutionary relationships of lectins of legumes, in terms of the protein processing properties and structures, since the species sheltered in this tribe they possess floral morphologic characteristics of transition between subfamily Caesalpinioideae and Faboideae (Herender, 1994).

Except for lectins derived from *Sophora japonica* (Ueno et al., 1991; Van Damme et al., 1997) and a preliminary report on *Swartzia pickelii* (Cavalcanti and Coelho, 1990), no lectin has been described in either of these groups. To describe novel plant lectins, the seeds of fifty species of native Amazonian Fabaceae, including representatives of Sophoreae and Swartziae, were screened for HA. Here, we report the occurrence of lectins in previously unstudied legume groups. In addition, we investigate the hypothesis that the plant lectins found in seeds from different subfamilies of Fabaceae in the Amazonian rain forest reflect the evolutionary history of these taxa.

MATERIAL AND METHODS

Botanical material: The mature seeds of fifty native Fabaceae species were collected throughout 2010 at various sites in the Amazonian rain forest, Brazil. Individual vouchers were prepared for each seed collection. These materials were deposited at the institutional herbarium of the National Institute for Research in the Amazon (MCTI-INPA) and were identified by specialist in Fabaceae. Table 1 lists the species that were studied. The seed coat was removed, and the seeds were powdered and stored at 4°C until they were used.

Chemicals: Experimental carbohydrates, electrophoresis reagents, α-lactose-agarose and DEAE Sepharose were obtained from Sigma-Aldrich Chemical Company (St. Louis, USA). Molecular weight markers were purchased from Promega Corp., Madison, WI, USA. All other chemicals employed in this study were of analytical grade.

Protein extracts: The powdered seeds were suspended in 150 mM NaCl (1:10, w/v) and stirred for 2 h at 25°C. The resulting suspensions were centrifuged at 20,000 x *g* at 4°C for 20 min. The precipitates were discarded, while the soluble phases were dialyzed in distilled water for 72 h at 4°C. The soluble protein fraction of each extract was recovered after an additional centrifugation step, as described above, and freeze dried. These lyophilized extracts were used in all further determinations.

Hemagglutinating assays: The extracts were screened for lectin activity by determining the hemagglutination (HA) titer of each sample in a 2% (v/v) rabbit or rat erythrocyte suspension in a U-bottomed microtitration plate. The protein samples were dissolved in Tris-buffered saline (100 mM Tris-HCl, pH 7.6, added to 150 mM NaCl) at 5 mg/mL and serially diluted in the plates using the same buffer before the erythrocytes (25 μL) were added. The assays were stored at 25°C, and the HA titers were recovered after 2 and 24 h. The inhibition of HA activity was evaluated as previously described (Ramos, 1997). Briefly, the samples that exhibited minimal HA activity were first incubated with decreasing concentrations of different carbohydrates (100 mM) and further mixed with 2% (v/v) erythrocyte suspension. The inhibition of hemagglutination was recorded after 24 h.

Chromatography: The samples were subjected to ion-exchange chromatography on DEAE Sepharose that had been equilibrated with 50 mM Tris-HCl, pH 8.0. After

loading, the samples were first eluted in the same buffer, followed by the stepwise addition of NaCl (0.1 M, 0.5 M and 1 M). The absorbance of the collected fractions was monitored at 280 nm. The fractions containing each recovered protein peak were assayed for their HA activity as described above.

Affinity chromatography was performed on α-lactose-agarose in buffered saline containing 5 mM $CaCl_2$ and 5 mM $MnCl_2$. The samples (200 mg) were applied to the column and initially eluted with buffered saline. After washing the unbound proteins, the column was eluted with 100 mM glycine, pH 2.6, containing 150 mM NaCl. Each protein fraction was assayed for HA after dialysis and lyophilization.

Electrophoresis: Polyacrylamide gel electrophoresis (SDS-PAGE) was used to examine the protein fractions from the chromatography peaks according to the Laemmli method (Laemmli, 1970). The protein samples were dissolved in 0.0625 M Tris-HCl, pH 6.8, under denaturing conditions (1% SDS and 1% β-mercaptoethanol) and loaded on a 12.5% polyacrylamide gel. The proteins were separated at 200 V and a maximum current of 15 mA at 20°C and observed by staining the gels with Coomassie brilliant blue R-250.

Table1. Phylogeny of Amazonian native Fabaceae screened for lectin activity, included classified subfamily and tribe.

Subfamily	Tribe	Specie	Subfamily	Tribe	Specie
Caesalpinioideae	Caesalpinieae	*Campsiandra laurifolia*	Mimosoideae	Parkieae	*Parkia panurensis*
Caesalpinioideae	Cassieae	*Cassia grandis*	Mimosoideae	Mimoseae	*Stryphnodendron guianense*
Caesalpinioideae	Cassieae	*Cassia leiandra*	Mimosoideae	Ingeae	*Zygia cauliflora*
Caesalpinioideae	Detarieae	*Copaifera venezuelana*	Mimosoideae	Ingeae	*Zygia inaequalis*
Caesalpinioideae	Detarieae	*Crudia oblonga*	Mimosoideae	Ingeae	*Zygia trunciflora*
Caesalpinioideae	Detarieae	*Cynometra bauhiniifolia*	Mimosoideae	Ingeae	*Zygia unifoliolata*
Caesalpinioideae	Cassieae	*Dialium guianense*	Faboideae	Phaseoleae	*Centrosema plumieri*
Caesalpinioideae	Cassieae	*Dicorynia paraensis*	Faboideae	Phaseoleae	*Centrosema triquetrum*
Caesalpinioideae	Caesalpinieae	*Dimorphandra caudata*	Faboideae	Dalbergieae	*Dalbergia spruceana*
Caesalpinioideae	Detarieae	*Hymenaea courbaril*	Faboideae	Phaseoleae	*Dioclea bicolor*
Caesalpinioideae	Caesalpinieae	*Caesalpinia ferrea var. ferrea*	Faboideae	Sophoreae	*Leptolobium nitens*
Caesalpinioideae	Detarieae	*Macrolobium multijugum*	Faboideae	Sophoreae	*Ormosia costulata*
Caesalpinioideae	Detarieae	*Peltogyne paniculata*	Faboideae	Sophoreae	*Ormosia discolor*
Caesalpinioideae	Cassieae	*Senna tapajozensis*	Faboideae	Sophoreae	*Ormosia grossa*
Caesalpinioideae	Caesalpinieae	*Tachigali hypoleuca*	Faboideae	Sophoreae	*Ormosia lignivalvis*
Mimosoideae	Mimoseae	*Anadenanthera peregrina*	Faboideae	Sophoreae	*Ormosia macrocalyx*
Mimosoideae	Mimoseae	*Dinizia excelsa*	Faboideae	Sophoreae	*Ormosia paraensis*
Mimosoideae	Mimoseae	*Entada polystachya var. polyphylla*	Faboideae	Sophoreae	*Ormosia smithii*
Mimosoideae	Ingeae	*Enterolobium schomburgkii*	Faboideae	Swartzieae	*Swartzia argentea*
Mimosoideae	Ingeae	*Macrosamanea spruceana*	Faboideae	Swartzieae	*Swartzia ingifolia*
Mimosoideae	Ingeae	*Inga pezizifera*	Faboideae	Swartzieae	*Swartzia laevicarpa*
Mimosoideae	Parkieae	*Parkia decussate*	Faboideae	Swartzieae	*Swartzia longistipitata*
Mimosoideae	Parkieae	*Parkia gigantocarpa*	Faboideae	Swartzieae	*Swartzia pendula*
Mimosoideae	Parkieae	*Parkia multijuga*	Faboideae	Swartzieae	*Swartzia polyphylla*
Mimosoideae	Parkieae	*Parkia nitida*	Faboideae	Swartzieae	*Swartzia sericea*

RESULTS AND DISCUSSION

Protein extracts: Screens for lectin activity frequently utilize different pH conditions for protein extraction and different erythrocyte sources for testing HA activity (Ainouz and Sampaio, 1991). In the present study, the proteins were extracted with 150 mM NaCl because lectin-containing extracts are commonly active under this condition. To evaluate the diversity of the extracted proteins, the extracts

were analyzed by electrophoresis. The protein profiles of the extracts that exhibited HA activity are shown in Figure 1. The extracts contained a wide range of proteins, suggesting that the protein extraction procedure was successful.

New lectins: The seeds of fifty native Amazonian Fabaceae species were screened for lectin activity based on the ability of their protein extracts to agglutinate rabbit or rat erythrocytes. The species were chosen based on their taxonomic positions or evolutionary relationships within the various legume tribes (Table 1). This strategy was chosen because numerous legume lectins have been described in species of Phaseoleae (notably from the subtribe Diocleinae) and Vicieae (mainly in *Pisum* and *Lathyrus*) and, to a lesser extent, in members of Loteae, Robineae, Crotalareae, Abreae and Dalbergiae, while other groups have been poorly investigated. The present study included several representatives of the more primitive groups of legumes (Subfamily Faboideae), especially Sophoreae and Swartziae, which have been the subject of few lectin studies.

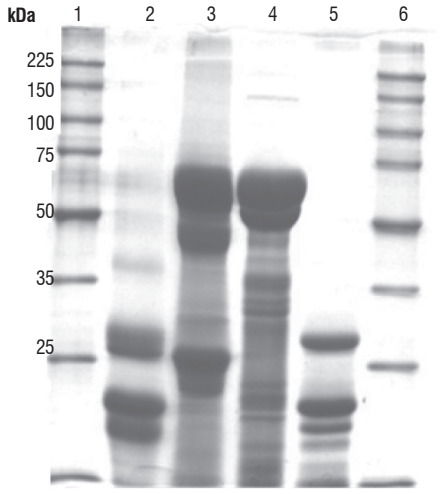

Figure 1. SDS-PAGE of protein extracts from seeds of amazonian Fabaceae. Electrophoresis was performed on a 12.5% gel, and the proteins were visualized by staining with Coomassie brilliant blue R-250. Lanes 1 and 6, molecular weight markers; lanes 2, 3, 4 and 5, crude extracts of *Dimorphandra caudata*, *Anadenanthera peregrina*, *Ormosia lignivalvis* and *Swartzia laevicarpa*, respectively.

HA activity was observed earliest in the extracts from *Anadenanthera peregrina, Dimorphandra caudata* and *Ormosia lignivalvis*, which belong to Mimoseae, Caesalpinieae and Sophoreae, respectively. Although hemagglutination was easily seen after 24 h, it was always weak. Rabbit erythrocytes were agglutinated by *Dimorphandra caudata* [titer: 2^4(HA = 16)], while rat erythrocytes were agglutinated by *Anadenanthera peregrina* [titer: 2^6(HA = 64)] and *Ormosia lignivalvis* [titer: 2^7 (HA = 128)]. The HA activity of these extracts was lost when the samples were heated at 100°C for 10 min before they were tested; therefore, proteins were likely to be responsible for the observed HA activity.

The *Swartzia* protein extracts exhibited a strong hemolytic effect, which inhibited the measurement of lectin activity and suggested the presence of hemolysins or saponins. However, because hemolysis persisted even after the extract was heated, saponins were likely responsible for the occurrence of hemolysis rather than hemagglutination (Konozy et al., 2002). Because *Swartzia* species are of great evolutionary importance within the legume family and because little consistent information is available regarding lectins in this group, the lectin activity of the *Swartzia* extracts was reexamined. The *Swartzia* extracts were fractionated by ion-exchange chromatography to separate the hemolytic agents from the putative lectins. The *Swartzia laevicarpa* protein extract was fractionated into three protein peaks. Further tests for HA activity showed the presence of lectin activity in the fraction that was eluted with 0.1 M NaCl, while hemolysis was observed in the fraction that was eluted with 0.5 mM NaCl (Figure 2A and B). These activities remained after the protein fractions were dialyzed and lyophilized. However, the HA activity was lost after heating, suggesting the presence of lectin. The protein profiles of the peaks obtained from ion-exchange chromatography were further examined by electrophoresis (Figure 3A). The HA fraction (DEAE-PII) showed very few protein bands, while PIII contained major proteins and retained its hemolytic activity (Figure 2B).

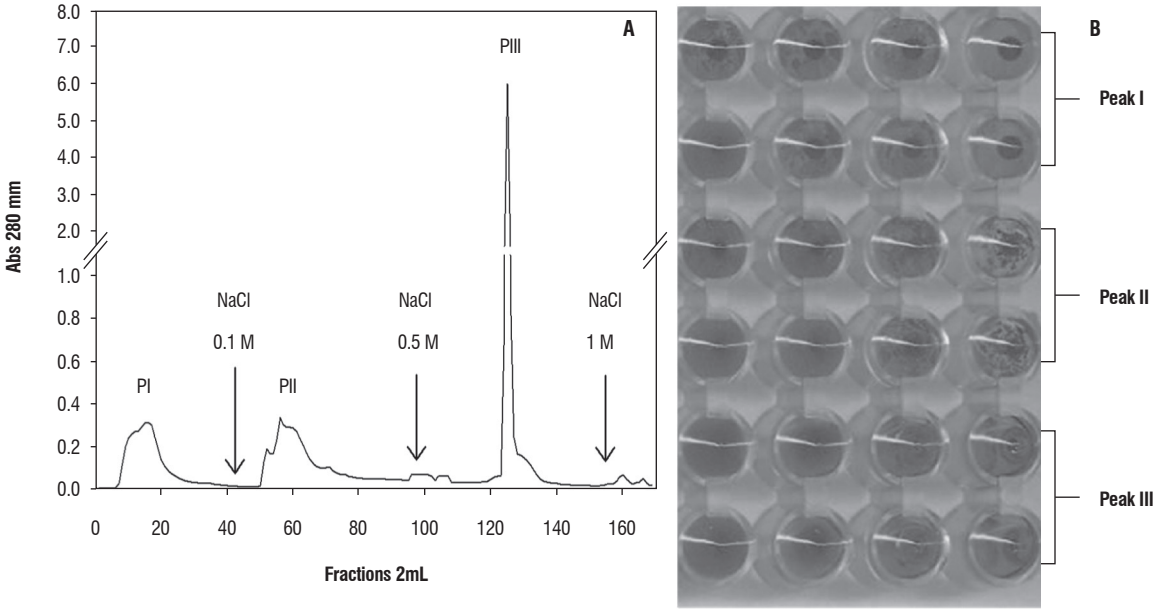

Figure 2. Anion exchange chromatogram of the *Swartzia laevicarpa* extract on a Sepharose fast flow column and hemagglutinating activity of peak I, II and III. A, For the analysis a 20-mL sample (2.5 mg/mL) was applied to a DEAE column (20 mL) that had been pre-equilibrated with 50 mM Tris-HCl, pH 8.0. Peak I (PI) was eluted with the same buffer, while PII and PIII were eluted by stepwise (0.1, 0.5 and 1.0 M) increases in NaCl concentration, as indicated by the arrows. Fractions (2 mL) were collected at a flow rate of 0.5 mL/min and monitored spectrophotometrically at 280 nm. B, Non-retained protein (PI), HA (Peak II) and hemolytic activities (PIII) of *Swartzia laevicarpa* extract fractions were separated by ion exchange chromatography and determined using 2% (v/v) rabbit erythrocytes.

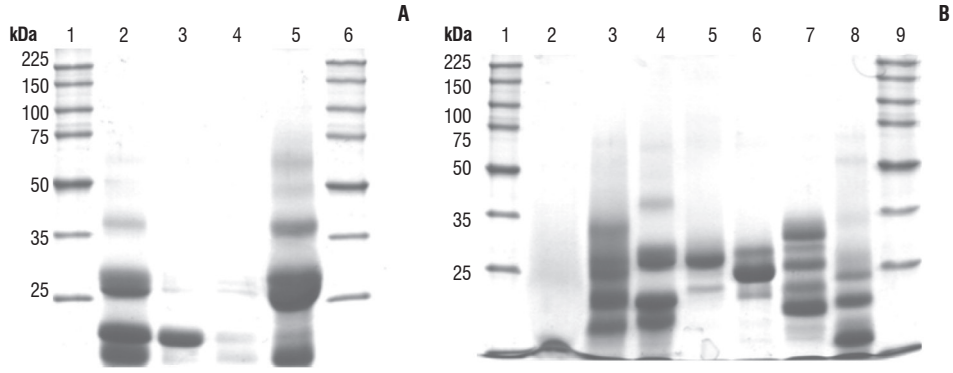

Figure 3. SDS-PAGE of *Swartzia* protein fractions in the presence of β-mercaptoethanol. A, *Swartzia laevicarpa*. Lane (3), DEAE-PI (- AHE); Lane (4), DEAE-PII (+ AHE); Lane 5, DEAE-PIII (hemolysis). B, Lanes 1 and 9, molecular weight markers; Protein profiles of *Swartzia* extracts: *S. argentea* (2), *Swartzia ingifolia* (3), *Swartzia laevicarpa* (4), *Swartzia longistipitata* (5), *Swartzia pendula* (6), *Swartzia polyphylla* (7) and *Swartzia sericea* (8). Samples were electrophoresed on 12.5% SDS polyacrylamide gel under reducing conditions.

As stated above, the active extracts exhibited weak HA despite the experimental conditions that were utilized. Because previous HA assays have used various sources of erythrocytes, the poor agglutination observed in this study and the hemolytic activity associated with these extracts may have discouraged the further study of these plant species in previous screens. Legume lectins often strongly agglutinate rabbit erythrocytes. For example, those from *Erythrina*, *Dioclea* and *Canavalia* exhibit strong agglutination (HA greater than 2024) of rabbit erythrocytes, and more than 30 lectins have been purified from these groups (Konozy et al., 2002; Konozy et al., 2003).

Carbohydrate-binding specificity: The agglutinating extracts were mixed with carbohydrates that are commonly used to investigate the primary specificity of lectins, such as glucose and galactose. As shown in Table 2, the HA activity of all of the extracts was inhibited in the presence of lactose. Galactose inhibited the extracts of *Anadenanthera peregrina, Ormosia lignivalvis* and *Swartzia laevicarpa*, but it did not inhibit those of *Dimorphandra caudata*. Glucose did not inhibit hemagglutination. These results suggest that all of the new lectins described here belong to the Gal/GalNac group.

Table 2. Hemagglutinating activity inhibition of the seed protein extracts of Amazonian native Fabaceae.

Species	Carbohydrates			
	Glucose	Mannose	Galactose	Lactose
[2]*Dimorphandracaudata*	-	-	-	+
[1]Anadenanthera peregrina	-	-	+	+
[1]Ormosialignivalvis	-	-	+	+
[2]*Swartzialaevicarpa* (PII)	-	-	+	+

Note: HA performed with (1) rat or (2) rabbit erythrocytes suspension (2 % v/v). Inhibition: (+), not inhibitory: (-).

Swartzia lectins: In the present study, seven *Swartzia* species were screened for lectin activity. All of these extracts exhibited strong hemolytic activity that persisted even after the extracts were heated. However, the detection of lectin activity in *Swartzia laevicarpa* extract after chromatographic separation suggested that lectins would be present in other *Swartzia* extracts. Therefore, the *Swartzia* protein extracts were compared by electrophoresis (Figure 3B). Surprisingly, the various *Swartzia* extracts exhibited distinctive protein profiles, and this direct comparison of their protein contents was discontinued. By following the same protocol used for *S. laevicarpa*, all of the *Swartzia* extracts were fractionated by ion exchange chromatography. Once more, the protein fractionation patterns differed strongly among the extracts, but hemolysis was still observed (data not shown). Surprisingly, HA was not observed in the protein fractions of *S. argentea*, *S. pendula*, *S. polyphylla* and *S. sericea*. However, the protein fractions of *S. ingifolia* and *S. longistipitata* exhibited HA. Their extracts were fractionated in two and four peaks, respectively (data not shown). Among all of the protein peaks that were examined, HA was observed only in PIII-300 mM NaCl (*S. ingifolia*) and PI-100 mM NaCl (*S. longistipitata*), and both were strongly inhibited by galactose (< 10 mM) and lactose (< 10 mM). We conclude that lectins are present in *Swartzia* species, but distinct protocols are needed to extract and purify these proteins. Because the HA of *S. laevicarpa* was more consistent and was clearly inhibited in the presence of lactose, its protein extract was further separated by affinity chromatography on an α-lactose-agarose column. As shown in Figure 4A, after the unbound proteins had been washed from the column, the protein fraction that was retained on the matrix was eluted by changing the pH conditions. The retained protein fraction (PII) still exhibited HA that was inhibited by galactose and lactose; however, the proteins that were eluted in PI did not exhibit these properties. Therefore, the lectin fraction was examined by electrophoresis, and a major protein band with an apparent molecular mass between 27 and 30 KDa was observed (Figure 4B). This protein is now the target of further purification and biochemical characterization studies. The results reported here suggest that *Swartzia* lectins are probably most inhibited by lactose and that affinity chromatography on immobilized lactose is likely the most effective technique to purify these new lectins.

Evolutionary implications: Detailed analyses of the amino acid sequences and three-dimensional structures of legume lectins have proven useful in elucidating the phylogeny of legume taxa (Loris et al., 1998; Oliveira et al., 2008). The events associated with the post-translational processing of legume lectins and the quaternary associations of their monomers also provide information regarding the evolutionary relationships of these proteins (Barre et al., 1994). For example, the seed lectin of *Vatairea macrocarpa* (tribe Dalbergiae) is the first galactose-specific lectin reported to exhibit post-translation processing similar to that seen in glucose/mannose-specific lectins in the sub-

tribe Diocleinae (Calvete et al., 1998; Konozy et al., 2002). Furthermore, the protein profiles of these lectins as observed by polyacrylamide electrophoresis are almost identical; the lectins are composed of three distinct bands corresponding to particular chain fragments produced by proteolytic processing (Konozy et al., 2002). These results suggest that the legume lectins belong to multiple distinct groups. For example, in terms of their biosynthesis, the lectins found in Phaseoleae (sub-tribe Diocleinae) and Dalbergiae may be more similar to each other than to other legume lectins that belong to more closely related taxa, such as Vicieae and Diocleinae. Because *Swartzia* represents primitive legume group, *Swartzia* lectins may provide novel insights regarding legume evolution and the relationships of legume lectins. The electrophoresis pattern and observed molecular weight of the putative *S. laevicarpa* lectin identified here are nearly identical to those of a seed lectin from *Sophora japonica* (Hankins et. al. 1987). The tribe Sophoreae is closely related to Swartziae and may have evolved from within the latter group.

The present study has successfully reported the occurrence of lectins in primitive legume groups (Subfamily Faboideae) and has provided new insights regarding the detection and purification of *Swartzia* lectins.

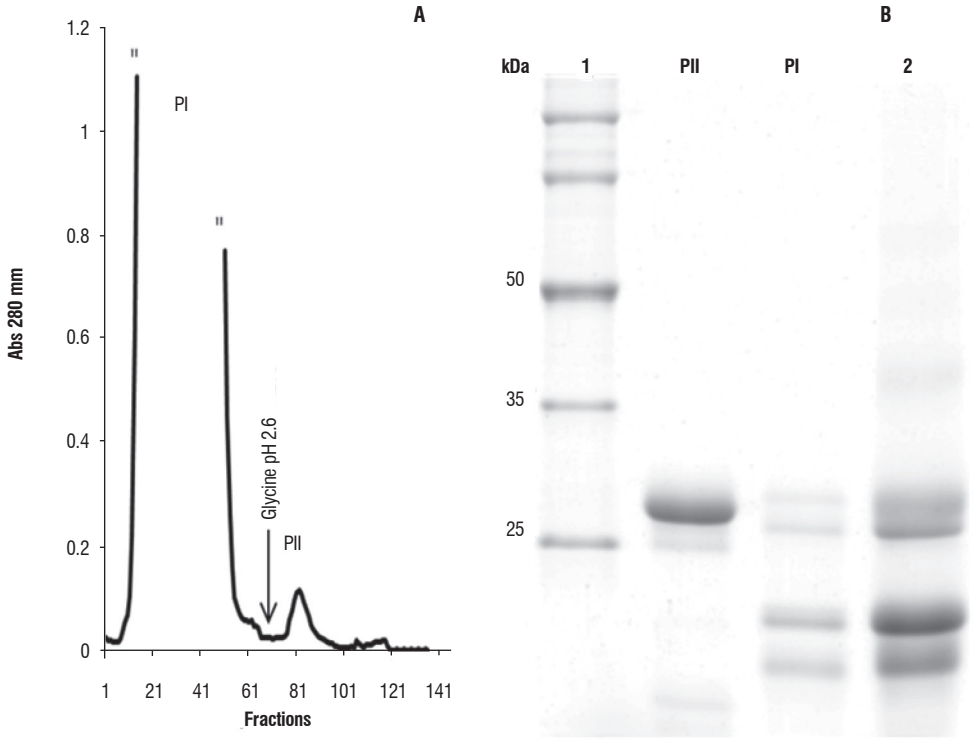

Figure 4. Affinity chromatography of the *Swartzia laevicarpa* protein extract on an α-lactose-agarose column and determination of the relative molecular mass of peak II. A, For analysis a 20-mL sample (10 mg/mL) was applied to a column (25 mL) that had been pre-equilibrated with 100 mM Tris-HCl, pH 7.6, plus 150 mM NaCl and 5 mM $CaCl_2/MnCl_2$. Peak I (PI) was eluted with the same buffer, while PII was eluted after the addition of 100 mM glycine-HCl and 100 mM NaCl, as indicated by the arrow. Fractions (2 mL for PI and 4 mL for PII) were collected at a flow rate of 20 mL/h and monitored spectrophotometrically at 280 nm. B, 12.5% polyacrylamide gel electrophoresis of the retained protein (PII), non-retained protein (PI) and total protein extract of *S. laevicarpa* (2). Molecular weight markers are the same as reported in Figure 1. Peak II exhibited HA, which was most inhibited by 25 mM lactose.

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