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Isolation and partial characterisation of galactose-specific lectins from African yam beans, *Sphenostyles stenocarpa* Harms

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Abstract

A new galactose-specific lectin was isolated from African yam bean (*Sphenostyles stenocarpa* Harms) by affinity chromatography on galactose-Sepharose 4B. SDS–PAGE analysis resulted in four polypeptide bands of approximately 27, 29, 32 and 34 kDa, respectively. Based on the analysis of carbohydrate content and native PAGE, it is likely that the *Sphenostyles* lectin is a tetrameric glycoprotein with M_r of approximately 122 kDa. N-terminal protein sequencing of purified lectins from four different *Sphenostyles* accessions shows that the four polypeptides have largely identical amino acid sequences. The sequences contain the conserved consensus sequence F-F–LILG characteristic of legume lectins, as well as *Phaseolus vulgaris* proteins in the arcelin-α-amylase inhibitor gene family. The lectin agglutinates both rabbit and human erythrocytes, but with a preference for blood types A and O. Using Western blotting, the lectin was shown to accumulate rapidly during seed development, but levels dropped slightly as seeds attained maturity. This is the first time a lectin has been purified from the genus *Sphenostyles*. The new lectin was assigned the abbreviation LECp.SphSte.se.Hga1.

Keywords: *Sphenostyles stenocarpa*; African yam beans; Leguminosae; Lectins; Purification; Sugar specificity; Seed development

1. Introduction

Lectins are a large and heterogenous group of proteins that have the ability to bind reversibly to mono- and oligosaccharides (Van Damme, Peumans, Pusztai & Bardoz, 1998). Although lectins have been identified and characterised from several plant species, taxa and organ types, the largest contribution to our understanding of the molecular and biochemical properties of plant lectins has come from studies of legume seed lectins (Sharon & Lis, 1990). Interest in seed lectins can also be attributed to the importance of legume seeds as a rich source of dietary protein, hence the need to study their protein composition. Although typical lectin levels in legume seeds range from 0.1–5% of total seed protein, values of up to 50% have been reported in some species such as those in the genus *Phaseolus* (Pusztai, Croy, Grant & Stewart, 1981). At present, detailed knowledge about the molecular, biochemical and physicochemical properties of several lectins, their carbohydrate binding, including sugar, specificities and their applications in agricultural and biomedical research is available (reviewed in Van Damme et al., 1998a).

The genus *Sphenostyles* belongs to the Leguminosae and the subfamily Papilionoideae. *S. stenocarpa*

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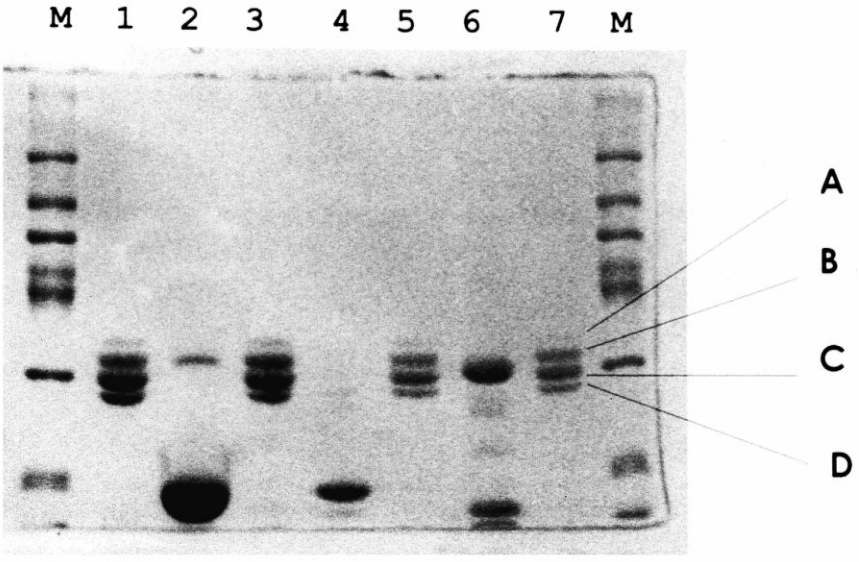

Fig. 1. SDS–PAGE (12%) of affinity purifed *S. stenocarpa* lectins. Each well contains 40 μg of protein. The four lectin subunits, namely A, B, C and D are labelled. Molecular mass reference proteins in M, in order of increasing molecular mass, are: lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5/19.7 kDa doublet), carbonic anhydrase (31 kDa), aldolase (40 kDa), ovalbumin (42.7 kDa), glutamate dehydrogenease (55 kDa) bovine serum albumin (66 kDA) and phosphorylase B (97.4 kDa). 1, EN953; 2, WGA; 3, EN971; 4, PSL; 5, EN982; 6, Con A; 7, UM971.

(Hochst. ex A. Rich) Harms, commonly known in West Africa as 'African yam bean' (AYB), is a popular grain legume that is also cultivated in other tropical areas of Africa for its highly proteinaceous seeds and tubers. Its nutrient composition and nutritive value have been well characterised (Nwokolo, 1987). Moreover, entomological studies at the International Institute of Tropical Agriculture based in Nigeria, W. Africa, have indicated that the pods and seeds of this edible legume may be resistant to the major pest of cowpea, namely the cowpea podborer (*Maruca vitrata*) (Omitogun O. G., Jackai L.E.N. and Thottappilly G., unpublished). Since some lectins are believed to have a role in plant defense (Gatehouse, Powell, Peumans, Van Damme & Gatehouse, 1995; Chrispeels & Raikhel, 1991), we are interested in the isolation and characterisation of this family of proteins from AYB. In the present study, we report for the first time the purification of *S stenocarpa* seed lectins by affinity chromatography, their sugar binding specificities, hemagglutinating activities and their expression during seed development.

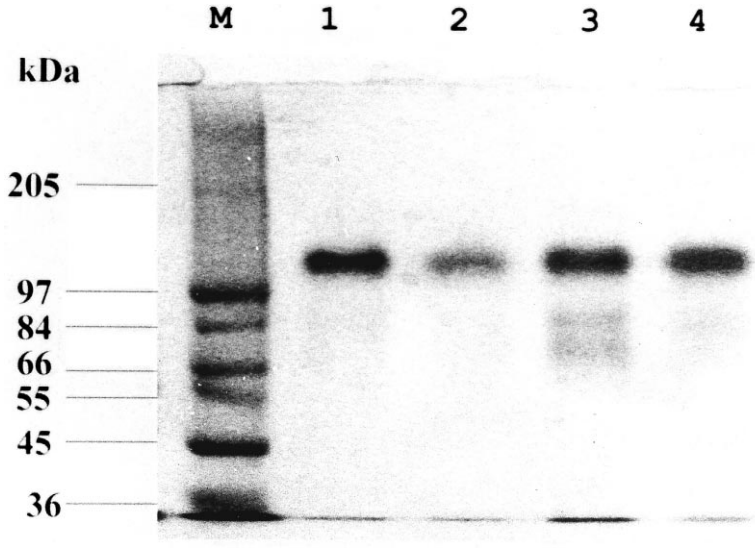

Fig. 2. Native PAGE (without 2-mercaptoethanol) of *Sphenostyles* lectins using 7.5% acrylamide gel. 1, EN953; 2, EN971; 3, EN982; 4, UM971. M, molecular weight reference marker. Proteins used in M are, in order of decreasing molecular weight, myosin, phosphorylase, fructose-6-phosphate kinase, bovine serum albumin, glutamic dehydrogenase, ovalbumin and glyceraldehyde-3-phosphate dehydrogenase.

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EN982 (B): D N F F S F G I K N F S S D D L I L Q S
EN971 (B): D N F F S F G I K N F S S D D L I L Q Q D
EN953 (C): D N F F S F G I K N F S S D D L I L Q Q
UM971 (D): D N F F S F G I K N F S S D D L I L Q P D A K V P
PEA:       D I Y F N F Q R F N E T . . N L I L Q R D A S V S
LECSJbmI:  S L S F T F N N F G P D Q R D L I L Q G D A H I P
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Fig. 3. Comparison of N-terminal sequences of B, C and D subunits of *Sphenostyles* lectin (this study) with pea (Mondaci & Dobres, 1993) and Japanese pagoda tree (LECSJbmI) (Peumans et al., 1997) lectins. The substituted glutamine or serine residues present in B and C subunits for proline in the D subunit is indicated in boldface.

2. Results and discussion

Lectins from four different *S. stenocarpa* accessions were purified by affinity chromatography on immobilised galactose Sepharose 4B and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE). Since lectins may run anomalously on SDS–PAGE gels, we used seed lectins from *Pisum sativum* (LECp.PisSat.se.Hmg1 or PSL, 17 kDa large β-subunit), *Triticum aestivum* (LECp.TriAes.se.Hch1 or WGA, 32 kDa subunit), and *Canavalia ensiformis* (LECp.CanEns.se.Hmg1 or Con A, 30 kDa subunit) to estimate the molecular mass of the *Sphenostyles* lectins (Fig. 1). Under reducing (with SDS and 2-mercaptoethanol [2-ME]) conditions, three major and one minor, polypeptide bands of approximately 27, 29, 32 and 34 kDa respectively, were observed in all four AYB accessions (bands A, B, C, D in Fig. 1). Except for one accession (EN98-2), the unbound protein fraction contained large amounts of polypeptides in the same size range as the bound lectins (data not shown). Hemagglutination activity was, however, absent from the non-lectin fractions. On average, yields of about 1.2 mg lectin g^{-1} seed meal were obtained. Futhermore, there was no evidence of the existence of another agglutinin in *Sphenostyles* seeds. SDS–PAGE in the absence of 2-ME also resulted in four bands. However, native PAGE under both non-reducing (without 2-ME, Fig. 2) and reducing (with 2-ME, data not shown) gave only a single band. Hence bands A, B, C and D may represent different subunits of a tetrameric hololectin of approximately 122 kDa.

Fig. 3 shows the N-terminal amino acid sequences of lectins from four *Sphenostyles* accessions. All four bands (A, B, C and D) shown in Fig. 1 were sequenced. The N-terminal sequence of at least 11 amino acid residues (DNFFSFIKNFS) were identical for all four subunits of the purified lectins. Hence sequencing cycles were terminated early in several runs. However, by comparing at least 20 amino acids residues for B, C and D subunits, one difference was observed, namely, substitution of Q (B and C subunits) in EN953 and EN971 by P (D subunit) in UM971 (Fig. 3). Moreover, the B subunit of EN982 lectin contained a serine instead of a glutamine residue present in the same subunit of EN971. This provides evidence for structural differences between the different subunits. Other differences that are likely to exist between the lectins from different accessions could be revealed through molecular cloning of the gene(s) encoding these proteins. The N-terminal sequences were also compared to a legume (pea) and a tree (Japanese pagoda) lectin (Mandaci & Dobres, 1993; Van Damme, Barre, Rouge & Peumans, 1997b). This comparison gave 55% and 44% identity, respectively,

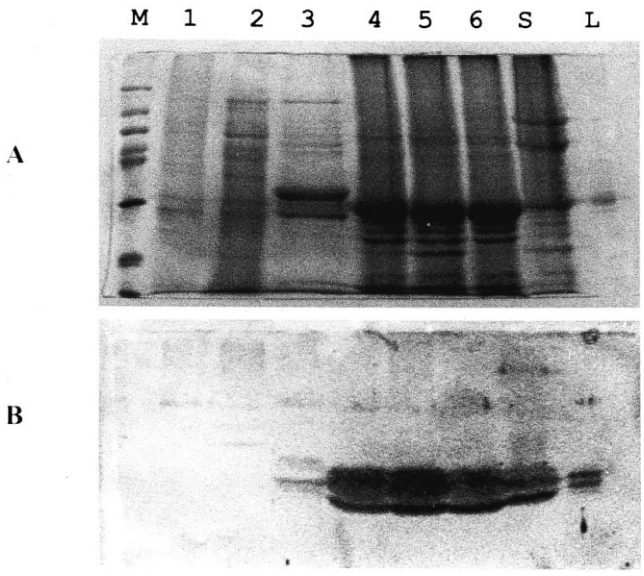

Fig. 4. Western blotting of total protein (A) from developing seeds of accession EN953 probed with antibody raised against purified lectin (B). The antibody was raised as described in Materials and methods. Numbers 1–6 represent weeks after flower fall. Approximately 100 μg of total protein was loaded in each well except L (contains 1 μg of affinity purified lectin). Well S contains total protein isolated from mature dry seeds.

Table 1
Agglutination of human and rabbit erythrocytes by purified *Sphenostyles* lectins

Erythrocytes	4b Relative activity[a]			
	EN95-3	EN97-1	EN98-2	UM97-1
A	100 (60)	100 (60)	100 (60)	100 (60)
B	40 (0)	40 (0)	40 (0)	60 (0)
AB	40 (0)	60 (0)	40 (0)	60 (0)
O	100 (100)	100 (100)	100 (100)	100 (100)
Rabbit	100 (40)	100 (60)	100 (60)	100 (80)

[a] The hemagglutinating activity of human type A erythrocytes was taken as 100. Agglutination assays utilised 10 μg of lectin incubated at room temperature in the presence of 3% trypsinised and untrypsinised (values in parentheses) erythrocytes.

when alignment was based on 25 amino acids (Fig. 3). Besides, the *Sphenostyles* lectin also has significant homology to other plant lectins (Chrispeels & Raikhel, 1991; Pratt, Singh, Shade, Murdock & Bressan, 1991; Van Damme et al., 1997b). In particular, the consensus sequence F-F–LILG for legume lectins, including *Phaseolus vulgaris* (common bean) proteins in the arcelin-α-amylase inhibitor gene family, can also be seen in the *Sphenostyles* lectin (Mirkov et al., 1994).

During seed development, the hull of AYB expands from about 2 cm at flower fall to an average length of 22 cm at maturity 6–7 weeks post-flowering (WPF). Maximum pod length is reached very early at about 3 weeks and thereafter, the hull merely thickens as the seeds also expand and mature. Changes in the levels of lectins synthesised during seed development were monitored through Western blotting of total protein extracted from developing seeds of EN953. As shown in Fig. 4, lectins were detectable 2 WPF and accumulated to reach a peak at 5 WPF. It is possible that the decline in lectin levels after 5 WPF is a result of degradation that can occur as the plant gets older. This observation has been reported before for galactose-specific lectins from winged bean (Barbieri, Lorenzoni & Stirpe, 1979; Shet & Madaiah, 1987). Interestingly, two bands of higher M_r were observed on blots from 2- and 3-week-old seeds instead of the four characteristic bands observed in older seeds. It is not clear what this means, but perhaps this may reflect developmental regulation at the level affecting the stability of the protein or post-translational modifications. Western blotting failed to detect the presence of lectins in tubers, stems, leaves and flowers (data not shown). However,

Table 2
Hemagglutination activities of crude *Sphenostyles* lectin extracts using rabbit erythrocytes

Accession	Hemagglutination titre (HT)[a]	Specific activity (HT per μg protein)
AG974	16	13.9
AG9841	256	1969.2
AG9844	128	266.7
AG9845	128	196.9
AG9846	32	15.7
AG9847	64	152.4
AG98417	256	1600.0
AG98418	512	8533.3
AG98413	512	8533.
EN9511	1024	7937.9
EN953	8	2.7
EN954	1024	11,770.1
EN96111	128	512.0
EN971	128	474.1
EN972	32	2.0
EN973	32	32.9
EN9731	256	1219.0
EN982	16	10.6
EN9821	128	512
EN9822	256	731.4
EN9849	32	46.3
EN98410	512	8533.3
EN98415	128	50.0
UM951	256	0.08
UM971	164	22.2
UM9711	128	216.9
UMZ9831	8	0.3
UMZ9832	32	1.3
UMZ9833	8	1.4

[a] HT was calculated as described in the Experimental.

Table 3
Inhibition of *Sphenostyles* lectins by monosaccharide sugars

Accession	Sugar (100 mM)[a] GC	MT	FR	GN	MN	MY	RM	ME	RB	AR	SR	GH	TR	FU
AG974	–[b]	–	–	–	–	–	–	–	–	–	–	–	–	–
AG9841	–	NA[c]	–	–	–	–	–	–	–	–	–	–	–	–
AG9844	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AG9845	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AG9846	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AG9847	–	–	–	–	–	NA	NA	–	–	–	–	–	–	–
AG98417	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AG98418	–	NA	–	–	–	–	NA	–	–	–	–	–	–	–
AG98413	–	NA	–	–	–	–	–	–	–	–	–	–	–	–
EN9511	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EN953	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EN954	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EN967	–	–	–	–	NA	–	–	–	–	–	–	–	–	–
EN96111	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EN971	–	–	–	–	NA	–	–	NA	NA	NA	–	–	NA	–
EN972	–	–	–	–	–	–	–	NA	NA	NA	–	–	NA	–
EN973	–	–	–	–	–	–	–	NA	NA	NA	–	NA	NA	–
EN9731	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EN982	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EN9821	NA	NA	NA	NA	NA	–	–	–	–	–	NA	NA	NA	–
EN9822	–	–	–	–	–	–	–	–	–	–	–	–	–	–
EN9849	–	NA	–	–	–	–	–	–	–	–	–	–	–	–
EN98410	–	NA	–	–	–	–	–	–	–	–	–	–	–	–
EN98415	–	NA	–	NA	–	–	–	–	–	–	–	–	–	–
UM951	–	NA	–	–	NA	–	–	–	–	–	–	–	–	–
UM971	–	–	–	–	–	–	–	–	–	–	–	–	–	–
UM9711	–	–	–	–	–	–	NA	–	–	–	–	–	NA	–
UMZ9831	–	NA	–	–	–	–	–	–	–	–	–	–	–	–
UMZ9832	–	–	–	–	–	–	–	–	–	–	–	–	–	–
UMZ9833	–	–	–	–	–	–	–	–	–	–	–	–	NA	NA

[a] GC, glucose; MT, maltose; FR, fructose; GN, *N*-acetyl-D-glucosamine; MN, mannose; MY, myoinositol; RM, rhamnose; ME, melibiose; RB, ribose; AR, arabinose; SR, sorbose; GH, glucosamine-hydrochloride; TR, trehalose; FU, fucose.
[b] –, Agglutination.
[c] NA, No agglutination.

agglutination assays using rabbit erythrocytes revealed the presence of low levels of galatose-specifc lectins in these organs. Absence or low levels of lectins in non-seed organs may be the result of immobilisation in membranes, partial enzymatic breakdown or changes in the expression of multiple lectin genes.

As shown in Table 1, the *Sphenostyles* lectin agglutinated both human and rabbit erythrocytes. However, blood groups A and O and rabbit blood were at least 1.5 times more readily agglutinated compared to types B and AB. The recently isolated *Vatairea macrocarpa* (Leguminosae) galactose-specific lectin exhibits similar preferences (Cavada et al., 1998). As expected, the sensitivity of the lectin was greatly enhanced following treatment with trypsin. In the absence of trypsin, the lectins from all four AYB accessions failed to agglutinate human blood groups B and AB. This behavior has been observed in other lectins, for example *Sambucus*

Table 4
Minimum inhibitory concentration of lactose, galactose and *N*-acetyl-D-galactosamine on purified *Sphenostyles* lectins

Accession	Minimum inhibitory concentration (mM)[a] Galactose	*N*-acetyl-D-galactosamine	Lactose
EN953	50	12.5	25
EN971	25	1.56	12.5
EN982	50	1.56	12.5
UM971	25	1.56	25

[a] Agglutination assays were performed with rabbit erythrocytes (3%) using 25 μg of purified lectins.

nigra agglutinin (Broekaert, Nsimba-Lubaki, Peters & Peumans, 1984).

Based on crude total protein extracts from mature seeds, hemagglutination titres (HT) were determined for 29 AYB accessions. As shown in Table 2, extensive variations were observed in crude as well as specific HT values. The lowest specific HT of 0.08 was in UM951, whereas the highest HT of 11,770 was in EN954. Such wide differences may be attributed to plant (varietal) differences, and possibly the inconsistencies inherent in the use of crude extracts rather than pure protein to determine HT values.

Several sugars (all sugars of the D-configuration except L-fucose) were tested for their ability to inhibit agglutination of AYB lectins (Table 3). Lectins from all accessions were readily inhibited by 100 mM of *N*-acetyl-D-galactosamine, galactose and lactose, hence these are not listed in Table 3. The minimum concentrations of these three sugars required for inhibition of agglutination are shown in Table 4. The concentrations of galactose and lactose required for 100% inhibition of agglutination of trypsin treated erythrocytes are rather high, although such levels are not uncommon for plant lectins (Peumans, Nsimba-Lubaki, Carlier & Van Driessche, 1984; Higuchi, Ohtana & Iwai, 1986; Cavada et al., 1998). The *Sphenostyles* lectin may therefore be regarded as a galactose-specific due to this sugar specificity (Van Damme et al., 1998b).

Other sugars which resulted in inhibition of agglutination were maltose, *N*-acetyl-D-glucosamine, mannose, myoinositol, melibiose, arabinose, ribose, rhamnose, and trehalose. Sorbitol and xylose did not cause inhibition both at 100 mM and 500 mM in all accessions (data not shown). It is likely that besides the minor variations in sugar specificities of extracts from different *Sphenostyles* accessions, all the lectins in the 29 accessions are fundamentally similar in their carbohydrate binding specificities. The total sugar content, determined using purified lectins, was 0.35% for EN953, 0.54% for EN971, 4.36% for EN982 and 1.59% for UM971.

The pH stability of the *Sphenostyles* lectin was tested over a range of 1–14 in 40 mM phosphate saline buffer (PBS) buffer. Agglutination ocurred only in the range of 2–10. For heat stability, temperatures of between 30–100°C were tested. Under these conditions, the lectins were stable in PBS between 30–70°C. Complete loss of activity occurred above 80°C.

Recently, a novel classification system has been described that allows unambiguous and uniform identification of plant lectins (Van Damme et al., 1998). Using this system, the affinity purified *Sphenostyles* seed lectin reported here has been assigned the abbreviation LECp.SphSte.se.Hga1. From this study, two roles may be suggested for LECp.SphSte.se.Hga1, namely, in plant defense against herbivores, and/or as a storage protein. Future work on LECp.SphSte.se.Hga1 will aim to clarify these observations, mainly through molecular characterisation of the gene(s) encoding LECp.SphSte.se.Hga1. The role of this lectin in defense against insect herbivores is currently under investigation, with emphasis on its effects on the development of the legume pod-borer, *M. vitrata*, pod-sucking bugs (*Clavigralla tomentosicollis*) and cowpea storage weevils (*Callosobruchus macullatus*).

3. Experimental

3.1. Plant material

Seeds of AYB were collected from local markets in Enugu, Umueze and Umuahia in Eastern Nigeria. These were either used directly for protein extraction or planted in the field at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria to monitor growth habit and for multiplication. Names were assigned to various seed collections based on differences in seed color. Field plants were used as sources of seeds, flowers, stems, leaves and tubers.

3.2. Reagents

All reagents were analytical grade. Phenyl Sepharose, Sepharose -4B, SP Sepharose Fast Flow, Sephadex G25 and Sephacryl S300 were purchased from Pharmacia (Uppsala, Sweden). Sugars, 1,3-diamino-propane (DAP) and polyvinyl sulphone were from Sigma (Germany). Purified WGA, PSL and Con A were also from Sigma. Human blood types O, A, B and AB were obtained with the assistance of staff from the Medical Unit at IITA clinic's laboratory.

3.3. Preparation of affinity adsorbent

Galactose-derivatised Sepharose-4B affinity matrix was prepared by divinyl sulphone coupling exactly as described previously (Peumans et al., 1997).

3.4. Purification of Sphenostyles seed lectins

Seeds of AYB were ground until the powder formed could pass through a 365 μM mesh. The seed meal was extracted in 0.2 M NaCl containing 1.0 g l^{-1} ascorbic acid at a concentration of 120 mg ml^{-1} by stirring for 2 h at room temperature (Sun & Hall, 1975). The homogenate was then centrifuged (9000 g, 20 min, 4°C) and the resultant supernatant adjusted to pH 7.5 with sodium hydroxide. This was centrifuged again for 10 min and the supernatant filtered through

coarse filter paper or a double layer of cheese cloth to remove particulate material. Ammonium sulphate (NH_4SO_4, 200 g l^{-1}) was then added and the soln filtered through Whatmann 3 MM filter paper. This soln was applied onto a column (2.6 × 20 cm, 5 cm ml^{-1}) of galactose-Sepharose 4B equilibriated with 2 M NH_4SO_4. Unbound proteins were washed with a soln of 2 M NH_4SO_4 until the A_{280} fell below 0.01. The lectin was then desorbed with either 20 mM DAP or 2 M NH_4SO_4 containing 0.2 M galactose, as described (Peumans et al., 1997). Using this procedure, all agglutinating activity present in the extract was retained on the column and could be eluted. Following this step, the lectin fraction was concentrated on a Phenyl Sepharose column and desorbed with DAP. The eluate was then adjusted to pH 3.8 with HCl prior to ion exchange chromatography using a column of SP Sepharose Fast Flow equilibriated with 50 mM formate buffer (pH 3.8). The lectin was desorbed with 0.5 M NaCl and, after addition of NH_4SO_4 to 1 M, applied onto a Phenyl Sepharose column as described above. Gel filtration was then performed on the resultant fraction using Sephacryl S300. Finally, the purified lectin was either desalted on a Sephadex G25 column or dialysed against water and lyophilised. Hemagglutination activity was monitored at each step of the purification procedure using trypsinised rabbit erythrocytes.

3.5. Total protein extraction and protein assays

The procedure for total protein fractionation was modified from (Sun & Hall, 1975). Tissues were homogenised with a Warring Blender prior to extraction in borate buffer (20 mM borate, 1 mM EDTA, pH 8.9) containing 0.5 M NaCl overnight, with stirring at 4°C. The extract was centrifuged (20,000 g, 15 min, 4°C) and the resultant supernatant dialysed for 24–48 h against several changes of distilled water. The dialysate was centrifuged again (20,000 g, 15 min, 4°C). Supernatants obtained were then frozen, lyophilised and resuspended in water at the appropriate concentration when needed. Protein concentration was estimated according to Bradford (1976) using bovine serum albumin as the standard.

3.6. Electrophoresis

SDS–PAGE was carried out using 12% acrylamide gels (Laemmli, 1970). For native PAGE, 7.5% acrylamide gels were used. Gels were either stained with Coumassie Blue R-250 or with Bio-Rad's Copper Stain and Destain reagent soln and calibrated using protein molecular weight standards.

3.7. N-terminal sequencing

Amino acid sequences for EN953 were determined at the Medical Department, Katholieke Universiteit Leuven, Belgium, whereas sequences for EN971, EN982 and UM971 were determined at the School Biological sciences, University of Durham, Durham, UK. In all cases, purified lectins were first separated by SDS–PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membrane. The desired polypeptide bands were then excised and sequenced on Applied Biosystems model 477A ABI protein sequencers with a 120A analyser.

3.8. Production of antisera and immunoblotting

Polyclonal antiserum against purified lectin was prepared by injecting rabbit with 1 mg of protein emulsified in Freund's complete adjuvant. Blood was collected from the ear vein of the rabbit 10 days after the booster injection. Serum was separated by centrifugation (1000 g, 10 min, 4°C). Protein transfer to nitrocellulose membrane and immunoblotting was carried out according to procedures outlined in Bio-rad technical manuals using the Mini Trans-Blot Transfer Cell system. The secondary antibody was goat anti-rabbit immunoglobulins coupled to alkaline phosphatase conjugate.

3.9. Hemagglutination activity

Hemagglutination assays were carried out on crude (total) protein or purified lectins according to the procedure described by Rüdiger (1993). Human or rabbit blood was centrifuged (1000 g, 10 min) and the supernatant discarded. Cells were then washed with PBS three times until clear before resuspending in PBS. Serial 2-fold dilutions of proteins were made in microtitre plates. Equal volume each (25 μl) of 3% trypsinised or untrypsinised erythrocytes and the protein sample were then mixed and incubated at room temperature. Agglutination was visualised by eye after 1 h by examining cells under a light microscope. The hemagglutination titre was calculated as the reciprocal of the concentration in the last well of the row in which agglutination was recognised. Tests were run at least in triplicates.

3.10. Sugar specificity

Sugar specificity of agglutinins was determined using a series of simple sugars at 100 mM. These were galactose, lactose, sorbitol, maltose, fructose, glucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, mannose, xylose, myoinositol, rhamnose, melibiose, ribose, arabinose, sorbose, glucosamine, trehalose and fucose.

In the absence of inhibition at 100 mM, assays were also conducted at 500 mM. The minimum sugar concentration that resulted in 100% inhibition of agglutination was determined for galactose, lactose and *N*-acetyl-D-glucosamine using purified lectins.

3.11. pH and heat stability

The pH and heat stability of lectins was determined essentially as described in Shet and Madaiah (1987). For pH stability, purified proteins (1 mg ml^{-1}) were incubated at 25°C for 24 h using different buffers in the pH range 1–14.

3.12. Quantitation of sugar content

Total carbohydrate content of purified lectins was estimated by the phenol-sulphuric acid method with reference to D-glucose (Dubois, Gilles, Hamilton, Rebers & Smith, 1956).

3.13. Developmental study

In order to follow the expression of lectins during seed development, flowers from field grown plants were tagged at anthesis. Pods were harvested at one-week intervals from the time of flower fall, frozen in liquid nitrogen and stored at −80°C until analysed.

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Appendix A

ISOLATION AND PARTIAL CHARACTERISATION OF NEW GALACTOSE-SPECIFIC LECTINS FROM AFRICAN YAM BEANS, *SPHENOSTYLES STENOCARPA* HARMS

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A new galactose-specific lectin was purified from *Sphenostyles stenocarpa* by affinity chromatography and further characterised through N-terminal sequencing, hemagglutination assays and expression during seed development.

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