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Isolation, purification and properties of lectin from the seed coat of *Mucuna* spp.

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ABSTRACT: Lectin isolated from *Mucuna* (cv Ukpo) seed coat was purified by affinity chromatography on porcine thyroglobulin-Sepharose followed by gel filtration. The purified lectin was found to be homogenous as shown by polyacrylamide gel electrophoresis and its single symmetrical elution from gel filtration. The molecular mass of the lectin is 20,000 ± 1,500 and is found to be a monomer by SDS gel electrophoresis. It agglutinated human erythrocytes A, B, O and AB blood group as well as animal erythrocytes. It is a thermostable glycoprotein which contains 13% carbohydrate and high proportion of acidic and neutral amino acids. Among the glycoprotein inhibitors tested, porcine thyroglobulin with the sequence of NeuAc (2 - 6/2 - 3) D-Gal B(1 - 4)D-GlcNAc was found to be the most potent inhibitor. However, its asialo counterpart was not inhibitory. The lectin was found only in the seed coat even in the immature stage. The lectin activity declined during maturation and was absent when completely matured and dried. This study showed that intact seed coat lectin activity was lost on incubation at 37°C for 62 days with 88% loss of water, while similar studies with scraped seed coat showed that the lectin activity was lost in 24 days with 84% dehydration.

Key Words: Lectins; *Mucuna* spp.; Seed coat; Glycoprotein inhibitors.

Introduction

Lectins are proteins that specifically and reversibly bind carbohydrates and agglutinate cells (1). They are very useful tools for the isolation and characterization of well defined glycan structure and cellular subsets (2). Many lectins have been grouped into distinct homologous proteins with common structural properties (3). Lectins are abundant in nature and are found in seeds of many plants (4 - 6). Most extensively studied are lectins from leguminous (3, 7). However, despite the wealth of information available on the properties of isolated plant lectins, their physiological function is not well understood and the localization of lectins in plants is an important pre-requisite in the evaluation of any lectin function (8). The appearance of lectin in almost all legume seeds occurs during the late stages of maturation of the seeds prior to dehyration and are located in the cotyledons (5, 9, 10). Apart from cotyledons, some have been found in embryo (11) and very small in the seed coat (9, 12). Studies of *Griffonia simplicifolia* Lentil (5, 9) and *Ricinus communis* (Euphorbeacaae) seeds (13), showed that there was no lectin in the immature seed

although it was found in ripe mature seeds. Recently some plants from monocotyledons and some from Gymnospermae have been studied because their extracts have strong carbohydrate binding properties (3, 14).

At present there has not been any report that demonstrated the presence of lectin exclusively either in seed coat or in immature seed (8).

This study describes the purification and properties of seed coat lectin and compares its carbohydrate specifically with other legume lectins earlier studied. The abolition of lectin activity in seed coat is also discussed.

Materials and Methods

Materials

Mucuna (cu Ukpa) seeds from the pods were collected from the author's garden. The seeds were planted in April and harvested in September - October. Seed coats were obtained by scraping the seed with pen knife. All reagents and chemicals were of analytical grade and were obtained from Sigma Company unless otherwise stated. Human blood were obtained from the healthy blood donors and were collected in citrate-dextrose. Rabbit, rats, duck and guinea-pig blood were collected by sacrificing the animals. The blood from sheep, goat and chicken were supplied from the slaughter house. The erythrocytes were washed with normal physiological saline (0.85%) and were treated with pronase P and neuraminidase as described in Ref. (15). 2% w/v cell suspension was prepared in saline.

Purification of lectin

Ten grams mature and immature seed coats were extracted with 100 cm^3 of saline and the extract was decolourized with a pinch of lead acetate and filtered. Then 2 cm^3 of the filtrate was layered on top of porcine thyroglobulin-Sepharose 4ß - conjugate column (1.5 x 5.5 cm) pre-equilibrated with 0.01M Tris-glycine buffer pH 8.0. The column was washed with the same buffer to remove the unbound protein and the bound protein was eluted with 0.01M glycine-HCl buffer pH 3.5. Each fraction (3.0 cm^3) was neutralized immediately with saturated $NaHCO_3$ solution. The active fractions were pooled, mixed, concentrated (16) and finally applied to a column of Sephadex G-100 (0.5 x 36cm) pre-equilibrated with 0.01M phosphate buffered saline pH 7.0 - 3.0 cm^3 fractions were collected. The active fractions were pooled, mixed, concentrated and stored at -20°C for further use.

Polyacrylamide disc gel electrophoresis was carried out using 10% gel at pH 8.5 in Tris-HCl buffer according to Hedrick and Smith (17) while SDS polyacrylamide gel electrophoresis was performed as in Ref. (18).

Molecular mass

Molecular mass was determined using polyacrylamide gel electrophoresis in non - denaturing system (17), SDS. gel electrophoresis (18) and gel filtration (16). The protein markers used were Bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (29,000), Trypsin-inhibitor (20,000) α-lactalbumin (14,000).

Haemagglutination assay

Haemagglutination assay was carried out as described by Ray and Chatterjee (8). 0.025 cm^3 solution of 2 fold serial diluted lectin in saline was added to equal volume of 2% suspension of human and animal erythrocytes and incubated for 1 hr. at room temperature. The agglutination was observed with naked eye. The titre was expressed as the reciprocal of the highest dilution of the lectin showing macroscopic agglutination.

Haemagglutination - Inhibition Test

Haemagglutination test was carried out as follows: 0.025 cm^3 of several different concentrations of carbohydrate inhibitors was added to equal volume of lectin solution, mixed and the mixture was incubated for 1 hr. at room temperature. At the end of the incubation period, 0.025 cm^3 of 2% suspension of red cells was added to each well and further incubated for 1 hr. The minimum concentration of sugar solution that showed inhibition was recorded.

Analytical method

Protein content of different lectin preparation was estimated using protein-dye binding modification (19). Bovine serum albumin was used as standard protein. Neutral sugar was estimated by the Phenol-H_2SO_4 method (20), with D-glucose as the standard. Amino acid analysis of the lectin was performed after hydrolysis of the sample (0.250 mg) with 6M HCl at 110°C for 24 hr (37). Tryptophan, was determined spectrophotometrically (21).

Effect of Cations, pH and Temperature

Mucuna lectin induced agglutination was tested in the pH range 3 - 6 in 10mM citrate-Phosphate buffer and pH 7 - 10 using 10mM Tris-Hcl buffer. The induced agglutination was also assayed in the presence of divalent cations, Ca^{2+}, Mg^{2+}, Mn^{2+} (30 - 100 mM) and at different temperature varying from 10°C to 90°C (at intervals of 10°C) for 30 min incubation. The heated solution was cooled to room temperature and then assayed.

Variation of lectin activity with maturation of Seed

Variation of lectin activity with maturation was measured using seeds of increasing weights. Fresh seeds of *Mucuna* of different weights ranging from 0.5g to 20g were used.

Seed coats of each group were scraped off and extracted (10% w/v) with 0.85% saline. The haemagglutinating properties of each extract was tested with normal human erythrocytes.

Role of water

A number of intact seeds of variable weights (13 - 16g) and scraped seed coat (3.9g) was incubated separately at constant temperate (37°C) for a few days. Among the incubated seeds, one or two were taken at a certain interval of time, scraped and extracted with saline. The activity of lectin and protein content were measured seperately at each time. The scraped seed coat (integument) was similarly treated.

Results and Discussion

Mucuna seed coat lectin was extracted using $(NH_4)_2SO_4$ precipitation and purified on porcine thyroglobulin-Sepharose affinity chromatography and eluted with 0.01M glycine-HCl buffer pH 3.5, followed by immediate meautralization. This was also passed through gel filtration on Sephadex G-100. This purification yielded the desired lectin with specific activity of 160 (titre per cm^3 per mg protein) and a purification fold of 100 with a yield of 4mg. The demonstration of lectin in the seed coat of *Mucuna* seeds is the first finding of the occurrence of lectin in the developing seed of legume. Studies in the development of Lentil (7) and phaseolus vulgaris lectins (12) indicated the presence of such proteins only in the ripening mature seeds. It has been reported that in *Dolichos biflorus* plant the lectin appeared abruptly 26 days after flowering with maximum level attained by 28 days (5). All lectins so far studied were found to be present in the cytoplasm of cotyledons. Using agglutination assays, the distribution of lectins in the seeds of dicotyledonous plants has been studied (22). The results showed that Maclura pomifera lectin started to accumulate during early seed development and attained maximum at 15 weeks with complete maturation of the seed (22). No lectin activity was found in dissected seed coat but the highest was found in the epicotyl. Similar high increase in lectin activity was reported in *Datura stramonium* (23) with growth of seeds. In contrast *Mucuna* seed coat lectin was extracted only from the seed integument and not from cotyledon. The disappearance of lectin in dried seed is perhaps due to loss of water. Hydration in addition to other factors

seems to be the most significant aspect for maintaining the three-dimensional confirmation of the lectin and also helps to release the lectin from the seeds as has been demonstrated for soybean lectin (24). The results of this study also confirm this.

Polyacrylamide gel electrophoresis at various pH, gel concentration and using various lectin concentration showed a single band and this seems to be homogenous. The homogeneity appears to be supported by its elution as a single symmetrical peak from gel filtration.

Haemagglutination assay

The haemagglutination pattern of normal and ensyme treated human and animal erythrocytes by purified lectin is shown in Table 2. The lectin agglutinated human A, B, O and AB erythrocytes equally well. This indicates that the lectin is a blood group non-specific one. It agglutinated chick, goat, rabbit and duck red cells but has no effect on guinea-pig and rat erythrocytes. Saracin lectin from Saraca indica has been reported to agglutinate the guinea-pig and rat erythrocytes (8). The average molecular mass estimated using various methods is 20,000 ± 1,500 while SDS gel electrophoresis shows a molecular mass of 19,000 ± 1,200. This indicates that the lectin is a monomeric protein with one polypeptide chain. The molecular mass of *Mucuna* lectin is higher than that of Saracin (8).

Table 1: Summary of purification procedures of *Mucuna* lectin

Fraction	Titre*	Protein(mg cm^{-3})	Specific activity++	Purification (fold)
Saline Extract	16	10	1.6	1
Decolorized with lead acetate	16	8	2	1.25
Affinity chromatography	32	0.8	40	25
Sephadex G-100	32	0.2	160	100

* Haemagglutination with normal human erythrocyte
++ Specific activity is expressed as titre cm^{-3} mg^{-1} protein.

Table 2: Haemagglutination pattern of various red cells

Erythrocyte	Titre		
	Untreated	Pronase treated	Neuraminidase Treated
Human A	8	16	16
B	32	32	16
O	16	32	16
AB	16	16	16
Chicken (local)	16	32	16
Goat	16	32	Nd.
Duck	16	32	-
Rabbit	16	8	8
Rat	8	4	4

Nd = Not determined

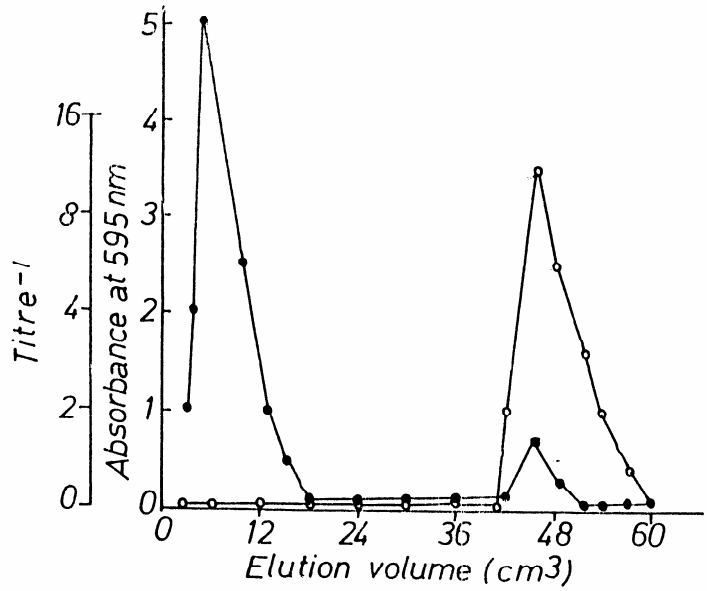

Fig. 1: Elution profile of Mucuna lectin from porcine thyroglobulin Sepharose column (1 x 5.0 cm) affinity chromatography. 2.0 cm^3 of decolourized extracts were applied to the column. After washing the column with 10 mM Tris-HCl buffer, pH 8.0 to remove the unreacted protein, the column bound protein was eluted with 10 mM glycine-HCl buffer, pH 3.5 and 3.0 cm^3 fractions were collected.
(●) Protein; (O) Lectin activity.

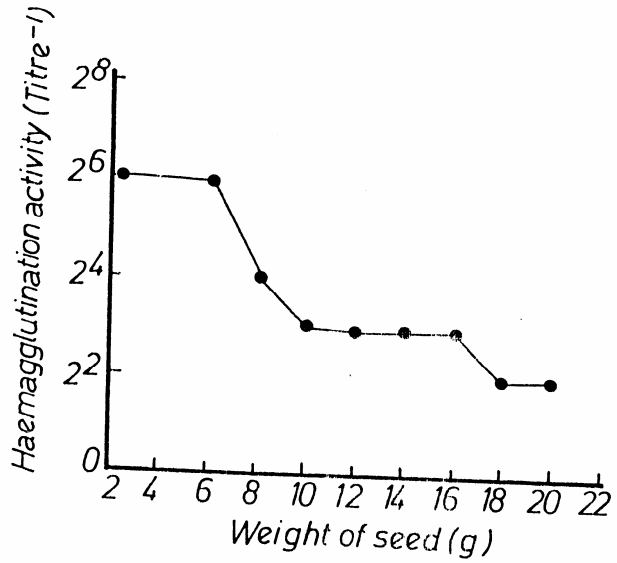

Fig. 2: Variation of lectin activity with maturation of the seed.

The *Mucuna* lectin activity was not remarkably affected by variation of pH of the medium or presence of divalent cations. The lectin retains its activity through pH range of 3 to 10 but activity gradually decreased at pH 9 and 10. The lectin is thermally stable up to temperature of 55°C.

Carbohydrate and amino acid analysis

Mucuna lectin is found to contain 13% neutral carbohydrate as determined by phenol-sulphuric acid reaction and this agrees well with the results obtained by GC analysis of neutral sugar 11% and amino sugar 1.5%. The glucan moiety of *Mucuna* lectin is composed of Arabinose (3.5g per 100g lectin), manose (5.0g), galactose (1.3g) and glucose (1.25g). The predominant amino acid is neutral amino acids alanine, glycine, serine and valine followed by acidic amino acids. It contains very low sulphur containing amino acids (Table 3). This contrasts with saracin (8) which contains no cysteine.

Table 3: Amino acid composition of lectin

Amino acid	g per 100g
Asp	7.5
Thr	6.6
Ser	8.5
Glu	15.3
Ala	15.2
Gly	20.1
Pro	3.02
Val	8.5
Met	0.5
Ile	3.2
Le	3.2
Tyr	1.0
Phe	2.0
His	0.51
Lys	6.00
Arg	1.80
Trp	5.20
Cys	0.05

Variation of lectin activity with maturation

The result of haemagglutination reaction by extracts of *Mucuna* lectin when tested separately showed that the lectin was located in the seed coat and not in cotyledon. The extracts of immature seed coats showed the highest agglutination activity when compared to the mature seeds. The decrease in lectin activity increase with the weight of the seed (Fig. 2).

Role of water in seed coat lectin

The role of water present in the seed coat in relation to lectin activity is shown in Fig. 3. Haemagglutinating activity of the lectin was abolished when the seeds lost 88.0% of water. However, the activity of the lectin was retained even at 73%dehydration (not shown). Fig. 3A shows the decrease in specific activity with increasing number of days, (a) while Fig. 3B showed the increase in percentage dehydration with number of days for intact seed coat. Lectin activity was lost after 66 days from initial. By contrast, the scraped seed coat lectin lost its activity after only 24 days (Fig. 3B (c). However, the loss of 84.0% water in the scraped seed coat caused the complete loss of lectin activity (Fig. 3B (d).

Haemagglutination-inhibition assay

Neither some of disaccharides nor free NeuAc, NeuAc -DGal-ß (1-4)-D-Glc, D-Gal ß-(1-4)-D-Glc NAc and D-Gal-ß(1-5) - D-Glc NAc inhibited *Mucuna* lectin induced agglutination. However, all asiale glycoproteins inhibited lectin agglutination (Table 4), indicating the specificity of the lectin towards complex carbohydrate chain of the glycoproteins employed. Porcine thyroglobulin is the most potent inhibitor of the lectin and contains at least two N-linked complex type Oligosaccharide chains (25). Likewise mucina were found to be good inhibitors. Porcine submaxillary mucin contains an O-linked oligosaccharide unit having a sequence of NeuAc -(2-6)-D-Gal-ß(1 - 3)-GalNAc (26) and the same unit is also present in bovine submaxillary mucin (27) and hog submaxillary mucin (28). However, bovine submaxillary mucin contains a large number of NeuAc (2 - 6) GalNAc units when compared with that of porcine submaxillary mucin. This seems to be responsible for the more inhibitory effect found in bovine mucin than in porcine submaxillary mucin (Table 4). Besides the mucins, fatiun moderately inhibited the lectin activity. Fetuin contains three O-linked and three N-linked Oligosaccharide chains (29) of which the former are two types, that is, NeuAC (2 - 3)-D-Gal ß(1 - 3) - D-GalNAc and NeuAC (2-3)-D-Galß (1 - 3) (Neuacα (2 - 6)-D-GalNAc. The second one is a complex type of triantenary carbohydrate chain with three NeuAC (α (2-6)/(2-3(-D-Gal-ß-(1-4)-GLcNAc sequence, (α 2-6/2-3) linkage being almost equal (30). It also contains another structure which differs from the previous one with subterminal Gal linked 1-3 to the GlcNAc unit with substitution at the C-6 hydroxyl group by NeuAc (8). The inhibitory effect of asiale fetuin may be due to the fact that α 2-3 linked NeuAC is less susceptible to acid hydrolysis when compared to α 2-6 linked. It has been shown that fetuin, after desialation, survived 5% NeuAc. By contrast in thyroglobulins NeuAc α (2-6)/2-3)D-Gal-ß(1-4)-GLcNAc sequence, the NeuAc is mostly α - 2-6 linked with small amount of α 2-3 linked (31). Acid hydrolysis leads to complete removal of α (2-6) linked NeuAc. making this glycoprotein non-inhibitory. Antithrombin III contains an alkaliabile O-chain (14) and was found to be a weak inhibitor. Although NeuAc is not inhibitory but when combines with N-acetyl/lactosylamine is responsible for binding (8). Non-inhibitory effect of neuraminyllactose is consistent with the conclusion that an acetamide group of C-2 of the reducing sugar in trisaccharide is necessary for binding. Lectin from *Sambucus nigra* (32) and *Bordetella portussis* toxin (31) have been reported to have specificity for terminal NeuAc α (2 - 6)D-Gal sequence irrespective of further sugar substitution on the galactose moeity. It therefore suggests that the combining site of *Mucuna* lectins is compelementary to the NeuAc α 2-6/2-3-Gal, (1 - 4)-GlcNAc sequence. α globulin, α macroglobulin and blood group substance are moderate inhibitors (Table 4). It has to be noted that assiale derivatives of glycoproteins are not inhibitory, except that of fetuin.

From this study, the exact carbohydrate specificity could not be deduced as lectins from different origins which recognised complex carbohydrate of various macro-molecules have been reported (8, 33 - 34).

In conclusion, this study has shown that *Mucuna* lectin is located only in seed coat and not ecotyledon and that the combining site of *Mucuna* seed coat lectin is complementary to the NeuAc α - (2 - 6)/(2 - 3)-D-Gal ß(1 - 4) D-Glc NAC sequence found in thyroglobulin.

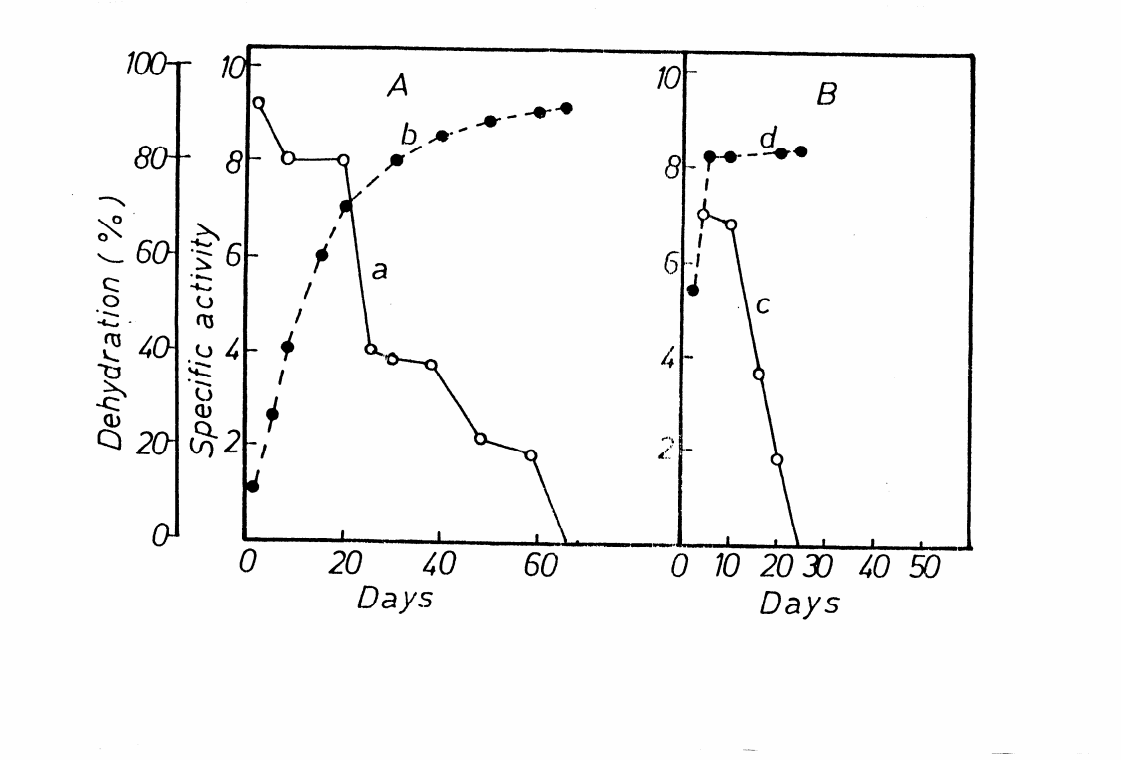

Fig. 3: The role of water on the activity of lectin. (A) Intact seed coat: (a) lectin activity versus number of days, (b) percentage dehydration versus number of days; (B) Scraped seed coat, (c) lectin activity versus number of days, (d) percentage dehydration versus number of days.

Table 4: Haemagglutination-Inhibition of *Mucuna* Lectin by glycoproteins

Inhibitor	Minimum inhibitory concentration (mg cm-3)*
Fetuin	1.3
Asailofeluin	1.25
Thyroglobulin (Procine)	0.08
Thyroglobulin (Bovine)	0.75
Submaxillary mucine (Procine)	3.20
Submaxillary mucine (Bovine)	1.25
Sumaxillary mucine (horse)	1.30
Erythrocyte mucoid (pig)	1.30
Erythrocyte mucoid (horse)	1.35
Glycophorin (Human)	3.10
r-globulin	0.75
Blood group O substance	0.76
Antithrombin III	5.25
Peptin A	5.25

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