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PURIFICATION AND PROPERTIES OF FOUR MONOCOT LECTINS FROM THE FAMILY ARACEAE

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Key Word Index— *Arisaema*; *Gonatanthus*; *Sauromatum*; monocot; Araceae; tuber; lectin; purification.

Abstract—Four new monocot lectins from the tubers of araceous plants, namely, *Arisaema consanguineum* Schott (ACA), *A. curvatum* Kunth (ACmA) and *Sauromatum guttatum* Schott (SGA) from the tribe Areae, and *Gonatanthus pumilus* D. Don (GPA) from the tribe Colocasieae have been purified by affinity chromatography on asialofetuin-linked amino activated silica beads. These lectins possess similar physicochemical and biological properties. All the lectins gave a single peak on HPLC size exclusion and cation exchange columns, and a single band on PAGE, (pH 4.5). In SDS–PAGE, all the lectins gave a single band corresponding to a subunit of M_r 13 000. All the lectins yielded multiple peaks on anion-exchange column, multiple bands on non-denatured PAGE (pH 8.3) and a family of bands on isoelectric focusing. The lectins agglutinate rabbit, rat and sheep red blood cells (RBCs) but are inactive towards human ABO erythrocytes. The haemagglutination activity of these lectins is inhibited by asialofetuin only, while simple sugars/derivatives including chitin, porcine mucin and fetuin did not react. In serological studies against rabbit anti-SGA serum, all four lectins produced immunoprecipitin lines. The lectins within each tribe were identical but the lectins belonging to the tribe Areae were only partially identical to the lectins from the tribe Colocasieae.

INTRODUCTION

Lectins have proved to be excellent and versatile macromolecular tools for the study of normal or transformed cell surfaces, for the isolation of glycoconjugates and for use in other areas of biomedical science [1, 2]. Most of the lectins which have been purified and characterized from plants have been obtained from dicotyledons. After the discovery of WGA [3], the first lectin from a monocot (Gramineae), more lectins were isolated from this and other monocot families such as the Liliaceae [4], Amaryllidaceae, Alliaceae [5–7] and Araceae [8]. Various Gramineae lectins from *Triticum aestivum*, *Secale cereale*, *Hordeum vulgare*, *Oryza sativa*, *Brachypodium sylvaticum* and *Agropyrum repens* [9] have been purified and characterized. Except for rice lectin [10], all Gramineae lectins have similar molecular structure, that is, they are dimers composed of M_r 18 000 subunits, and recognize GlcNAc, oligomers of GlcNAc and GalNAc. Amaryllidaceae lectins are dimers or tetramers composed of M_r 13 000 subunits, specific for mannose and mannose oligomers, mixtures of complex isolectins, and do not agglutinate human erythrocytes. With the exception of *Colocasia esculenta* [11] and *Alocasia indica* lectins [12], the araceous lectins have not been studied in purified form.

In this paper we describe, for the first time, the purification by affinity chromatography, and some properties, of four monocot lectins from the tubers of *Arisaema consanguineum*, *A. curvatum*, *Gonatanthus pumilus* and *Sauromatum guttatum* belonging to the family Araceae.

Abbreviations: RBC, red blood cell; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; PBS, phosphate buffered saline; ACA, *Arisaema consanguineum* agglutinin; ACmA, *A. curvatum* agglutinin; GPA, *Gonatanthus pumilus* agglutinin; SGA, *Sauromatum guttatum* agglutinin; WGA, wheat germ agglutinin.

RESULTS AND DISCUSSION

The specific inhibition of haemagglutination by asialofetuin was the basis for using asialofetuin-linked amino activated silica as the common affinity matrix for the purification of these monocot lectins. The affinity purification of ACA, ACmA, GPA and SGA is summarized in Table 1. All four lectins, after binding to an immobilized asialofetuin column, were eluted by 0.1 M glycine–HCl, pH 2.5, as a single peak (Fig. 1). Because the purification factor was low, we checked the suitability

Table 1. Affinity purification of araceous lectins

Plant species	Total protein* (mg)		Total haemagglutinating units (H.U.)†		Specific activity (H.U./mg protein)		Recovery (%)
	CE	AP	CE	AP	CE	AP	AP
Arisaema consanguineum	251	50	54 459	26 463	217	529	49
A. curvatum	504	111	18 018	12 012	36	108	67
Gonatanthus pumilus	855	122	16 0413	84 196	188	690	53
Sauromatum guttatum	335	35	35 9442	24 3056	1073	6944	68

*Calculated for 100 g of fresh tubers.

†Tested with rabbit RBCs. CE = Crude extract. AP = Affinity purified lectin.

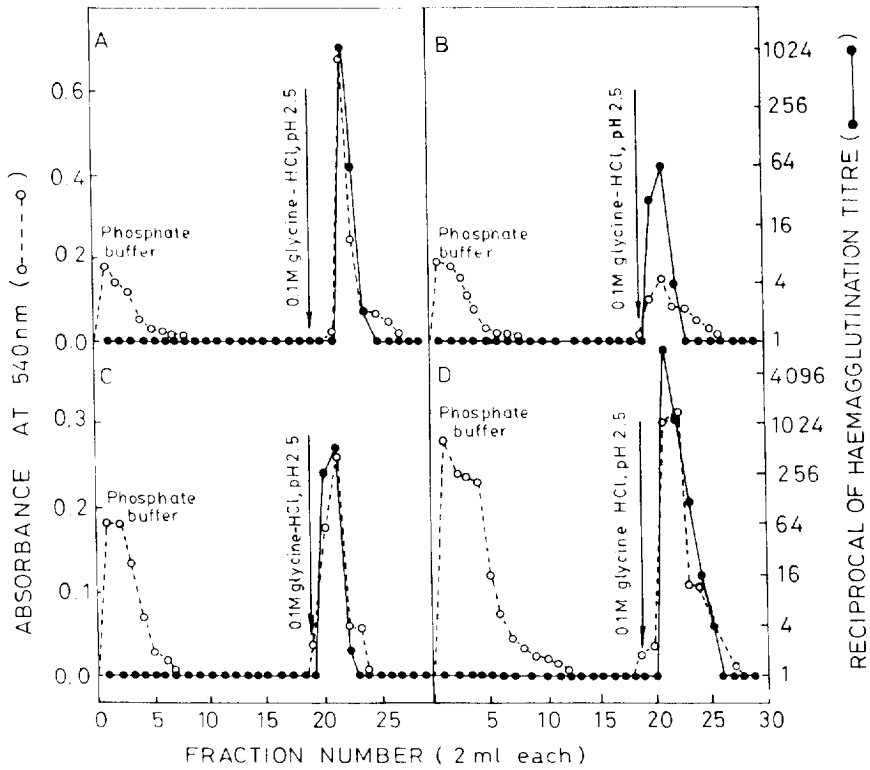

Fig. 1. Affinity chromatography of ACA (A), ACmA (B), GPA (C) and SGA (D) on asialofetuin-linked amino activated silica column (0.8 cm × 3 cm). Total crude protein loaded was 22, 16, 15 and 23 mg, respectively. After removing the unbound proteins with 0.01 M PBS (pH 7.2), lectin was eluted with 0.1 M glycine–HCl buffer (pH 2.5); flow rate = 40 ml hr^{-1}; o- - - -o absorbance at 540 nm [24]; ●——● reciprocal of haemagglutination titre against rabbit erythrocytes.

of the affinity system by testing the homogeneity of the purified lectins by various methods, i.e. PAGE at pH 4.5 (Fig. 2(B)), SDS–PAGE (Fig. 2(A)), gel filtration on Biogel P-200 and cation exchange chromatography. Furthermore, compared to the elution of dialysed crude extracts as multiple peaks by size exclusion on a 300 SW HPLC column and the formation of multiple bands in SDS–PAGE (Fig. 2(A)), the corresponding purified lectins yielded a single peak on HPLC and a single band in SDS–PAGE. A reasonably high amount of lectin activity was recovered in the purified lectins. Interestingly, although all four of the araceous lectins constituted only a small proportion of the total weight of tubers, they represented a considerable proportion of the tuber protein suggesting that such a high lectin content may fulfil some physiological role in the plant.

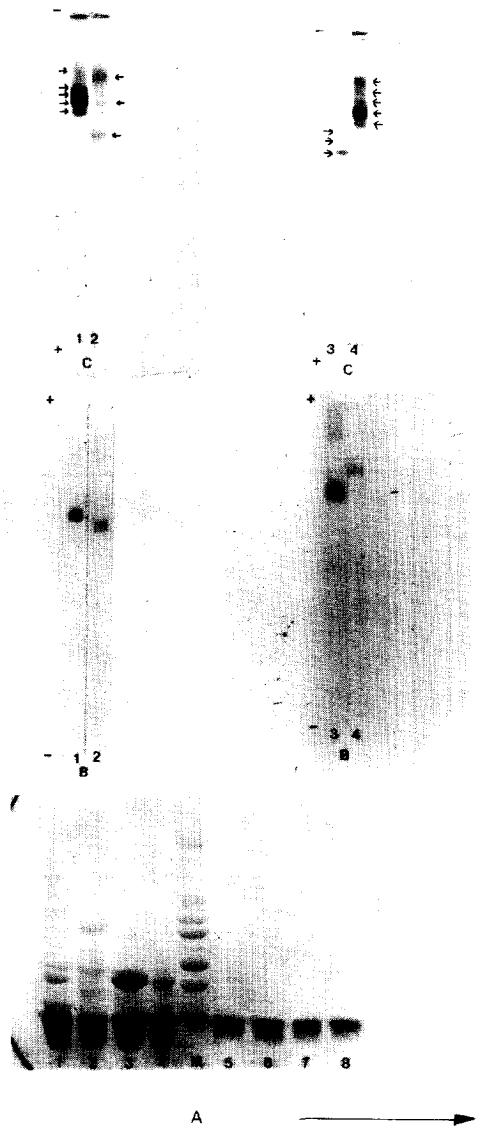

Fig. 2. (A) SDS–PAGE of crude tuber extracts (Lanes 1–4) and affinity purified lectins (Lanes 5–8) of *Arisaema consanguineum* (Lanes 1, 5), *A. curvatum* (Lanes 2, 6), *Gonatanthus pumilus* (Lanes 3, 7) and *Sauromatum guttatum* (Lanes 4, 8) using a 12.75% gel in the presence of 2% β-mercaptoethanol (3.5 mA gel^{-1}, 3 hr). Protein load: crude protein = 100 μg each, purified lectin = 40 μg each. Lane M, M_r markers (from top to bottom): albumin, bovine (M_r 66 000); albumin, egg (M_r 45 000); glyceraldehyde-3-phosphate dehydrogenase (M_r 36 000); carbonic anhydrase (M_r 29 000); trypsinogen (M_r 24 000); trypsin inhibitor (M_r 20 100); and α-lactalbumin (M_r 14 200). Gels were stained with Coomassie brilliant blue. (B) Discontinuous PAGE (pH 4.5) using 7.5% gel (10 mA gel^{-1}, 6 hr); protein load = 80 μg. Lane (1) ACA, (2) ACmA, (3) GPA, and (4) SGA. (C) Discontinuous PAGE (pH 8.3) using 10% gel (2 mA gel^{-1}, 6 hr); protein load = 50 μg. Lane (1) ACA, (2) ACmA, (3) GPA, and (4) SGA.

The native M_r of ACA, ACmA and SGA was 49 000 while that of GPA was 43 000, as estimated on a calibrated Biogel P-200 column. In SDS–PAGE, both under reducing (Fig. 2A) and non-reducing conditions, each lectin moved as a single band corresponding to M_r 13 000. The values of the native and subunit M_r of the lectins suggest that they are composed of four identical subunits which are not held together by disulphide linkages. The behaviour of purified lectins in PAGE, pH 8.3 (Fig. 2(C)), and their charge microheterogeneity revealed by isoelectric focusing (IEF) (Fig. 3) and anion exchange chromatography (Fig. 4) suggests that, like *Alocasia indica* lectin [12], these lectins exist as a complex mixture of isolectins differing in charge. Such a charge heterogeneity and the occurrence of isolectins has been reported for lectins from various sources, e.g. WGA [13] and lectins from Amaryllidaceae and Alliaceae [5], where the heterogeneity has a genetic basis [14]. Our observations on araceous lectins corroborate those of a lectin from *Bandeiraea simplicifolia*, which also showed multiple bands in PAGE under basic conditions and IEF [15]. Besides genetic reasons, other factors that can lead to the observed charge microheterogeneity are variations in individual tubers or their ageing and heterogeneity of the oligosaccharide chains [15].

These lectins, like *Alocasia indica* lectin from the family Araceae [12], differed distinctly from other monocot lectins [7, 16] in carbohydrate binding specificity. Thus, none of the members of a series of mono-, di- and trisaccharides, chitin, porcine mucin or fetuin, could inhibit the haemagglutination activity of the araceous lectins. However, desialylated fetuin at a minimal concentration of 125 μg ml^{-1} (2.8 μM) was able to inhibit the haemagglutination by ACA, ACmA and SGA, while the minimal inhibitory concentration of asialofetuin for GPA was 62.5 μg ml^{-1} (1.4 μM). The ability to recognize asialofetuin but not fetuin indicates the masking of recognition sites by sialic acid in fetuin. Fetuin, a complex serum glycoprotein, bears sialylated carbohydrate chains, i.e. 80% asparagine-linked oligosaccharides terminating in *N*-acetyllactosamine (Gal-β-1,4-GlcNAc) and 20% Ser/Thr-linked oligosaccharides having T-disaccharide (Gal-β-1,3-GalNAc) terminals [17]. Interestingly, although asialofetuin binds to the araceous lectins, the simple sugars constituting the oligosaccharide chains of asialofetuin, i.e. Gal, GalNAc, GlcNAc and Man, even when tested at a final concentration of 2 M, failed to recognize the lectins. Moreover, a mixture of GlcNAc oligomers in the chitin hydrolysate, when tested at a final concentration of 1 mg ml^{-1}, also did not inhibit haemagglutination by any of the four lectins. Thus, T-disaccharide, *N*-acetyllactosamine or mannose oligomers present in the oligosaccharide chains of asialofetuin might constitute the acceptor site for these lectins from the family Araceae. These lectins did not agglutinate normal or neuraminidase-treated human ABO erythrocytes. However, all four lectins agglutinated rabbit, rat, sheep and goat erythrocytes, except SGA which could not agglutinate goat erythrocytes even after desialylation (Table 2).

Unlike Amaryllidaceae and Alliaceae monocot lectins [5], ACA, ACmA, GPA and SGA, are glycosylated proteins having a sugar content of 0.71, 1.58, 4.10 and 0.47%, respectively. Both ACA and ACmA could withstand temperatures of 60° for 15 min while GPA and SGA toler-

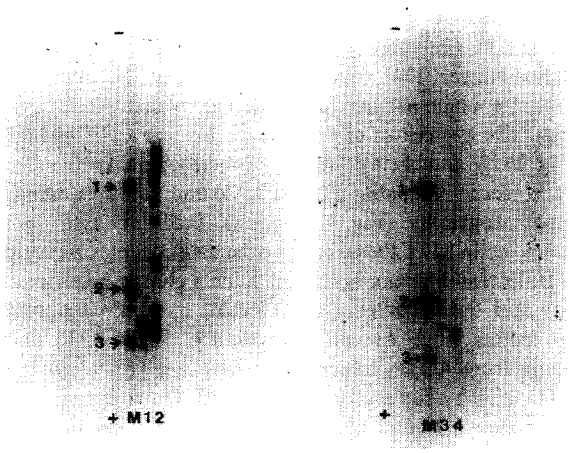

Fig. 3. Isoelectric focusing of non-denatured lectins on 5% polyacrylamide gel using carrier ampholine of pH range 3.5–10.0 (20 mA gel^{-1}, 12 hr); protein load = 25 μg; lane M = positions of p*I* marker proteins; (1) carbonic anhydrase I, human erythrocytes (p*I* 6.6); (2) carbonic anhydrase II, bovine erythrocytes (p*I* 5.9); (3) trypsin inhibitor, soybean (p*I* 4.6); Lane (1) ACA, (2) ACmA, (3) GPA, (4) SGA.

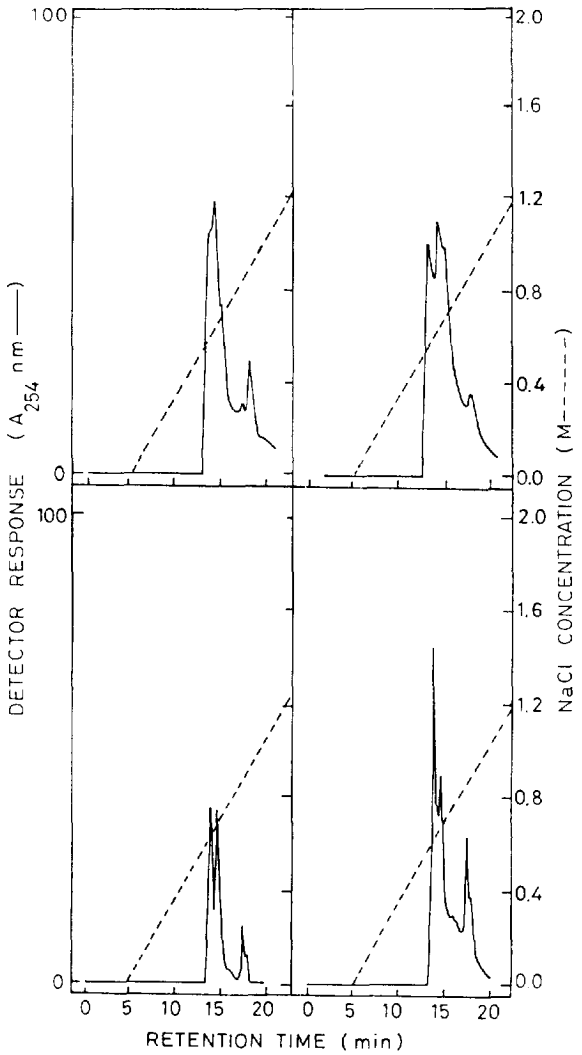

Fig. 4. Anion exchange HPLC of ACA (A), ACmA (B), GPA (C), and SGA (D) on DEAE-5PW column. Each lectin (10 μg) chromatographed. 0.01 M Tris–HCl buffer (pH 8.3) was used as running buffer to wash the column and the lectins were eluted by an ascending linear gradient of NaCl (0–2 M) prepared in the running buffer. Flow rate = 1 ml min^{-1}.

ated temperatures of 65° and 55° for 15 min without any loss of haemagglutination activity. GPA and SGA retained 25% of their haemagglutination activity, even after incubating in a boiling water bath for 15 min, while the residual activity in the case of ACA and ACmA was 50 and 12%, respectively. None of the lectins required metal ions for haemagglutination activity.

Taxonomically, ACA, ACmA and SGA belong to the tribe Colocasieae, while GPA and *Alocasia indica* lectin are from the tribe Areae [18]. All four araceous lectins studied, as well as *Alocasia indica* lectin, cross-reacted with rabbit anti-SGA serum (Fig. 5). Serological identity between members of the same tribe and partial identity between members of different tribes, i.e. Areae and Colocasieae, reflect the evolutionary divergence between these tribes of the family Araceae. In our preliminary investigations, all the four lectins were found to possess mitogenic potential for human blood lymphocytes. Thus, this investigation along with our previous study on *Alocasia indica* lectin [12], shows that the Araceae family is a new source of related lectins which may prove to be useful tools for glycoconjugate research.

EXPERIMENTAL

Materials. *Arisaema consanguineum* Schott, *A. curvatum* Kunth, *Gonatanthus pumilus* D. Don and *Sauromatum guttatum* Schott grow wild in temperate regions. Tubers of these plants were collected from the Kumaon Hills, India, in the month of September. Amino activated silica beads (1000 Å pore size: 100 μ diameter) were purchased from Clifmar Associates, U.K. All sugars/derivatives, glycoproteins, M_r markers for SDS–PAGE and gel filtration chromatography, and p*I* markers for isoelectric focusing were the products of Sigma Chemical Co., U.S.A. Biogel P-200 for gel filtration chromatography was supplied by Bio-Rad, U.S.A.

Lectin purification. *Arisaema consanguineum* (ACA), *A. curvatum* (ACmA), *Gonatanthus pumilus* (GPA) and *Sauromatum guttatum* (SGA) agglutinins were purified by affinity chromatography using asialofetuin immobilized on amino activated silica, as previously described [12, 19]. For immobilization of asialofetuin, 1 g of amino activated porous silica beads were mixed with 20 ml 1.25% glutaraldehyde soln for 2 hr. The beads were then washed with 50 ml 0.1 M $NaHCO_3$ buffer, pH 8.3, and mixed with 20 mg asialofetuin dissolved in 10 ml 0.1 M $NaHCO_3$ buffer by means of a roller mixer. After estimating the unbound asialofetuin in the supernatant, it was found that 16 mg of asialofetuin was immobilized on

Table 2. Agglutination of erythrocytes

	MEALC ($\mu g\,ml^{-1}$)							
	ACA		ACmA		GPA		SGA	
Type of erythrocyte	UT	NT	UT	NT	UT	NT	UT	NT
Rabbit	4.65	4.65	1.85	1.85	1.78	1.78	0.144	0.144
Rat	74.38	0.58	59.06	0.06	68.31	0.02	1.140	0.005
Goat	NA	148.95	NA	29.53	NA	273.24	NA	NA
Sheep	5.95	9.30	236.25	1.85	273.24	17.06	NA	0.143

MEALC = Minimum erythrocyte agglutinating lectin concentration; UT = untreated RBCs; NT = neuraminidase-treated RBCs; NA = no agglutination.

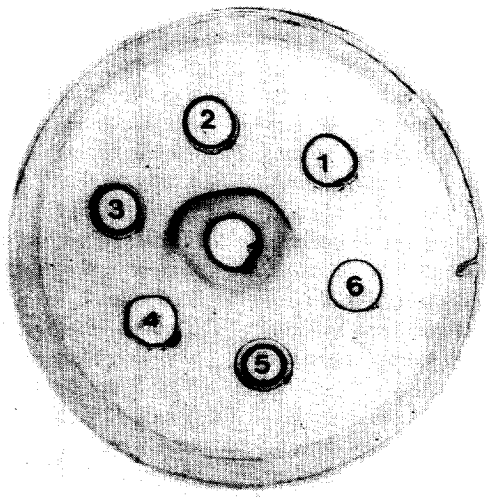

Fig. 5. Double immunodiffusion of purified araceous lectins against 25 μl anti-SGA antiserum (central well). Ten μg of SGA (1), ACA (2), ACmA (3), *Alocasia indica* lectin (4, 5), and GPA (6) were applied in the peripheral wells.

the silica. To block the unreacted groups, ligand-linked silica beads were washed with 0.2 M glycine in $NaHCO_3$ buffer by means of a roller mixer for 6 hr. Finally, the column was equilibrated with 0.01 M PBS and used for lectin purification.

Immediately after collection, tubers of all species were processed for the prepn of crude extracts. Fresh tubers were washed, peeled, chopped, crushed in a waring blender in 0.01 M PBS, pH 7.2, and kept overnight. The crushed tubers were again homogenized in the blender for maximum protein extraction and after centrifugation at 20000 *g* for 30 min, the clear supernatant was extensively dialysed against 0.01 M PBS at 4°. Dialysed crude extract was applied to the affinity column (0.8 cm × 3 cm) and recirculated (× 3) to ensure complete adsorption of lectin. The unbound proteins were washed off with 0.01 M PBS and the bound lectin eluted with 0.01 M glycine–HCl buffer, pH 2.5. Eluted frs were immediately neutralized with 2 M Tris–HCl buffer, pH 8.8, and dialysed exhaustively against 0.01 M PBS at 4°C.

Haemagglutination and sugar inhibition assays. Agglutination assays and their inhibition were carried out as described earlier [8]. The series of sugars used in the hapten inhibition assay included 3 pentoses, 17 hexoses or their derivatives, 4 disaccharides, 2 trisaccharides, 1 polysaccharide, i.e. chitin ($4\,mg\,ml^{-1}$) and 3 serum glycoproteins, i.e. fetuin ($4\,mg\,ml^{-1}$), asialofetuin ($2\,mg\,ml^{-1}$) and porcine mucin ($4\,mg\,ml^{-1}$). Chitin hy-

drolysate, containing a mixture of GlcNAc oligomers, was also tested at a final concentration of 1 mg ml^{-1}.

Homogeneity and M_r determination. Homogeneity and molecular mass of native ACA, ACmA, GPA and SGA lectins were determined by gel filtration chromatography on a calibrated Biogel P-200 column (1.6 cm × 79 cm; V_o = 45.4 ml) using 0.01 M PBS, pH 7.2. The purity of the affinity purified lectins was also checked on a Waters 650 Advanced Protein Purification System using a 300 SW HPLC analytical size exclusion column (0.8 cm × 30 cm). Dialysed crude tuber extracts were also chromatographed on a HPLC size exclusion column equipped with a 254 nm UV detector.

Electrophoretic analyses. Polyacrylamide tube gel electrophoresis using a discontinuous buffer system was carried out on a 7.5% (w/v) gel at pH 4.5 [20] and 10% (w/v) gel at pH 8.3 [21, 22]. The subunit M_r of the four lectins was estimated by discontinuous SDS–PAGE in the presence and absence of 2% β-mercaptoethanol using 12.75% (w/v) tube gels [23]. Isoelectric focusing of the purified lectins was performed in 5% polyacrylamide gel using carrier ampholine of pH range 3.5–10.0 as described in the LKB Laboratory Manual no. 2001.

Ion exchange chromatography. Ion exchange chromatography of lectins was carried out on a Waters 650 HPLC System using a DEAE-5PW (0.8 cm × 7.5 cm) and a SP-5PW (0.8 cm × 7.5 cm) ion exchange column. Lectins dialysed against 0.01 M Tris–HCl, pH 8.3, and 0.05 M acetate–acetic acid, pH 4.5 (running buffers), were applied to the DEAE-5PW and SP-5PW columns, respectively. A linear gradient of increasing NaCl concentration (0–2 M) in running buffers was used to elute the lectins bound to the anion and cation exchange columns.

Production of antisera and immunodiffusion. Antibodies against the four araceous lectins were raised in rabbits. Each lectin (1 mg), emulsified in Freund's complete adjuvant, was injected intramuscularly into different rabbits. Three booster injections, each at an interval of 7 days, were given. Rabbits were bled a week after the final injection and the serum sepd.

Immunodiffusion of ACA, ACmA, GPA and SGA as well as *Alocasia indica* lectin against antisera of all the four lectins was performed in petri dishes containing a layer of 1.25% noble agar. After formation of precipitin lines, gels were washed repeatedly with 5% sodium citrate soln to remove soluble proteins and finally stained with 0.1% amido schwarz dye.

Metal ion requirement. Lectins were demetallized by prolonged dialysis against 0.1 M EDTA followed by 1 M HoAc and tested for haemagglutination titre.

Heat stability. Thermal stability of the lectins was ascertained by incubating the purified lectins at elevated temps ranging between 50° and 95° (5° intervals) for 15 min at each temp.

Protein and carbohydrate determination. Protein estimation was carried out according to Lowry *et al.* [24]. Total neutral sugar content was determined by reaction with anthrone reagent [25].

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