Carbohydrate Research, 191 (1989) 305–314
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

PURIFICATION OF ANT-EGG GLYCOPROTEIN AND ITS INTERACTION WITH JACALIN*

SUDIPA RAY AND BISHNU P. CHATTERJEE†
Department of Biological Chemistry, Indian Association for the Cultivation of Science, Jadavpur, Calcutta 700 032 (India)
(Received August 17th, 1988; accepted for publication, February 28th, 1989)

ABSTRACT

Ant (*Oecophylla smaragdina* Fabr.) eggs contain a glycoprotein which was extracted with phenol–saline and purified on jacalin–Sepharose 4B. The glycoprotein contained D-mannose, D-galactose, D-glucose, and 2-acetamido-2-deoxy-D-glucose, and gave precipitin bands with the α-D-galactosyl-specific lectins from *Artocarpus integrifolia* (jacalin), *Artocarpus lakoocha* (artocarpin), *Agaricus bisporus*, and *Maclura pomifera*. The presence of non-reducing α-D-galactosyl end-groups was corroborated by methylation analysis and enzymic degradation. The formation of the jacalin–glycoprotein complex was dependent on time, pH, and the ionic strength of the medium.

INTRODUCTION

The interaction of lectins with soluble polysaccharides and glycoproteins, or such particulate antigens as cells, often results in precipitation or agglutination owing to the attachment to specific carbohydrate determinants. The interaction of concanavalin A with polysaccharides[1–5] may be analogous to the antibody–antigen system. The interaction of jacalin, the lectin produced by *Artocarpus integrifolia*, with the glycoprotein from ant eggs was demonstrated first by the agar gel-diffusion technique[6] and the quantitative precipitin reaction[7]. We now report on the purification and properties of ant-egg glycoprotein.

EXPERIMENTAL

Ant (*Oecophylla smaragdina* Fabr.) eggs, used as avian food and bait for fish, were purchased from a local market. All operations were carried out at 4°, unless otherwise stated.

*Carbohydrate–Lectin Interactions, Part I.
†Author for correspondence.

0008-6215/89/$ 03.50

Lectins from *A. integrifolia*[8] (jacalin), *Artocarpus lakoocha*[9] (artocarpin), and *Arachis hypogaea*[10] (peanut) were prepared by the rivanol precipitation method. Concanavalin A was obtained from Calbiochem, and lectins from *Agaricus bisporus*, *Maclura pomifera*, *Glycine max*, *Dolichos biflorus*, and *Helix pomatia* were purchased from Sigma. Jacalin was conjugated[11] to cyanogen bromide-activated Sepharose-4B (Pharmacia). The lectin from *Crotalaria striata* was purified to homogeneity on 2-acetamido-2-deoxy-D-galactose-conjugated starch[12].

Purification of ant-egg glycoprotein (AEG). — Ant eggs (400 g) were homogenised in 0.15M NaCl using a blender (Remi), and the slurry was stirred overnight, then centrifuged in a refrigerated Sorvall RC-5B centrifuge at 12,000 r.p.m. for 40 min. The clear supernatant solution was treated with 1 vol. of aqueous 90% phenol at 65° for 30 min. The hydrophilic part that accumulated at the upper phase after centrifugation was collected, dialyzed extensively against distilled water, and lyophilized. Part (10 mg) of the residue (2.6 g) was applied to a column (1 × 2.0 cm) of jacalin-conjugated Sepharose 4B pre-equilibrated with PBS (10mM phosphate buffer containing 150mM NaCl, pH 7.0). The column was washed with the same buffer until the absorbance of the effluents at 280 nm became 0.02, and the adsorbed material was then eluted with PBS containing 0.3M D-galactose. The fractions that reacted positively with phenol–H_2SO_4, after concentration by Centricon 10 (Amicon), were combined, dialyzed against distilled water, and stored at 4° until used.

Gel electrophoresis. — Disc gel electrophoresis in 10% polyacrylamide gel was performed[13] at pH 8.9 in Tris–glycine buffer, with staining using 0.05% Coomassie Brilliant Blue in aqueous 7% acetic acid and destaining with aqueous 7% acetic acid. The protein markers were ovalbumin (mol. wt. 45,000), carbonic anhydrase (29,000), cytochrome C (12,400), aprotinin (6,500), and myoglobin (fragment III, 2,510).

High-voltage electrophoresis. — A Shandon L-24 apparatus was used with Whatman 3MM paper, 0.05M sodium barbital (pH 8.5), and pyridine–acetic acid (pH 4.0) buffers, and a potential gradient of 36 V/cm. Glycoprotein was detected with 0.1% ninhydrin in acetone.

Antiserum. — Phenol-extracted AEG (5 mg/mL) was mixed with Freund's complete adjuvant (Difco Laboratories) and injected intramuscularly into rabbits once per week for 4 consecutive weeks followed by a booster in Freund's incomplete adjuvant after 2 weeks. The animals were bled by cardiac puncture in the following week. The blood was allowed to clot, and the serum was collected and preserved at −20° until used.

Immunoelectrophoresis. — A Desaphor HL electrophoresis apparatus (Desaga) was used as described[14], with a 3% agarose gel in 0.05M sodium barbital buffer (pH 8.5) at 3 V/cm for 20 min. The gel plates were developed overnight with antiserum (100 μL), fixed, stained as for gel electrophoresis, and destained[15].

Immunodiffusion. — Ouchterlony plates were prepared as described[16]. Each well was filled with 10 μL of test sample (5 mg/mL) and the plates were developed overnight at 25° in a moist chamber.

Treatment of glycoprotein with α-D-galactosidase. — A solution of purified AEG (20 mg) in 0.5M citrate–phosphate buffer (2 mL, pH 5.2) was incubated[17] with α-D-galactosidase (0.09 U/mL) for 48 h at 37°. The mixture was then heated at ~100° for 2 min and centrifuged, and the supernatant solution was dialyzed against distilled water overnight and lyophilized. An Ouchterlony double-diffusion test was performed on the residue as described above.

Carbohydrate analysis. — Total neutral carbohydrate was determined by the phenol–H_2SO_4 method[18], using D-glucose as standard. Individual neutral sugars were determined after hydrolysis with M H_2SO_4 for 16 h at 100° with *myo*-inositol as the standard. The hydrolysate was neutralised ($BaCO_3$) and the neutral sugars were analysed as their alditol acetates by g.l.c. (Hewlett–Packard Model 5730 A) on 3% of ECNSS-M or 3% of OV-225 on Gas Chrom Q (100–120 mesh). For determination of amino sugars, the glycoproteins were hydrolysed with 4M HCl for 4 h at 100° with *myo*-inositol as the standard. The acid was removed by co-distillation with methanol, the residue was stored over KOH pellets under vacuum, and the amino sugars were analyzed as the alditol acetates[19] by g.l.c. on 3% of Poly A-103 on Gas Chrom Q (100–120 mesh).

Methylation. — Purified AEG was methylated twice by the method of Hakomori[20]. The product had no i.r. absorption for hydroxyl at 3600–3300 cm^{-1}. The methylated glycoprotein was hydrolysed with 2M trifluoroacetic acid for 20 h at 100° and the products were analysed as the alditol acetates by g.l.c. on 3% of ECNSS-M on Gas-Chrom Q (100–120 mesh) and 3% of OV-225 on Gas-Chrom Q (100–120 mesh).

Interaction with lectins. — Interaction of the purified AEG with jacalin, artocarpin, peanut, and concanvalin A was assayed by a turbidimetric method[21]. A solution of AEG (2 mL, 1 mg/mL) in 0.2M acetate buffer (pH 3.8) was stirred with a solution of the lectin in 0.15M NaCl (50 μL, 10 mg/mL). The development of turbidity was monitored at 405 nm spectrophotometrically (Hitachi Model 100-60).

Effect of pH. — To a solution of purified AEG (2 mL, 1 mg/mL) in each of various buffers (0.5M glycine–HCl, 0.5M acetate, 0.5M PBS, and 0.5M Tris), with pH values in the range 1.5-10.5, was added a solution of jacalin in 0.15M NaCl (50 μL, 10 mg/mL). Each mixture was stirred and incubated for 15 min at room temperature, and the absorbance was measured at 405 nm against a blank containing glycoprotein.

Effect of concentration of salt and organic compounds. — Turbidimetric measurements were performed on a mixture of a solution of AEG in 0.2M acetate buffer (2 mL, 0.5 mg/mL; pH 3.8) and a solution of jacalin (25 μL, 10 mg/mL) in PBS with incorporation of increasing concentrations of NaCl, $CaCl_2$, $MnCl_2$ (10–200mM). Formation of the jacalin–AEG complex was also tested in the presence of KCl, KBr, KI, and KSCN (0.02–2M); 200mM $NaIO_4$, $NaNO_2$, Na_2SO_4, and K_2SO_4; 200mM urea; and 100mM methanol, ethanol, 1-propanol, 1-butanol, ethyleneglycol, and glycerol. The absorbance was measured at 405 nm as described previously.

RESULTS

Purification of AEG. — The saline extract of ant eggs, on treatment with hot phenol, gave a glycoprotein (AEG) which was purified by elution from jacalin–conjugated Sepharose 4B, with 0.3M D-galactose. Purified AEG gave a single band in polyacrylamide gel electrophoresis at pH 8.9, whereas the phenol-extracted

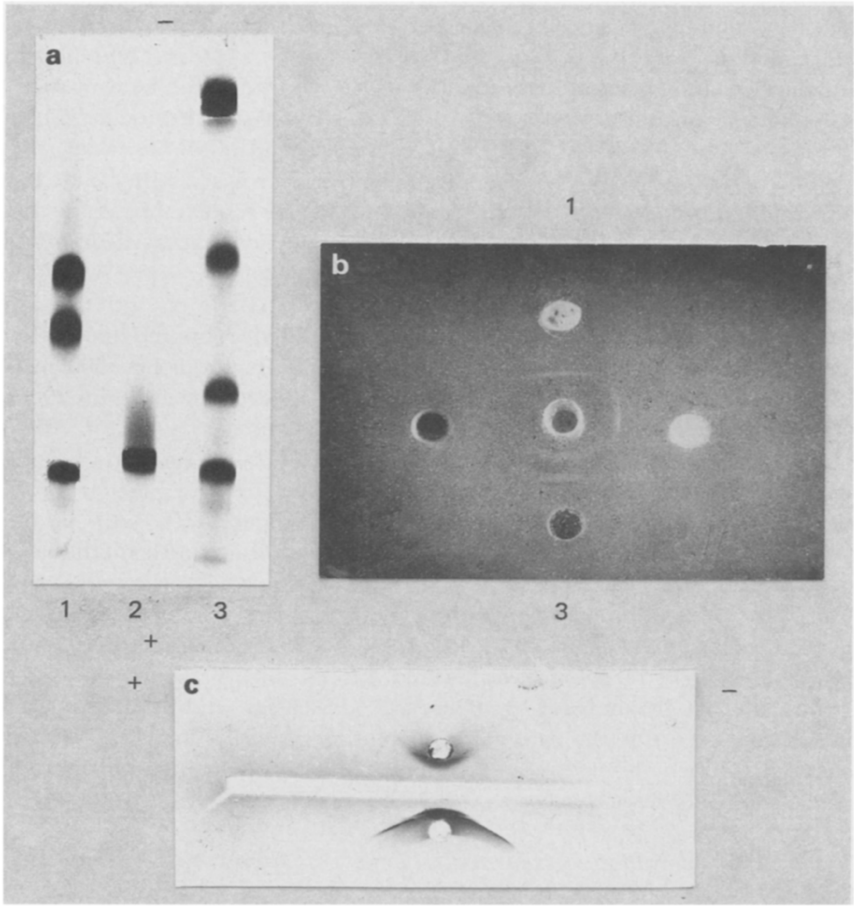

Fig. 1. (a) Polyacrylamide disc gel electrophoresis of purified ant-egg glycoprotein (AEG) in 10% gel with 100–250 μg of sample at pH 8.9: lane 1, phenol-extracted AEG; lane 2, purified AEG; and lane 3, protein markers. The migration of glycoprotein was from the top. The gel was stained with Coomassie Brilliant Blue. (b) Immunodiffusion pattern of AEG with antiserum to the glycoprotein (middle well): 1, retarded AEG fraction; 2, unretarded AEG fraction; and 3, phenol-extracted AEG. The wells are numbered clockwise from the top (well 1). (c) Immunoelectrophoresis of AEG. Upper and lower wells were filled with affinity-column-retarded and -unretarded AEG fractions. The trough was filled with rabbit antiserum to the glycoprotein.

TABLE I

CARBOHYDRATE COMPOSITION OF ANT-EGG GLYCOPROTEIN

Sugar	*Glycoprotein fractions*	
	Affinity-column retarded (g/100g)	*Affinity-column unretarded (g/100g)*
Gal	4.24	3.96
Glc	1.49	15.95
Man	0.46	
GlcNAc	2.02	1.98

material gave three bands (see Fig. 1a) and produced a single arc with its rabbit antiserum (see Fig. 1c). The molecular weight was estimated to be ~5,000. A single band was also obtained on immunodiffusion (see Fig. 1b). The unretarded material from the column also cross-reacted. High-voltage electrophoresis at pH 8.5 gave a single spot that migrated towards the anode, whereas at pH 4 it migrated towards the cathode.

Carbohydrate analysis. — The glycoprotein (Table I) contained galactose, glucose, mannose, and 2-acetamido-2-deoxyglucose. The retarded and unretarded AEG fractions from jacalin-conjugated Sepharose 4B contained 7.6 and 20% of neutral carbohydrate, respectively, as determined by the phenol–H_2SO_4 method, (*cf.* 6.19 and 19.91%, respectively, as determined by g.l.c.). The retarded AEG fractions contained D-mannose, D-galactose, and D-glucose in the ratios 1:9.1:3.23,

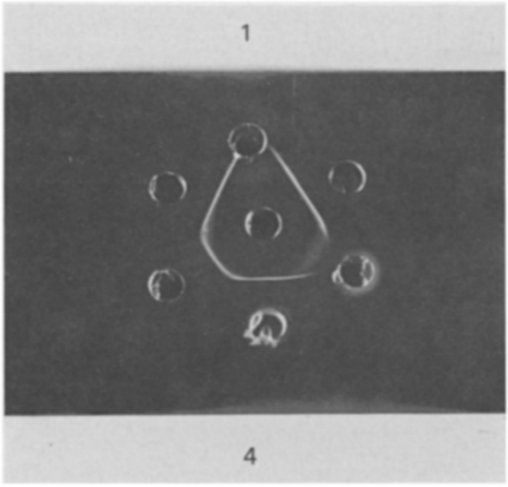

Fig. 2. Precipitin reaction of purified AEG (middle well) with lectins: 1, Concanavalin A; 2, *A. integrifolia*; 3, peanut; 4, *A. bisporus*; 5, *M. pomifera*; and 6, *A. lakoocha*. The wells are numbered clockwise from the top (well 1). Each well was filled with 20 μL of test substance (5 mg/mL).

whereas the unretarded AEG fraction contained D-galactose and D-glucose in the ratio 1:4. Each of these fractions also contained 2-acetamido-2-deoxy-D-glucose.

Ouchterlony double diffusion. — Purified AEG precipitated the lectins jacalin, artocarpin, *A. bisporus, M. pomifera,* peanut, and concanavalin A (see Fig. 2). Of these lectins, jacalin, artocarpin, *A. bisporus,* and *M. pomifera* showed a reaction identity, whereas peanut lectin reacted in a different way as evidenced by spur formation. The glycoprotein did not precipitate the lectins from *D. biflorus, G. max, C. striata,* and *H. pomatia,* whereas, with wheat-germ lectin, a sharp precipitation band appeared (not shown). After treatment with α-D-galactosidase, the glycoprotein did not precipitate the lectins.

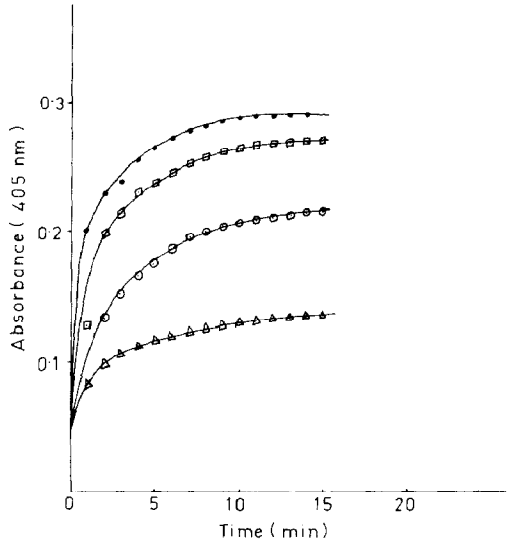

Fig. 3. Interaction of purified AEG with jacalin (—●—), artocarpin (—□—), peanut lectin (—○—), and concanavalin A (—▲—) at pH 3.8; 2 mg of AEG and 0.5 mg of lectin in a total volume of 2.05 mL were taken in each measurement.

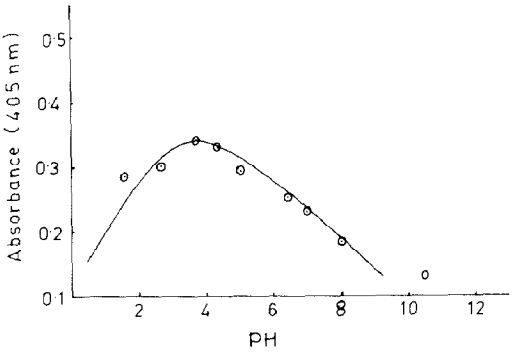

Fig. 4. Effect of pH on the AEG–jacalin interaction. The conditions were as described in Fig. 3.

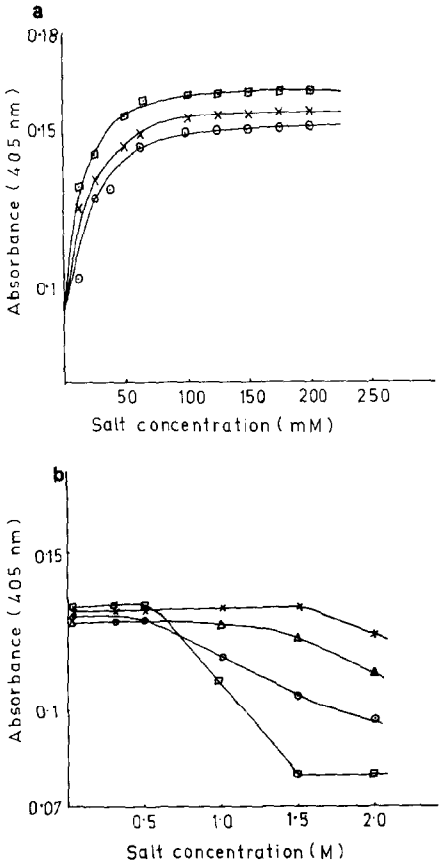

Fig. 5. (a) Effect of NaCl (—O—), $MnCl_2$ (—×—), and $CaCl_2$ (—□—) on the AEG–jacalin system: 1 mg of AEG and 0.25 mg of jacalin in a total volume of 2.025 mL were taken in each measurement. (b) Effect of KCl (—×—), KBr (—△—), KI (—O—), and KSCN (—□—) on the AEG–jacalin system. The conditions were as in (a).

Methylation analysis. — Methylated purified AEG gave 2,3,4,6-tetra-*O*-methyl-D-galactose (1 mol), 2,3,4-tri-*O*-methyl-D-galactose (4.6 mol), 2,4,6-tri-*O*-methyl-D-galactose (2.7 mol), 2,3,4,6-tetra-*O*-methyl-D-glucose (1.0 mol), 2,4,6-tri-*O*-methyl-D-glucose (0.8 mol), 4,6-di-*O*-methyl-D-glucose (1.0 mol), and 2,3-di-*O*-methyl-D-mannose (0.9 mol). The g.l.c.–m.s. data for the alditol acetates of the above compounds will be published elsewhere.

Interaction with lectins. — Fig. 3 shows the interaction of AEG with jacalin, artocarpin, peanut lectin, and concanavalin A. The turbidity reached a maximum in 14 min. Jacalin interacted most strongly and concanavalin A least strongly.

Effect of pH. — Binding between jacalin and AEG was pH-dependent (see Fig. 4) with an optimum at pH 3.8.

Effect of salts and organic compounds. — The jacalin–AEG interaction in-

TABLE II

EFFECT OF DIFFERENT SALTS AND ORGANIC COMPOUNDS ON ANT-EGG GLYCOPROTEIN–JACALIN INTERACTION

Compounds	*Concentation (mM)*	*Absorbance (405 nm)*
Saline	150	0.128
Sodium sulfate	200	0.141
Potassium sulfate	200	0.134
Sodium nitrite	200	0.133
Sodium periodate	200	0.111
Urea	200	0.045
Methanol	100	0.160
Ethanol	100	0.157
1-Propanol	100	0.160
1-Butanol	100	0.155
Ethylene glycol	100	0.164
Glycerol	100	0.163

creased with increasing strength of the salt (see Fig. 5a), and $CaCl_2$ was the most effective. Fig. 5b shows the effect of various potassium salts on the jacalin–AEG interaction. KI and KSCN inhibited the precipitation (the latter strongly at 1.5M), whereas the chloride and bromide salts had no effect even at 2M. Na_2SO_4, $NaNO_2$, and K_2SO_4 increased the turbidity, whereas $NaIO_4$ and urea caused a decrease. Ethanol increased the precipitation, but other alcohols, ethylene glycol, and glycerol had no effect (see Table II).

DISCUSSION

Glycoproteins, as well as polysaccharides, are readily detected by the Ouchterlony double-diffusion technique using lectins. Isolation and fractionation of glycoproteins and polysaccharides can be effected by affinity chromatography on lectins.

The earlier study of the precipitin reaction of ant-egg glycoprotein with jacalin prompted purification of the glycoprotein on immobilized jacalin. The glycoprotein (AEG) purified by this method was homogeneous by several standard physical methods. The AEG fraction retarded on the column contained glucose, galactose, mannose, and 2-acetamido-2-deoxyglucose. The line of identity in the precipitin band exhibited by the α-D-galactosyl-specific lectins jacalin, artocarpin, *A. bisporus,* and *M. pomifera* indicated the presence of terminal non-reducing α-D-galactosyl units. The above results were corroborated by methylation analysis, which gave 2,3,4,6-tetra-*O*-methyl-D-galactose, and by degradation with an α-D-galactosidase followed by precipitation studies. The spur formation by peanut lectin (Fig. 2) suggests reactivity with the glycoprotein that was different from those of the other lectins. This behaviour might be due to the presence of *N*-acetyl-lactosamine residues in the AEG fraction bound to the column, as peanut lectin also cross-reacts

with this disaccharide[22]. The reactivity of peanut lectin is not due to its specific carbohydrate receptor, β-D-Gal-(1→3)-D-GalNAc, since the retarded AEG fraction did not contain GalNAc (see Table I). This result was further supported by the fact that the 2-acetamido-2-deoxy-D-galactosyl-specific lectins, such as those from *H. pomatia, D. biflorus, G. max*[23], and *C. striata*[12], did not react with retarded AEG fraction.

The parameters optimal for jacalin–AEG precipitation, which reached a maximum in the system in 14 min, were investigated by turbidimetry. The interaction of the retarded AEG fraction with peanut lectin and concanavalin A was weaker, but precipitation was complete in the same time, whereas artocarpin behaved similarly to jacalin. Unlike the concanavalin A–polysaccharide interaction, in which maximum precipitation occurs at pH 6.1–7.2, the jacalin–AEG precipitation was maximal at pH 3.5–4.

Increase in ionic strength of the salt in the reaction system facilitates the reactivity of charged groups, thereby increasing the precipitation of the jacalin–AEG complex. The significant change in turbidity with various inorganic salts indicated that changes in ionic composition had pronounced effects on the binding. The same concentration of sulfate and nitrite enhanced the interaction, whereas periodate and urea caused inhibition. Periodate can oxidise some amino acids and will cleave sugars having vicinal hydroxyl groups, whereas urea is a protein denaturant and can cause cleavage into sub-units.

The effect of various anionic species of the same metal cation showed that the anions had more pronounced effects than variation in ionic strength. This effect was also observed in the interaction of protein antigens and antibodies[24]. The effect of anions on the lectin–glycoprotein interaction followed the familiar lyotropic series $CNS^- > I^- > Br^- > Cl^-$ and was related to their energies of hydration. The energies of hydration of Cl^-, Br^-, and I^- ions are 65,000, 57,000, and 47,000 cal/g mol, respectively[25], and the value for CNS^- is lower[26] than that of I^-. Decrease in the energy of hydration of an anion facilitates its approach and binding with a positive group in the protein/glycoprotein, thereby inhibiting the interaction. The inhibition of the jacalin–AEG interaction by some anions emphasised the importance of charged groups in the above reaction.

Unlike the concanavalin A–polysaccharide interaction, the presence of alcohols in the jacalin–AEG system increased the precipitation. Increase in the number of -OH groups and $-CH_2$ units decreased the binding between concanavalin A and polysaccharides[5]. The reverse effect was found for the jacalin–AEG system (see Table II). Thus, hydrogen bonding may not play a significant role in the binding of jacalin and ant-egg glycoprotein.

ACKNOWLEDGMENTS

We thank Dr. H. Ahmed for his help in this investigation. One of the authors (S.R.) acknowledges the Council of Scientific and Industrial Research for a fellowship.

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