Biochimica et Biophysica Acta, 705 (1982) 396–404
Elsevier Biomedical Press

BBA 31282

PURIFICATION AND PARTIAL CHARACTERIZATION OF AN AGGLUTININ FROM *PHASEOLUS COCCINEUS* VAR. 'ALUBIA'

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(Received December 21st, 1981)

Key words: Agglutinin; Hemagglutination; (Phaseolus coccineus)

An agglutinin from seeds of 'alubia', a Mexican strain of *Phaseolus coccineus*, has been purified by affinity chromatography using physically entrapped stroma. The protein appears to be homogeneous by electrophoresis, molecular sieve chromatography and ultracentrifugation. A molecular species of approx. M_r 112000, with S values of 6.25, 4.52, 4.63 and 4.65 at pH 2.5, 4.5, 7.0 and 9.5, respectively, consisting of four similar subunits (28 kDa), and containing 20% w/w glucosamine, is found to be responsible for the hemagglutinating capacity of 'alubia' extracts. No sugar able to inhibit agglutination has been found. The possibility that hemagglutination by *Ph. coccineus* var. 'alubia' involves cell receptors other than simple carbohydrate structures must therefore be considered.

Introduction

The hemagglutinating capacity of seed extracts is routinely tested in the presence of sugars. If inhibition occurs, it is usually concluded that agglutination is caused by a lectin showing affinity for the carbohydrate in question. There are many examples, however, where inhibition is not observed even in relatively high concentrations of sugar (0.1 M), and/or glycoprotein (1% w/w). Little attention has been paid to the properties of this kind of agglutinin, though they show remarkable biological activities such as immunosuppression and mitogenicity [1]. A probable reason is the diffuculty of designing simple and efficient methods for the purification of these materials. We have recently applied a procedure taking advantage of the large affinity of agglutinins for the cell membrane, allowing their rapid isolation from other substances lacking this property [2]. For this purpose, red cell membrane ghosts (stroma) are physically entrapped in chromatographic columns constituting an affinity adsorbent. Its efficiency has been demonstrated by the purification of the agglutinins from two different seed extracts [2]. We now report the characterization of a hemagglutinin from *Phaseolus coccineus*, var. 'alubia' purified by this method.

Materials and Methods

Seeds of *Ph. coccineus*, var. 'alubia', classified at the agricultural school of Chapingo, Mexico, were kindly provided by Dr. F. Cordoba (CIB, La Paz, B.C.S., Mexico). Sepharose 6B and Sephacryl S-200 were from Pharmacia Fine Chemicals, Uppsala, Sweden, Ampholine-PAG plates, pH range 3.5–9.5, came from LKB (Stockholm, Sweden). All other chemicals were of analytical purity purchased from commercial sources.

Agglutinin extraction. The procedure for the extraction of the agglutinins from 'alubia' seeds and the preliminary fractionation by salting-out with

solid ammonium sulphate, has been described previously [2].

Affinity chromatography on stroma columns. The preparation of red-cell membrane ghosts (stroma) and their entrapment on columns has been reported before [2]. The different fractions obtained after chromatography of the protein mixture precipitating in the range of 40–80% solid ammonium sulphate saturation of 'alubia' crude extract (F_{40}^{80}) on stroma columns are: FA, portions of proteins with no affinity for stroma and no hemagglutinating capacity; fraction FB, adsorbed hemagglutinating material eluted from the column by distilled water; fraction FC, adsorbed hemagglutinating material eluted from the column by 0.2 M glycine-HCl, pH 2.5.

Gel filtration on Sepharose 6B. For the removal of non-hemagglutinating materials desorbed from the columns along with the active fractions, FB and FC were separately chromatographed on columns (2.0 × 80 cm) packed with Sepharose 6B, equilibrated with 0.1 M ammonium acetate, pH 4.5, at 20°C, at the flow rate of 9 ml/h. The new active fractions thus collected were designated FB-6B and FC-6B, respectively.

Hemagglutination studies. The double dilution procedure with a 2% suspension of blood group A, B or O red cells in microtiter plates was employed in hemagglutination tests. Buffers of different pH and ionic strength were adjusted to physiological osmotic pressure with D-glucose according to data obtained from Ref. 3.

Electrophoretic studies. Electrophoreses in the presence and absence of SDS were carried out in PAA 4/30 gradient plates (Pharmacia Fine Chemicals, Uppsala, Sweden). Two series of protein standards (high molecular weight and low molecular weight kits from Pharmacia Fine Chemicals, Uppsala, Sweden) were used as references: thyroglobulin (669000), ferritin (440000), catalase (232000), lactate dehydrogenase (140000) and bovine serum albumin (67000), as high molecular weight (HMW) protein standards; phosphorylase *b* (94000) bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20100) and α-lactalbumin (14400), as low molecular weight (LWM) protein standards. Conditions for electrophoresis were as recommended by the booklet accompanying these products. Electrophoresis at pH 4.0 in 0.4 M glycine/acetate buffer was performed as described in Ref. 4. Isoelectric focusing was carried out according to LKB instructions for use of ampholine PAG plates in an LKB Multiphor apparatus. The pH gradient on the plate was determined with an Ingold (Lot 403-30-M8) surface electrode before the staining-destaining step.

Molecular sieve chromatography on Sephacryl S-200. To determine the number and size of the subunits constituting the agglutinin, the fractions FB-6B and FC-6B were separately chromatographed on a Sephacryl S-200 column (1.0 × 130 cm) equilibrated with 6 M guanidinium-HCl, pH 5, at 20°C [5]. For reference, a mixture of ^{14}C-alkylated proteins prepared according to Ref. 6 was employed using labelled iodo[^{14}C]acetamide; insulin (3000–4000), CM-Cys toxin isolated from *Naja naja* snake venom [22] (7500), RNAase (14000), chymotrypsinogen (25700), and human serum albumin (66000). The total specific activity of this mixture, containing about 3 mg/ml of each protein, plus unreacted iodoacetate, was 13.6 · 105 dpm/ml. 100 μl of the mixture were added to the sample (5 mg FB-6B or FC-6B, in 100 μl 6 M guanidinium-HCl, pH 5), and finally mixed with 50 μl of a 2% solution of Dextran blue 2000 before application to the column at a flow rate of 6 ml/h. The chromatography could be followed spectrophotometrically and the distribution of the radioactivity in the samples (ml/tube) was determined in a Beckman LS 100C scintillation counter. For this, 200-μl aliquots were mixed with 5 ml Instagel (Packard Instruments, S.A., Zurich, Switzerland) in Beckman poly-Q vials. The results are expressed as distribution coefficients, K_d ($K_d = W_e - W_0/W_t - W_0$); where W_0, W_e and W_t represent the elution volumes of Dextran blue 2000, the protein in question and free ^{14}C-labelled iodoacetate, respectively. K_d values are plotted against $N^{0.555}$, where N is the number of amino acid residues of the protein, according to the method suggested by Porath [7].

Fluorescence and CD studies. Fluorescence emission spectra and the relative fluorescence values of the agglutinin at various pH values were recorded on an AMINCO SPF-500 spectrofluorometer used in the ratio mode, in quartz cuvettes of 1 cm pathlength at room temperature. CD spectra were

also obtained at room temperature (20°C) with an automatic spectropolarimeter, JASCO JA-41, (Japan Spectroscopic Co., Tokyo), using regular cells of 1.0 and 0.25 cm pathlength and a protein solution of 3 mg/ml in different buffers: 0.05 M glycine-HCl, pH 2.5; 0.05 M acetate, pH 4.5; 0.5 M phosphate, pH 7.0 (0.02 M Phosphate-buffered saline containing 0.9% NaCl, pH 7.4); and 0.05 M glycine-NaOH, pH 9.5. Protein concentrations were verified by ultraviolet adsorption at 280 nm and by determining the total amino acid content by the ninhydrin method. Ellipticity is expressed as molar ellipticity [θ], at wavelengths above 250 nm, and as mean residual ellipticity, $\theta_{w,r}$, below 250 nm.

Amino acid analysis. A 0.5-mg sample was hydrolyzed under vacuum with 2 ml 6 M HCl at 110°C in sealed tubes for 24, 48 and 72 h. The samples were analyzed on an automatic amino acid analyzer Durrum D500. The values for threonine and serine were corrected as recommended in Ref. 8. Tryptophan was determined spectrophotometrically using molar absorptivity values of 5554 $M^{-1} \cdot cm^{-1}$ for tryptophan, 1260 $M^{-1} \cdot cm^{-1}$ for tyrosine and 150 $M^{-1} \cdot cm^{-1}$ for disulfide bridges at 278 nm [6].

Carbohydrate analysis. The reaction with anthrone for determining total hexose was employed as in Ref. 9 using D-galactose as standard. The amino sugars were determined from amino acid analysis data.

Ultracentrifugation studies. Ultracentrifuge studies were performed on an analytical centrifuge MSE Centriscan 75 equipped with both schlieren and ultraviolet absorption optics. Sedimentation coefficients were calculated as recommended in the instrument manual and the molecular weight from the sedimentation equilibrium data (15000 rpm, 20 h) according to Ref. 10. A partial specific volume of 0.7 for 'alubia' agglutinin was assumed and the study was carried out on solutions containing 0.5–1.5 mg/ml of the agglutinin at 20°C.

Results and Discussion

Specificity of 'alubia' agglutinin

Some studies on the hemagglutination, mitogenicity, and immunosuppressive properties of *Ph. coccineus* have been previously reported [1,2,11–14]. Two hemagglutinins have been characterized [12], and one mitogen of relatively low erythroagglutinating potency has been described [14]. The hemagglutinins were found to be inhibited by the sugar *N*-acetyl-D-galactosamine and by a glycopeptide lacking this hexosamine, but this puzzling observation was unfortunately not discussed [12]. The hemagglutinating properties of the mitogen, on the other hand, were not inhibited by any monosaccharide, while its lymphocyte-stimulating ability was completely abolished by α-D-mannoside [14]. In our case we have tested 18 different monosaccharides, including those which are known to be the carbohydrate determinants in the ABO blood group system, alone or in combinations, as well as polysaccharides and several glycoproteins against the agglutination of blood groups A, B and O cells, but none of them were able to inhibit this agglutinin (Table I). Accordingly, if 'alubia' agglutinin does recognize a carbohydrate structure at all, this must display a special stereochemical configuration uniquely characteristic of the corresponding receptor on the cell surface. Our attempts to isolate such a structure have so far been unsuccessful. Most probably, the extraction procedures employed (enzymatic digestions or solubilization of membrane components by detergents) alter the composition or inhibitory capacity towards the agglutinin [13,21]. More recently it was found that fetuin does possess inhibitory capacity towards 'alubia' agglutinin. This effect, however, is lost upon fetuin digestion with papain; that is, the mixture of glycopeptides thus obtained does not inhibit. In sum, although 'alubia' agglutinin does not possess a clear affinity for soluble glycoproteins and carbohydrates, it does bind to the cell membrane. Whether this may indicate the existence of complex receptors carrying functional carbohydrates, or mechanisms of interaction between cells and 'alubia' agglutinin not involving carbohydrates at all, remains unclear.

Yields and properties of the purified samples

The purification of 'alubia' agglutinin has been performed by affinity chromatography on physically entrapped stroma [2], (Fig. 1). Since no sugar inhibiting this agglutinin has been found, its elution is carried out by changing the salt concentration and pH of the medium.

TABLE I

OLIGOSACCHARIDE AND GLYCOPROTEINS LACKING INHIBITORY EFFECT TOWARDS THE HEMAGGLUTINATING CAPACITY OF *Ph. COCCINEUS* VAR. 'ALUBIA'

Mixture: D-glucose, D-galactose, D-fucose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine. Hemagglutination titer was adjusted to 8 against red cells of all blood groups (A, B and O).
All carbohydrates came from Sigma (St. Louis, MO, U.S.A.). The glycoproteins were from NB Co. (Cleveland, OH, U.S.A.), except blood-group substances A, B and O, which were prepared according to Ref. 15.

Monosaccharide (0.1 M)	Disaccharide (0.1 M)	Polysaccharide (1% w/v)	Glycoprotein (1% w/v)
L-Fucose	Melibiose	Dextran	Blood group substances A, B, 0
D-Fucose	Lactose	Chitin	Porcine gastric mucin
D-Glucose	Saccharose		Bovine placenta
D-Galactose	Cellobiose		Placenta powder
D-Fructose	Isomaltose		Submaxillary powder
Arabinose	Trehalose		Pancreas powder
α-Methyl-D-mannoside			
α-Methyl-D-glucoside			
N-Acetyl-D-glucosamine			
N-Acetyl-D-galactosamine			
N-Acetyl-D-mannosamine			
N-Acetyl-D-neuraminic acid			
Glucosamine (HCl)			

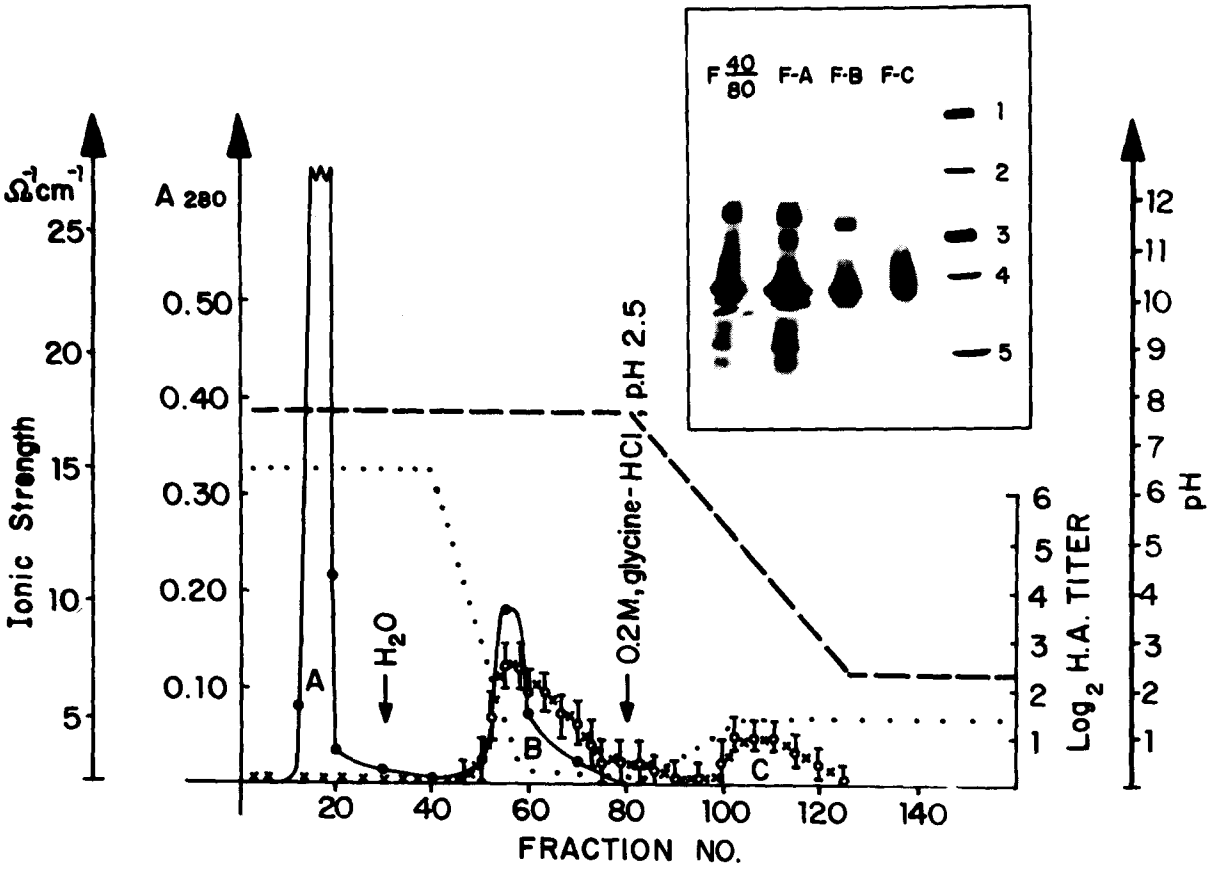

Fig. 1. Affinity chromatography of 'alubia' extracts on immobilized stroma. (Insert: electrophoresis of the collected fractions at pH 8.4: (1) Thyroglobulin, 669000; (2) ferritin, 440000; (3) catalase, 232000; (4) lactate dehydrogenase, 140000; (5) bovine serum albumin, 67000) ------, pH; · · · · · ·, conductivity; ●——●, absorbance; ×××, hemagglutination titer.

Some non-hemagglutinating material emerging from the column along with the active fractions during elution have been efficiently removed by gel filtration on Sepharose 6B. The hemagglutination capacity, yields and purification degree of the different fractions have been summarized in Table II.

As previously discussed, the remarkable ability of distilled water to disrupt the agglutinin-stroma complex has been attributed to a weakening in the hydrophobic bonding taking place in the formation of the complex [2]. Indeed, very recently we have observed that ethylene glycol mixtures also reduce drastically the adsorption of 'alubia' agglutinin on stroma columns, further supporting such an assumption. On the other hand, the data presented in Table III showing the quenching of the relative fluorescence of the agglutinin can be regarded as evidence for the accessibility of the fluorescent aromatic structures of the protein to the medium. The corresponding CD spectra of this agglutinin under similar conditions confirm these observations (Fig. 3).

Electrophoretic analysis

The homogeneity of the fractions eluted from the stroma column has been studied by electrophoresis. As mentioned above, a gel filtration step has been included to remove non-hemagglutinating material accompanying the fractions FB and FC. After this step, both fractions appear as single bands at pH 8.4 and 4.0 under non-denaturing conditions (Fig. 2). As seen in Fig. 2, the proteins in FB-6B and FC-6B have migrated to the same position, corresponding to a molecular species of approx. M_r 112000. By SDS-polyacrylamide gel electrophoresis (Fig. 4), in contrast, the M_r for the subunit is found to be about 30000. This approximation may be higher than the true value due to the fact that many glycoproteins bind anomalous amounts of detergent molecules [16]. A further estimation of the size of the agglutinin subunits has been obtained by molecular sieve chromatography of the different fractions on columns of Sephacryl S-200 equilibrated with 6 M guanidinium-HCl at pH 5 and 20°C. As shown in Fig. 5, a value for N of about 257 residues (approx. 28 kDa) for the subunit correlates well with that of the native protein as determined by electrophoresis under non-denaturing conditions (Fig. 2). Hence it may be concluded that the 'alubia' agglutinins are molecules of 112 kDa composed of four similar subunits of about 28 kDa.

Finally, by isoelectric focusing the fractions FB-

TABLE III

QUENCHING OF RELATIVE FLUORESCENCE OF 'ALUBIA' AGGLUTININ AT 330 nm BY pH

Protein concentration, 0.1 mg/ml of FB-6B or FB-6C; excitation at room temperature.

Buffer	pH	Excitation wavelength (nm)		
		270	280	290
0.2 M glycine-HCl	2.5	28.8	30.2	23.0
0.2 M acetate	4.5	23.4	25.3	20.6
0.2 M phosphate	7.0	25.8	25.8	19.4
0.2 M glycine-NaOH	9.5	32.2	35.9	26.6
Distilled water	6.0	24.3	26.5	20.7

TABLE II

PURIFICATION OF 'ALUBIA' AGGLUTININ

Agglutinin was purified from 100 g of seeds. Yield (%) and purification factor are in relation to F_{40}^{80}.

Fraction	Weight (mg)	Yield (%)	Minimum hemagglutination dose	Purification factor
F_{40}^{80}	1500.0	100	204 mg/ml	
FB	217.0	14	64	3.2
FC	77.5	5	24	8.5
FB-6B	77.5	5	7.9	25.8
FC-6B	46.5	3	3.9	52.3

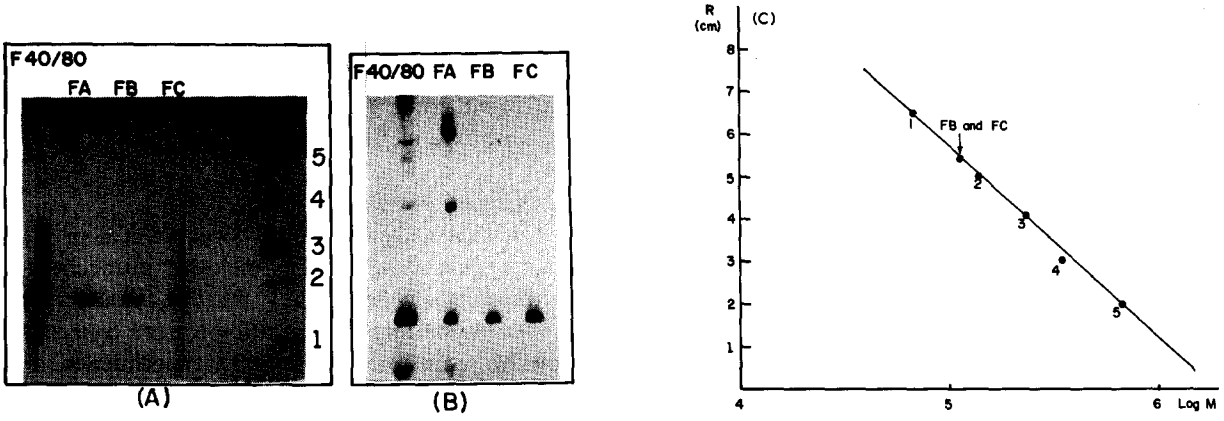

Fig. 2. Electrophoresis at pH 8.4 of the hemagglutinating fractions obtained after gel filtration on Sepharose 6B of the materials present in peaks A, B and C in Fig. 1. (A), Electrophoresis at pH 8.4: (1) thyroglobulin, 669000; (2) ferritin, 440000; (3) catalase, 232000; (4) Lactate dehydrogenase 140000; (5) bovine serum albumin, 67000). (B), electrophoresis at pH 4.0.

6B and FC-6B appear to be composed of the same number of protein bands (at least five different bands can be observed) positioned in the region between pH 4.5 and 5.5, suggesting a close similarity between both fractions. When either FB-6B or FC-6B are rechromatographed on stroma columns, two subpopulations of molecules according to the conditions of elution (distilled water or 0.2 M glycine-HCl, pH 2.5) are again obtained. Therefore FB-6B and FC-6B are believed to be the same. This has been confirmed by chemical analysis of the samples, as shown below.

Amino acid analysis

The amino acid composition of fractions FB-6B and FC-6B is given in Table IV. Most relevant is their lack of cysteine and their relatively high content of aspartic acid, which explains the low isoelectric point observed for this agglutinin (between pH 4.5 and 5.5) The amino acid moiety of 'alubia' agglutinins corresponds to only about 65% of the total weight of the sample. With the exception of glycine, the relative portion of the amino acid residues in both fractions is very similar. The difference in glycine amount noted is most likely due to buffer contamination.

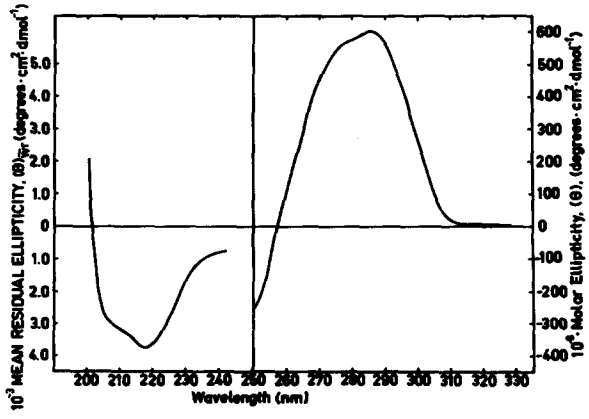

Fig. 3. CD spectra of 'alubia' agglutinin.

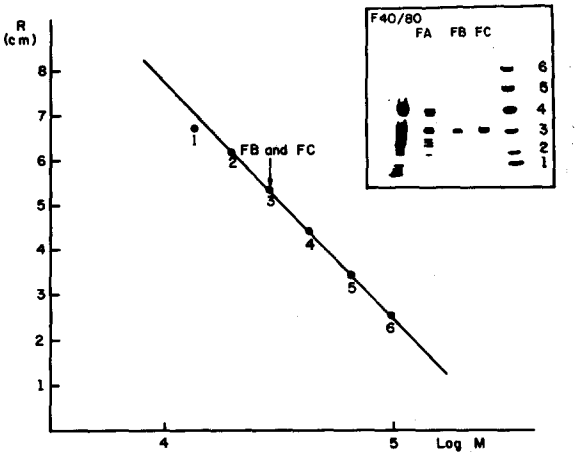

Fig. 4. Electrophoresis as described in Fig. 2 but in the presence of SDS at pH 8.4: (1) α-lactalbumin, 14400; (2) soybean trypsin inhibitor, 20100; (3) carbonic anhydrase, 30000; (4) ovalbumin, 43000; (5) bovine serum albumin, 67000; (6) phosphorylase *b*, 94000.

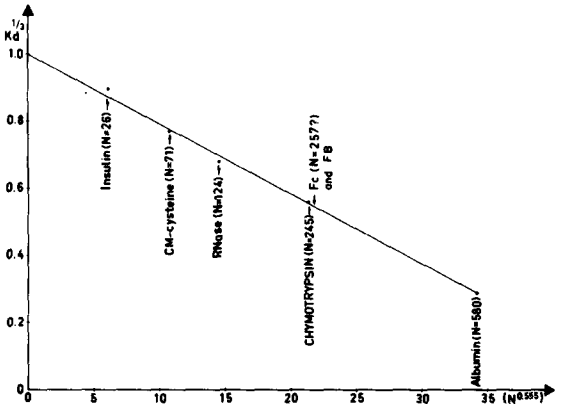

Fig. 5. Estimation of M_r of 'alubia' agglutinin subunits by molecular sieve chromatography on Sephacryl S-200. Both fractions FB-6B and FC-6B were run in the same column with similar results.

Carbohydrate analysis

From the amino acid analysis, the presence of glucosamine in fractions FB-6B and FC-6B has been established and found to be about 1.6%. No galactosamine has been detected. Total carbohydrate referred to D-galactose as standard was found to be 18% in weight, as determined by the anthrone method in both fractions.

TABLE IV

AMINO ACID COMPOSITION OF *PHASOLEUS COCCINEUS* VAR. 'ALUBIA' AGGLUTININS

	Residues/100 amino acids		Total number of residues/100000 g protein	
	FB-6B	FC-6B	FB-6B	FC-6B
Asp	14.99	15.07	72	72
Thr	10.22	10.16	48	48
Ser	13.16	13.03	60	60
Glu	7.58	7.71	36	36
Pro	4.77	4.77	20	20
Gly [a]	7.98	11.62	32	32
Ala	7.82	7.80	32	32
Val	8.69	8.80	40	40
Ile	5.18	5.13	24	24
Leu	10.06	10.07	48	48
Tyr	2.37	1.95	12	8
Phe	6.82	6.82	32	32
His	0.85	0.90	4	4
Lys	5.07	5.18	24	24
Arg	2.43	2.51	12	12
Trp [b]	2.9	2.9	12	12

[a] Excluded from the calculations due to uncertainty in determination possibly caused by contamination from the buffer. The value adopted for the total amino acid composition of the protein was based on fraction FB.

[b] Calculated spectrophotometrically [8].

Centrifugation studies

Further study on the physicochemical properties of 'alubia' agglutinins included ultracentrifugation analysis. In Table V the $S_{20,w}$ values, after the corresponding extrapolation to zero concentration in sedimentation velocity experiments, and the molecular weights calculated from sedimentation equilibrium data at different pH values, are listed. Since all the collected data for both fractions are practically identical, the table refers to them by the general term of 'alubia' agglutinin.

In the absence of substantial conformational changes of the 'alubia' agglutinin molecules, as concluded from spectrofluorometry and CD studies, the size differences found at various pH values are probably due to significant variations in their state of hydratation. As shown in other examples [17], these changes may modify the apparent Stokes radius and the frictional ratio coefficient, leading to distinct changes in hydrodynamic properties of the molecule.

Hemagglutination studies with 'alubia' agglutinin

Despite their relatively high hemagglutinating titer (Table II), the agglutinin from 'alubia' seeds is not inhibited by oligosaccharide solutions. This raises questions concerning the nature of their receptor on the cell membrane. As described previously, available evidence points to the possible

TABLE V

SEDIMENTATION COEFFICIENTS AND MOLECULAR WEIGHTS OF 'ALUBIA' AGGLUTININ AT DIFFERENT pH VALUES

Conditions: 0.5–1.5 mg/ml of FB-6B of FC-6B in different buffers at 20°C.

Buffer	pH	$S_{20,w}$	M_r
0.05 M glycine-HCl	2.5	6.25	110000
0.5 M acetate	4.5	4.52	86500
0.05 M phosphate	7.0	4.63	94600
0.05 M glycine-NaOH	9.5	4.65	106000

participation of hydrophobic bonding in their interactions with stroma. For example, the adsorption capacity of stroma columns is drastically affected by the ionic strength of the medium (Fig. 2). Furthermore, 3 M urea and 50% ethylene glycol in phosphate-buffered saline also diminish the ability of 'alubia' agglutinins to bind to this adsorbent (unpublished results). Trying to establish the nature of the interaction between these agglutinins and native cells, we have studied the hemagglutination phenomenon under different experimental conditions that included variations in pH and salt concentration. As shown in Figs. 6 and 7, it is clear that under conditions where both the agglutinins and the red cell membrane possess a similar charge the agglutination phenomenon still occurs. There is, nevertheless, an obvious effect of the change in ionic strength at physiological pH when the salt concentration is drastically reduced (Fig. 7). One may therefore presume that the role of NaCl, besides affecting the osmotic pressure of the cell, is primarily to neutralize the charges on the interacting molecules, and next to enhance their corresponding hydrophobicity, thus favouring agglutination. This is possible, since a considerable fraction of the agglutinin consists of hydrophobic amino acids which may determine the interacting properties of these molecules. In this context, it may be pertinent to mention that in many glycoproteins the carbohydrate moiety may induce a specific folding that allows a maximum exposure of the hydrophobic regions of their polypeptide chains [18,19], thus enhancing their ability to interact with other compounds hydrophobically.

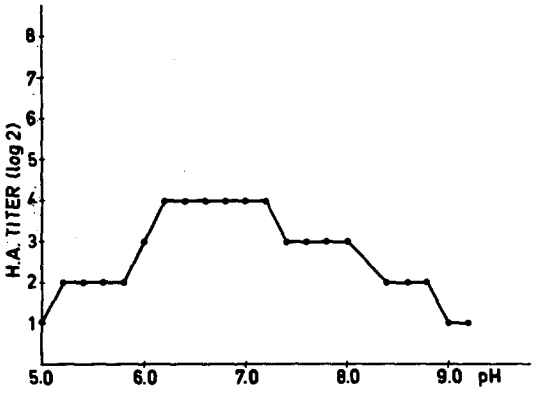

Fig. 6. Effect of pH on hemagglutination by 'alubia' agglutinin.

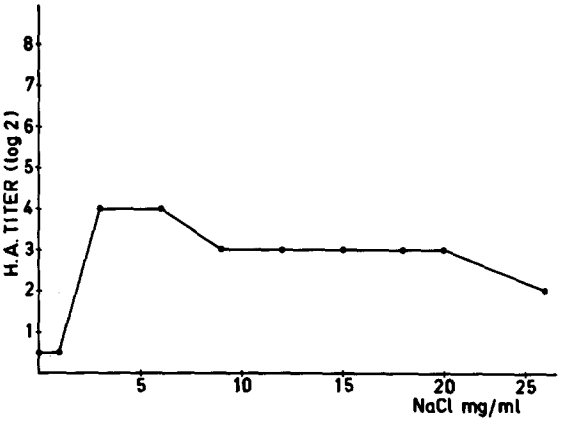

Fig. 7. Effect of salt concentration of hemagglutination by 'alubia' agglutinin.

The nature of the cell receptor for 'alubia' agglutinin

It is known that in affinity systems involving immobilized lectins the presence of detergents in the chromatography buffer diminishes nonspecific adsorption [20,21]. In a recent paper [13] dealing with the isolation of the receptor for *Ph. coccineus* on human red cells, however, adsorbed material could not be removed by carbohydrates from a column containing the immobilized agglutinin as a ligand. This is an agreement with the fact that sugars seem ineffective in competing for the binding of *Ph. coccineus* agglutinin to the cell membrane. Curiously, and although the isolated receptors were shown to be very similar in carbohydrate composition to glycoproteins commonly present on cell surfaces, they were unable to inhibit the hemagluttination induced by this agglutinin. In our case, the fact that *Ph. coccineus* var. 'alubia' agglutinin are removed from stroma columns under two different sets of experimental conditions first suggested to us the presence of either two different kinds of receptor on the cell membrane, or of two agglutinin in 'alubia' extracts. After being able to establish the identity of fractions FB-6B and FC-6B by different criteria, we propose the existence of heterogeneous binding sites on stroma to account for the chromatographic behaviour of the agglutinin on this adsorbent. In addition to a study of the kinetics of interaction between stroma receptors and 'alubia' agglutinin, it would be interesting to try to relate the chemical

and physical properties of the receptor to the biological properties of 'alubia' agglutinin. Work in this direction is in progress in our laboratories.

Acknowledgements

We would like to thank Dr. Felix Cordoba for the continuous supply of seeds employed in this work. Thanks are due to Lars Goran Overstedt and Gunnar Johansson for ultracentrifugation and spectrofluorometry studies. We are grateful to Dr. I. Sjoholm and P. Edman for the CD analysis, and to Gosta Forsling and Per Korsnas for the valuable advice in the preparation of the manuscript.

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