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PURIFICATION OF A LECTIN FROM *AMARANTHUS LEUCOCARPUS* BY AFFINITY CHROMATOGRAPHY

E. ZENTENO and J.-L. OCHOA

Centro de Investigaciones Biológicas de Baja California, A. C., Box 128, La Paz, Baja California Sur 23000, México

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Key Word Index—*Amaranthus leucocarpus*; Amaranthaceae; lectin; agglutinin; stroma columns; erythrocytes.

Abstract—A lectin of M_r *ca* 45,000 per subunit from *Amaranthus leucocarpus* seeds, has been isolated and purified by affinity chromatography using a stroma column. It is a glycoprotein (10% w/w carbohydrate) containing six *N*-acetyl-D-glucosamines, four D-galactoses, one D-glucose and traces of xylose residues for each three D-mannose residues per molecule. Its amino acid composition reveals a predominance of acidic residues (aspartic and glutamic) and of glycine and alanine. In addition, the lectin contains an unusual amount of essential aminoacids such as methionine, tryptophan and lysine. Electrophoretically and chromatographically homogenous, it focuses as a multiple-band protein in the pH range of 4.8–5.2. It agglutinates the different human blood groups of the ABO system equally well, albeit being inhibited by *N*-acetyl-D-galactosamine in a specific fashion. In contrast to *A. caudatus* hemagglutinin *A. leucocarpus* lectin is inhibited by serum glycoproteins such as fetuin, it is mitogenic and is not toxic.

INTRODUCTION

Lectins are carbohydrate-binding proteins (glycoproteins) of nonimmune origin with no enzymatic activity [1–3]. Due to their specific binding properties, lectins have been used in studies of cell surface architecture, blood-group typing, isolation and characterization of oligosaccharide structures, etc. [4–6]. Lectins also show other interesting biological effects such as immunosuppression, mitogenicity and cytotoxicity; hence they are widely used in immunology, cell biology and cancer research [2, 5, 6]. Lectins are commo to all kingdoms and in the case of plants, are particularly isolated from legumes, Solanaceae and cereal families [2, 3] for commercial purposes. From the Amaranthaceae, however, only one species *Amaranthus caudatus* has been reported as containing a toxic agglutinin lacking serological specificity and not being inhibited by serum glycoproteins [7]. This lectin proved also to be non-mitogenic with hog lymphocytes, reacted weakly with isolated erythrocyte membrane proteins and therefore was regarded as a cell surface-specific reagent [7].

In Mexico, *A. leucocarpus* (syn. *A. hypochondriacus*) is the most popular cultivar of the Amaranthaceae family and is used to prepare a confection called 'alegria'. The high nutritional value of *A. leucocarpus* seeds [8] results from a considerable amount of protein of unusual amino acid composition. As we have observed earlier hemagglutinating activity with *A. leucocarpus* extracts [9], we considered it of interest to isolate and characterize the substance responsible for such an effect.

RESULTS

A. leucocarpus seeds produced *ca* 8% of protein saline extract which showed an equal hemagglutination capacity towards the different ABO blood groups yielding a HA titre of 64 at a protein concentration of 12 mg/ml. The treatment of the red cells with neuraminidase or trypsin enhanced their susceptibility to agglutination by the extract (HA titre with neuraminidise treated cells 256), as occurs with many other lectins ([2, 3], see also [7]).

The *Amaranthus* extract was purified in a column packed with blood group 'A' stroma (Table 1, Fig. 1). As judged by SDS electrophoresis and by electrophoresis at pH 4.5, the hemagglutinating material eluted by distilled water appeared homogeneous. At this stage, 80% of the original activity was recovered with an increase in specific activity of 16 times. From electrophoresis it was concluded that the M_r of the protein subunits correspond to M^+ *ca* 45000 ± 2000. The subunit lectin possessed a marked tendency to aggregate as a function of pH, consequently several bands appeared in polyacrylamide gel electrophoresis at higher pH values. For the same reasons, and possibly due to microheterogeneity in carbohydrate composition, the isoelectric focusing electrophoresis pattern of the purified lectin showed at least four bands located in the pH range 4.8–5.2.

The amino acid composition and carbohydrate content of *Amaranthus* lectin is listed in Table 2. The number of amino acid residues was calculated assuming a subunit M_r of 45 000. Accordingly, if an average M_r of 115 is given to the amino acids, then the amino acid composition accounts for only 80% of the total weight of the material. If the value of the anthrone analysis is correct, 10% moisture is an acceptable estimation and the overall chemical composition described in Table 2 is a good approximation of the nature of the *Amaranthus* lectin molecule. The carbohydrate composition of *A. leucocarpus* lectin is apparently similar to animal glycoproteins [10] and, therefore, this fact could be regarded as an interesting finding that deserves special attention in plant glycoprotein studies [11].

The pH and temperature effect on the stability of the *Amaranthus* lectin indicates that activity is destroyed by heating at 100° for five min and yet it is quite stable to temperature and pH variations (Table 3). It is also interesting that the hemagglutinating activity is not

Table 1. Purification of *Amaranthus* lectin on physically immobilized stroma

Fraction*	Protein (mg)	Hemagglutinating (HA) units	Specific activity†	Protein %	Yield HA units %	Purification factor
Crude	812	44800	55	100	100	1
FA	503	—	—	62	—	—
FB	40	35800	895	5	80	16
FC	8	—	—	1	—	—

*From 10 g of seeds. For definitions and chromatographic conditions see Experimental and Fig. 1.
†Against normal erythrocytes blood group O (H).

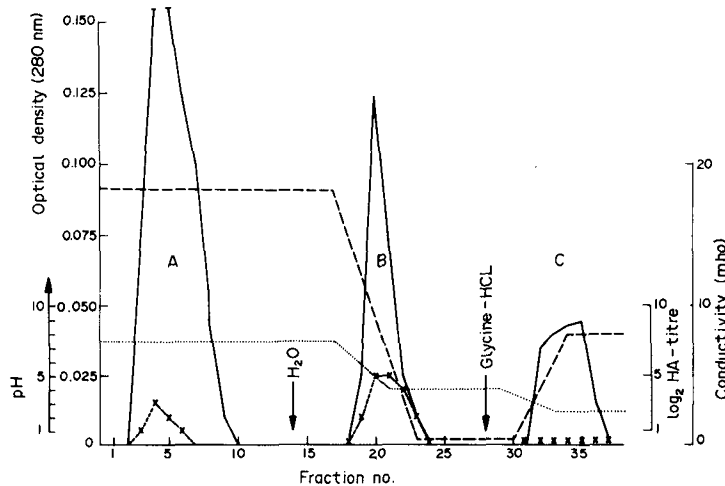

Fig. 1. Chromatography of *Amaranthus* crude extract on a stroma column. A sample of 2 ml with a total activity of 12 000 units was applied to a PBS equilibrated column (4 x 3 i.d. cm) with a total adsorption capacity for this lectin of 10 000 hemagglutinating units at a flow rate of 20 ml/hr and room temperature. Samples of 3 ml were collected and their ionic strength (– – –), pH (. . . .), hemagglutinating activity (x – – x), and optical density at 280 nm (———) determined. Three main fractions were obtained: (A) material not adsorbed and hemagglutinating activity in excess, (B) fraction eluted with distilled water and (C) fraction eluted with 0.2 M glycine–HCl, pH 2.5.

modified by the presence or absence of divalent metals, or by moderate quantities of polarity-reducing agents, such as urea (3 M) and ethylene glycol (25%).

Biological properties

Amaranthus leucocarpus lectin shows important mitogenic activity against mouse spleen lymphocytes (Table 4). This effect appears as a concentration-dependent response. As shown here, the mitogenic activity of 4 μg of *A. leucocarpus* lectin is similar to that obtained using 2 μg of concavalin A as a control. It is also clear that neither the crude extract nor the purified lectin are cytotoxic. After this assay, the cell viability was determined according to the trypan blue-staining and found to be higher than 88%.

Inhibition studies

The inhibition assay showed that the monosaccharide *N*-acetyl-D-galactosamine (GalNAc) selectively inhibited the agglutination capacity of the *Amaranthus* extract. Other sugars, such as L-fucose, D-glucose, D-mannose, D-galactose, lactose, D-galactosamine, D-glucosamine, D-mannosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine and neuraminic acid, were ineffective. Amongst the glycoproteins tested fetuin and sub-maxilliary mucin were relatively good inhibitors. Fetuin showed the best inhibition of the hemagglutinating activity of the *A. leucocarpus* lectin (Table 5). Its hydrolysis under mild alkaline reductive conditions [12] caused the loss of fetuin inhibitory capacity towards the *Amaranthus* lectin (Table 5). Heat treatment of the glycoproteins tested for inhibition of the lectin at 80° for one hr, enhanced the inhibitor power probably due to the exposure or adoption of the appropriate conformation of the sugar moiety for binding on one hand, and the partial release of terminal sialic acid residues that are precluding the binding to the GalNAc residues, on the other [10]. This effect is more pronounced under acidic conditions [12] and seems to be common for all the glycoproteins tested (Table 5).

DISCUSSION

In this paper we demonstrate that physically entrapped erythrocyte stroma on chromatographic columns is useful for the purification of chemically specific agglutinins such as *A. leucocarpus* lectin. This chromatographic system is based on the selective removal of biomolecules showing a biospecific affinity for cell membrane components. Their elution may be carried out either by a non-biospecific

Table 2. Amino acid composition and carbohydrate content of *Amaranthus* lectin

	nmol/Sample	Residues per subunit
Asp	7.4	37
Thr	4.6	23
Ser	4.8	24
Glu	7.3	37
Pro	0.4	2
Gly	7.0	40
Ala	7.5	38
1/2 Cys	0.6	3
Val	4.0	20
Met	1.3	7
Ileu	3.1	16
Leu	4.8	24
Tyr	2.4	12
Phe	2.6	13
His	1.3	7
Lys	1.6	8
Arg	2.2	11
Trp	0.3	2
Carbohydrate*		
Gal		4
Man		3
Glc		1
Xyl		traces
GlcNAc		6

*Per three mannose residues.

Table 3. Effect on the hemagglutinating activity of *Amaranthus* lectin* by physicochemical parameters and chemical agents

	Titre		Titre
Control	32	Ethylene glycol (25%)	32
EDTA (dialysis)	32	Ca^{2+} (5 mM)	32
Heated 65°, 15 min	32	Mg^{2+} (5 mM)	32
Heated 65°, 30 min	8	Mn^{2+} (5 mM)	32
Heated 100°, 1 min	16	Zn^{2+} (5 mM)	32
Heated 100°, 5 min	0	—	—
Urea (3 M)	16	pH range 4–8	32

*Protein concentration of extract 6 mg/ml.

method, with deforming buffers (i.e. low pH, low or high ionic strength, etc.) [13], or by suitable carbohydrate solutions competing for the biospecific sugar-binding-site of the lectin in question. In our case we chose a non-biospecific elution method (distilled water and/or 0.2 M glycine, pH 2.5) as being more economical than the use of *N*-acetyl galactosamine solutions.

A. leucocarpus agglutinin behaves very similarly to *A. caudatus* agglutinin with regard to blood-group and carbohydrate specificity [7]. However, while *A. caudatus* agglutinin was shown to be cytotoxic and non-mitogenic [7], we found that *A. leucocarpus* is not toxic in *in vitro* tests and shows mitogenic activity towards mouse lymphocytes (Table 4). *A. caudatus* reacts very poorly with serum proteins [7], *A. leucocarpus* is efficiently inhibited by fetuin (Table 5).

It is known that fetuin possesses three GalNAc units located in three small oligosaccharide chains which act as the linkages between each carbohydrate chain and the peptide backbone [14–16]. Such a linkage (denoted as the *O*-linkage) is labile and susceptible to hydrolysis under mild alkaline reductive conditions. It is not surprising therefore that fetuin loses its inhibitory capacity against *A. leucocarpus* lectin after treatment with sodium borohydride (Table 5). Moreover, since one of the three GalNAc residues of fetuin is also substituted by a sialic acid residue the increase in the inhibiting power of the asialo-fetuin (Table 5) is explained by the unmasking of another group capable of binding to the lectin.

The *A. leucocarpus* lectin binding site on the red cell surface may resemble the structure of the short carbohydrate chains of the fetuin molecule [17]. This is particularly true in the case of glycophorin [18] and may help to explain the erythrocyte agglutination effect induced by the lectin. The actual mechanism of such a phenomenon, however, must await further characterization of both the lectin and its receptor. Work in this direction is in progress at our laboratory.

EXPERIMENTAL

Seeds of *A. leucocarpus* (syn. *A. hypochondriacus*) were purchased at the local market in Tulyehualco, Mexico. Standard sugars, as well as the glycoproteins, were obtained from Sigma and Sephadex G-25 Superfine from Pharmacia. Other reagents were of analytical grade. Blood from healthy donors was a gift from the local Hospital J. M. Salvatierra.

Extraction. Finely ground seeds (10 g) were suspended in 100 ml of saline soln (0.9% NaCl in dist H_2O) and stirred for 2 hr at 4°. The pH was adjusted to 4 with 4 M HOAc and the suspension allowed to stand overnight at 4°. The clear supernatant obtained by centrifugation (3000 *g*, 10 min) was filtered through a glass fibre membrane (GF/A Whatman) and stored at 4° for further studies.

Preparation of stroma column. Erythrocyte membrane residues (stroma) were obtained by lysis of human red blood cells group A according to ref. [19] and packed in chromatographic columns as described in ref. [13]. Prior to chromatography, the column was washed ×4–5 with the buffers employed for elution: dist H_2O, 0.2 M glycine pH 2.5 and 9.5, and PBS. The total adsorption capacity of the column was calculated from the total number of hemagglutinating units eluted after satn with a given lectin soln.

Hemagglutination assays. The test was carried out using a 2% red cell suspension in PBS (0.02 M K buffer pH 7.4 with 0.9% NaCl and 0.002 M NaN_3) according to the two-fold serial dilution procedure in microtiter plates. The hemagglutination titre is defined as the reciprocal of the max. diln. showing visible agglutination; a hemagglutinating unit is the min. amount of material capable of inducing agglutination; the sp. act. is considered to be the number of hemagglutinating units per mg of protein.

Inhibition of hemagglutination induced by *Amaranthus* lectin was studied with different carbohydrate and glycoprotein solutions in PBS as follows: 25 μl of a lectin soln of four hemagglutinating units were added to 25 μl of two-fold serially diluted carbohydrate (0.1 M) or glycoprotein (1.0% w/v) solns and after standing for 30 min, mixed with the erythrocyte suspension. The min. concn of either sugar or glycoprotein capable of fully inhibiting the lectin was noted.

Table 4. Mitogenic activity of *Amaranthus* lectin

Reagent	cpm of ^{3}H-TdR incorporated*	Relative response
None	1,1	1
A. leucocarpus crude extract		
5 μg	6200	5.5
25 μg	23900	21.4
50 μg	11300	10.3
A. leucocarpus lectin		
1 μg	19700	17.7
4 μg	24900	22.4
40 μg	13600	12.6
Concanavalin A		
2 μg	29700	26.6
Lipopolysaccharide (from *Vibrium cholerae*)		
200 μg	19450	17.5

*Average of three experiments with a standard deviation of ±5%.

Table 5. Minimum concentration (mg/ml) of carbohydrate and native or modified glycoproteins necessary to inhibit four hemagglutinating units of *Amaranthus* lectin

Glycoproteins	Native	Heating at 80°	Heating at 80° in 0.025 N H_2SO_4	0.1 M NaOH	0.1 M NaOH 0.1 M $NaBH_4$
Fetuin	1.25	0.625	0.312	2.5	5.0
Gamma-globulin	*	5.0	*	*	*
Lactoglobulin	*	5.0	2.5	*	*
Mucin	5.0	5.0	2.5	5.0	5.0
GalNAc	.065	—	—	—	—

*Ineffective at 10 mg/ml.

Mitogenic activity and cell viability. Lymphocytes obtained from mice (2-month-old, 25–30 g each) spleen by Ficoll-paque (Sigma) gradient centrifugation (3000 *g*, 30 min, room temp. were washed × 3 with balanced Hank's soln (HBSS, Microlab, México) and cultured in McCoy media (Microlab, Mexico) supplemented with 10% heat-inactivated calf serum and penicillin, and streptomycin G (100 U and 100 μg, respectively per ml of culture medium). Triplicate portions of 100 μl of cell suspension (cell density: 4×10^6 cells/ml) were then estimated by addition of 10 μl of mitogen at various concns in culture medium in Microtest, II Plates (No. 3040, Falcon). Following a period of 48 hr of estimulation under a 5% CO_2-humidified air atmosphere at 37°, 0.4 μCi per well of ^{3}H-thymidine (^{3}H-TdR: 10 μCi/mM) were added and the incorporation of label assessed by harvesting the cells with an automated Sample Harvester (Mod. 24V, Brandel, Gaithersbury, MD, U.S.A.) after 3 days. The material thus collected and dried on glass fibre filters (Whatman GF/A 11) was suspended in vials containing 3 ml of toluene for scintillation counting. Cell viability was determined by mixing 10 μl of the original cell suspension with 100 μl of 0.04— Trypan Blue (Sigma) in 0.9% NaCl soln, in the presence or absence of the mitogen. After incubating for 2 min at room temp. the cells were counted under an optical microscope.

Physicochemical studies. The temp stability of the lectin was determined after incubation at 60 and 100° for several intervals of time. The effect of pH on the hemagglutination phenomenon induced by the *Amaranthus* lectin was determined either directly, using glutaraldehyde treated erythrocytes [20] in the range 4–9, or indirectly, by an adsorption–desorption procedure using a stroma column. The buffer used was 0.2 M glycine adjusted to the desired pH value with 0.2 M HCl or 0.2 M NaOH. The effect of divalent metals on the hemagglutining capacity of the lectin was estimated by mixing the metal soln with a previously dialysed lectin soln against 0.1 M EDTA in PBS and the agglutination assay carried out as indicated above.

Protein determinations were done spectrophotometrically or according to the method ref. [21]. The carbohydrate content of the purified agglutinin was estimated by the anthrone method [22] with Dextran M_r 10 500 as std. The corresponding carbohydrate composition was determined by GC as described in ref. [23] using myo-inositol as int. std.

Electrophoresis on polyacrylamide gradient gels was run under non-denaturant conditions in 0.02 M Tris–glycine buffer with 0.1% SDS in the same buffer, or at pH 4.5 in tubes of 10 × 0.5 cm i.d. using 0.2 M glycine–HOAc as buffer and run at 4 m Am/tube until the track dye (Bromophenol Blue) left the gel.

Isoelectric focusing electrophoresis was done using Ampholine PAGE plates in an LKB Multiphor apparatus. The pH gradient after focusing was determined with surface electrode before the staining-destaining step.

Amino acid analysis. Samples of *ca* 9 μg in 50 μl, were hydrolysed under vacuum with 2 ml of 6 M HCl at 110° in sealed tubes for 20, 48 and 72 hr and analysed on an automatic analyser. The values for serine and threonine were extrapolated to zero time hydrolysis and for leucine, isoleucine and valine, to infinite time hydrolysis as recommended in ref. [24]. Proline was calculated after performic acid oxidation of the sample as suggested in ref. [25] and half-cysteine value corrected by a factor of 1.82 obtained after determination of half-cysteine on a known sample by a similar procedure. Tryptophan was determined spectrophotometrically using molar absorptivity values of 5690 M/cm, and 1280 M/cm for tryptophan and tyrosine at 280 nm, respectively, and of 4815 M/cm and 385 M/cm for tryptophan and tyrosine at 288 nm as suggested in ref. [26].

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