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Isolation of two blood type A and N specific isolectins from *Moluccella laevis* seeds

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Extraction of *Moluccella laevis* seed flour with phosphate-buffered saline, followed by ammonium sulfate (40%) precipitation and affinity chromatography of the dialysed precipitate on a column of galactose-bound Sepharose, afforded a single protein peak with high agglutinating activity for human A^{MM} and O^{NN} erythrocytes and poor activity for B^{MM} and O^{MM} cells. Gel filtration of the affinity-purified lectin on a column of Superose 12 gave two peaks that differed markedly in their activities for A^{MM} and O^{NN} erythrocytes. Whereas the ratio of A/N activities in the slow-moving peak (isolectin I) was 0.25–0.5, that of the fast-moving peak (isolectin II) was 2–3. Molecular mass estimation by gel filtration on Sephadex G-150 gave a value of 130 kDa for isolectin I and 240 kDa for isolectin II. SDS-polyacrylamide gel electrophoresis of isolectin I without or with mercaptoethanol revealed that it contains a subunit of 67 kDa, consisting of two chains (46 and 28 kDa, after reduction) held together by S-S bonds, and two noncovalently linked subunits of 42 and 26 kDa. The same subunits are also present in isolectin II, where the 67 kDa seems to be predominant. The *M. laevis* isolectins are unique in that they are specific for determinants of two different blood group systems, and also because they contain both covalently and noncovalently linked subunits. The pattern of inhibition by different sugars of the two blood type activities of the isolectins, assayed with either A^{MM} or O^{NN} erythrocytes, was similar. Of the monosaccharides tested, *N*-acetylgalactosamine was the best inhibitor, being 300–600 times more active than galactose. Methyl α-galactoside was a better inhibitor than the corresponding β-galactoside. The sugar specificity fits well with the specifity of the isolectins for blood type A erythrocytes, but does not account for their type N specificity.

Lectin; Subunit; Disulfide bridge; Blood type specificity; Sugar specificity

1. INTRODUCTION

The presence of an agglutinating activity in extracts of seeds from the plant *Moluccella laevis* (Bells of Ireland) of the genus Labiatiae was first described by Bird and Wingham [1,2] some 20 years ago. They noted that the blood type specificity of the extract was unusual in that it reacted with both type A and type N erythrocytes. They also reported that the anti-A and anti-N activities were simultaneously adsorbed on formaldehyde-treated erythrocytes of either blood type, and concluded that *Moluccella laevis* seeds contain a lectin with A + N specificity. The carbohydrate specificity of this extract was subsequently examined with a small number of mono- and oligosaccharides and was found to be directed towards *N*-acetylgalactosamine [2,3]. However, the lectin was not purified and, therefore, no information was available on its physicochemical properties, nor was it clear whether both the anti-A and anti-N activities reside in the same molecule as originally suggested [2].

Among the many lectins examined, only very few are blood type N specific, and there are no other reports on a lectin with a dual specificity of the kind ascribed to the *Moluccella laevis* lectin. We therefore undertook the purification and characterization of this lectin.

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Abbreviations: HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline, pH 7.4

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2. MATERIALS AND METHODS

2.1. *Materials*

Seeds of *Moluccella laevis* were obtained initially from Thompson and Morgan (Ipswich, England) and subsequently from Weller Flowerseed Company (Guadelupe, CA). Divinylsulfone was from Sigma (St. Louis, MO). Freshly drawn and typed human blood was obtained from the Blood Bank, Sheba Hospital, Tel Hashomer, and used within one month. *N*-Acetylfucosamine (monosaccharides are of the D configuration unless otherwise noted) was synthesized in our laboratory. Galactose-derivatized Sepharose 4B was prepared by the divinylsulfone method of Porath and Ersson [4] as described [5]. All other materials were of the highest purity available.

2.2. *Protein estimation*

The method of Lowry et al. [6] was routinely used with bovine serum albumin as standard. Alternatively, protein was estimated by absorption at 280 nm, assuming an OD of 1.0 for 1 mg/ml.

2.3. *Gel electrophoresis*

Electrophoresis in the presence of SDS was carried out on 8% and 10% gels in the discontinuous buffer system of Laemmli [7]. The proteins were prepared for electrophoresis by boiling in sample buffer for 5 min without or with 5% mercaptoethanol. The gels were stained for protein either by Coomassie brilliant blue or by the silver method [8].

2.4. *Hemagglutinating activity*

The hemagglutinating activity of the lectin and the inhibitory activity of sugars were measured by the serial dilution technique in microtiter plates [5]. A unit of activity is defined as the lowest concentration of lectin that gives visible agglutination.

2.5. *Gel filtration*

A commercially made Pharmacia column (100 ml) of Superose 12 prep grade was used. The column was operated on a HPLC system (LKB, Bromma, Sweden) at room temperature. It was equilibrated with PBS and elution was with the same buffer. Fractions of 0.25 ml were collected at a flow rate of 6 ml/h.

2.6. *Molecular mass analysis*

Molecular masses were estimated by gel filtration on a column (1.8 × 90 cm) of Sephadex G-150 operating in PBS at room temperature. The column was calibrated with the following proteins: soybean agglutinin (120 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), bovine chymotrypsinogen A (25 kDa) and bovine ribonuclease A (13.7 kDa).

3. RESULTS

Moluccella laevis seeds were finely ground, defatted by extraction with petroleum ether and air-dried at room temperature. The flour was treated with acetone (250 ml/50 g) overnight in the cold, the acetone removed by filtration and the flour air-dried again. It was then extracted with PBS (500 ml/50 g flour) overnight in the cold and the mixture centrifuged at 12000 rpm for 15 min. Solid ammonium sulfate was added to the supernatant (24.3 g/100 ml) to give 40% saturation. The mixture was stirred for 2 h at room temperature and kept overnight in the cold. The precipitate formed was collected by centrifugation, dissolved in 0.2 M galactose in 1 M NaCl and dialysed overnight in the cold against the same solvent, followed by dialysis against PBS. The dialysate was clarified by centrifugation (12000 rpm, 20 min) and one-fifth of the supernatant applied to a column (1.8 × 10 cm) of galactose-derivatized Sepharose 4B equilibrated with PBS. The column was washed with the same buffer until the absorbance at 280 nm of the effluent was less than 0.05 and the bound lectin was eluted with 0.2 M galactose. Elution was followed by monitoring the absorbance at 280 nm, the fractions containing protein were collected, dialysed extensively against distilled water and lyophilised or concentrated by ultrafiltration. The individual fractions each agglutinated both A^{MM} and O^{NN} erythrocytes, at an approximately constant ratio of activities (A:N = 0.25–0.5). In some experiments a linear gradient of the sugar (obtained by mixing 100 ml of 0.2 M galactose into 100 ml PBS) was applied. Under these conditions too, a single symmetrical peak was obtained and the curves of hemagglutinating activity, as measured with A^{MM} and O^{NN} cells, overlapped with each other and with the curve of protein (fig.1). The recovery of activity of the affinity purified lectin was about 50% and was the same with respect to erythrocytes of both blood types. The yield was 25 mg (by wt) of lectin with a specific activity of 25–50 units/mg when assayed with A^{MM} erythrocytes and about 100 units/mg when assayed with O^{NN} or B^{NN} cells. With O^{MM} and B^{MM} erythrocytes, the specific activity was 10–20 times lower than for the A^{MM} cells.

Gel filtration on Superose 12, under conditions of HPLC, gave two protein peaks, a slow-moving (isolectin I) and a fast-moving (isolectin II) one (fig.2). Isolectin II was enriched in anti-A activity (ratio anti-A:anti-N = 2–3), whereas higher anti-N activity was found in isolectin I (ratio anti-A:anti-N = 0.25–0.5). Gel filtration on Sephadex G-150 gave a molecular mass of 130 kDa for isolectin I and 240 kDa for isolectin II. Upon SDS-PAGE of the isolectins, three bands were observed

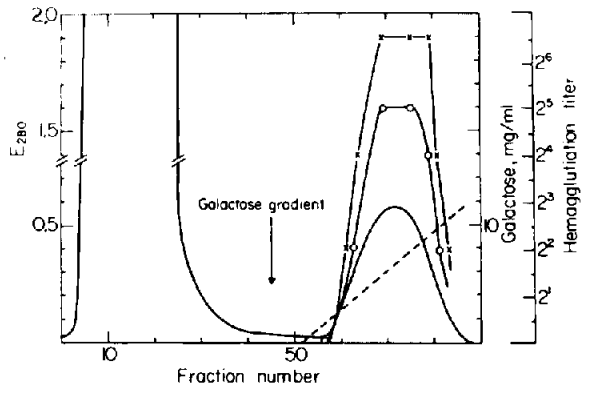

Fig.1. Affinity chromatography of *Moluccella laevis* lectin on a galactose-Sepharose column. Approximately one-fifth of the dialysed ammonium sulfate precipitate obtained from 50 g defatted flour was applied to the column (1.8 × 10 cm), which was then washed with PBS followed by a linear gradient of galactose and fractions of 2.5 ml were collected. Solid line indicates absorbance at 280 nm. (×—×) and (○—○), hemagglutination titer tested with type O^{NN} and A^{MM} human erythrocytes, respectively; broken line indicates concentration of galactose.

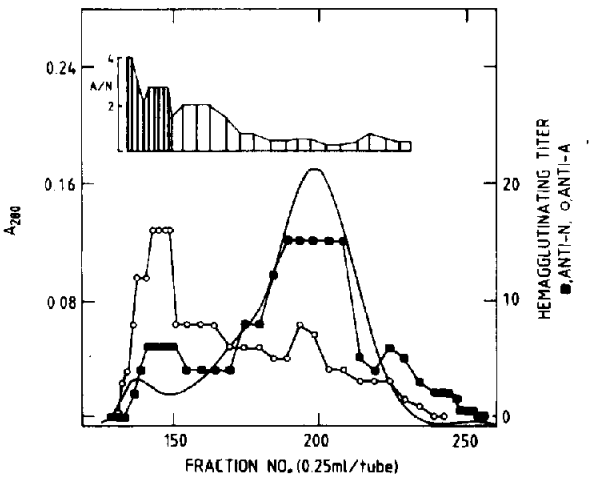

Fig.2. Gel filtration of affinity-purified *Moluccella laevis* lectin by HPLC. The affinity-purified lectin (~1.5 mg protein in 1.85 ml PBS) was fractionated on a column of Superose 12. (Bottom) Solid line indicates absorbance at 280 nm; the first peak (○—○) corresponds to isolectin II and the second (■—■) to isolectin I. (Top) Ratios of anti-A to anti-N activities.

with apparent molecular masses of 67, 42 and 26 kDa (fig.3, lanes 2 and 3). The bands of isolectin I were of approximately equal intensity (lane 3), whereas in the case of isolectin II, the 67 kDa band was considerably stronger than the two other, equally intense, bands (lane 2). Reduction of the isolectins by mercaptoethanol prior to SDS-PAGE afforded also three bands in each case, of molecular masses 46, 28 and 26 kDa (lanes 4 and 5). At most, only traces of other bands could be seen on the electrophoretogram. The affinity-purified lectin gave the same band as the isolectins on SDS-PAGE (lane 1). In a separate experiment (not shown) the individual bands of the affinity-purified lectin were electroeluted from an SDS-PAGE, reduced by mercaptoethanol, and analysed again by SDS-PAGE. The reduction converted the 67 kDa band into two bands of 46 and 26 kDa; the 42 kDa band was converted to a 46 kDa, while the 26 kDa band remained unchanged.

Among the monosaccharides tested, *N*-acetylgalactosamine was by far the best inhibitor of both activities of the isolectins when assayed with either A^{MM} or O^{NN} cells (table 1). It gave complete inhibition at about 0.01 mM concentration, and was 300–600 times more inhibitory than galactose. Although fucose was not inhibitory at a con-

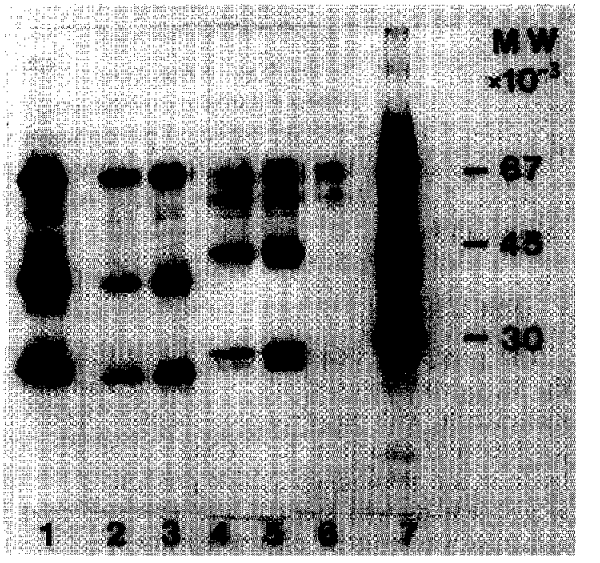

Fig.3. SDS-PAGE of *Moluccella laevis* isolectins. Electrophoresis was in a 10% gel. Proteins were visualized by silver staining. Lanes: 1, affinity-purified *Moluccella laevis* lectin; 2 and 4, isolectin II; 3 and 5, isolectin I; 6, 5% 2-mercaptoethanol; 7, molecular mass markers: bovine serum albumin (67 kDa), ovalbumin (45 kDa) and *Erythrina corallodendron* (30 kDa). Samples in lanes 1–3 were unreduced, and those in lanes 4–7 were reduced with 5% 2-mercaptoethanol prior to electrophoresis.

Table 1

Inhibition[a] by sugars of the agglutination of human blood type O^{NN} and A^{MM} erythrocytes by *Moluccella laevis* lectins

Sugar[b]	Isolectin I		Isolectin II		Affinity-purified
	O^{NN}	A^{MM}	O^{NN}	A^{MM}	O^{NN}
Galactose[c]	1.0 (2.5–4)	1.0 (5–10)	1.0 (2)	1.0 (5)	1.0 (7)
GalNAc	400	600	400	300	500
MeαGal	10	8.5	7.5	5	8
MeβGal	1	0.85	0.75	0.5	1
FucNAc	50	50	60	40	70
pNPαGalNAc	1400	1600	800	900	2100

[a] Inhibitory activity is presented relative to galactose, which was arbitrarily set as 1.0

[b] No inhibition by fucose, L-fucose, mannose or methyl α-mannoside at 25–30 mM. No inhibition with 100 mM 4-methylumbelliferone

[c] Minimum concentration of galactose (in μmol/ml) required for complete inhibition of four hemagglutinating units is given in parentheses

centration of 25–30 mM, *N*-acetylfucosamine was a very good inhibitor (minimal inhibitory concentration 0.1 mM), stressing the importance of the 2-acetamido group in binding to the lectin. Methyl α-galactoside was a better inhibitor than methyl β-galactoside. Of the sugars tested, *p*-nitrophenyl α-*N*-acetylgalactosamine was the most powerful inhibitor, 2–4 times stronger than *N*-acetylgalactosamine.

4. DISCUSSION

The procedure for the affinity purification of the *M. laevis* lectin is similar to that of many other plant lectins with one exception, namely the use of a solution of galactose in 1 M NaCl to dissolve the lectin in the ammonium sulfate precipitate for the subsequent dialysis step. It was observed that if galactose was omitted, no activity was found in the dialysate. Attempts to purify the lectin by affinity chromatography on Sepharose, acid-treated Sepharose [9] or lactose-derivatized Sepharose [10] were not successful, since the lectin did not bind to the unsubstituted Sepharose and only weakly to the column containing lactose. Good purification was, however, obtained on a column of galactose-derivatized Sepharose. On this column, the lectin emerged as a single peak even when elution was done with a gradient of galactose, suggesting similar affinity for this sugar of the different fractions.

Freshly prepared affinity-purified lectin had a low solubility in PBS (about 2 mg/ml). Moreover, after a few days storage in the frozen state, the solution became turbid, probably because of polymerization of the protein, as has been observed with other lectins (e.g. soybean agglutinin [11]). Only solutions clarified by centrifugation were used for further experiments.

The results of gel filtration on Superose 12 clearly show that the affinity-purified lectin consists of two closely-related isolectins, both of which are blood type A and N specific, although with different relative activities with respect to erythrocytes of the two blood types. From SDS-PAGE analysis in the absence and presence of mercaptoethanol, it may be tentatively concluded that isolectin I is made up of three noncovalently associated subunits, 67, 42 and 26 kDa. Isolectin II is made up of the same subunits, although apparently in a different ratio. The 42 kDa band gave, after treatment with mercaptoethanol, a band of 46 kDa. An increase in the apparent molecular mass after reduction has been observed with the isolated light and heavy chains of IgG [12] and other proteins containing intramolecular S-S bonds [13]. It can therefore be assumed that the 42 kDa band contains such bonds. The 67 kDa band gave, after treatment with mercaptoethanol, two bands of 46 and 28 kDa, suggesting that the two chains of the largest subunit of the isolectins are linked by S-S bridges. S-S linked subunits have been found in other lectins, e.g. *Ricinus communis* agglutinin and lima bean lectin, but we are not aware of a lectin consisting of a mixture of covalently and noncovalently linked subunits. At this stage it is not known which of the subunits possess carbohydrate binding sites. Furthermore, we cannot conclude whether the isolectins consist of subunits which are anti-A + N, or whether they are a mixture of molecular species made up of varying proportions of anti-A and anti-N subunits. The latter case would be analogous to what has been observed with other lectins, such as PHA [14], *Griffonia simplicifolia* lectin I [15] and *Maclura pomifera* lectin [16].

The high specificity of the lectin for α-*N*-acetylgalactosamine fits well with the A blood type

specificity of the *Moluccella laevis* lectin, but does not account for its N specificity. In this context it should be mentioned that the N-specific *Vicia graminea* lectin is not inhibited by any monosaccharide, only by glycopeptides containing the disaccharide Galβ1 $\longrightarrow$ 3GalNAc in clusters on adjacent amino acid residues [17].

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