RESEARCH COMMUNICATION

Isolation, by affinity chromatography and gel filtration in 8 M-urea, of an active subunit from the anti-(blood-group A + N)-specific lectin of *Moluccella laevis*

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The lectin from *Moluccella laevis* seeds agglutinates specifically blood-type-A and -N erythrocytes, and both activities are inhibited by micromolar concentrations of *N*-acetyl-D-galactosamine. The lectin consists of three subunits: a 67 kDa heterodimer, made up of two S-S-linked polypeptides of 28 and 46 kDa, and two non-covalently linked moieties of 26 and 42 kDa, the latter migrating after reduction with an apparent molecular mass of 46 kDa. Here we demonstrate that affinity chromatography of a crude protein fraction from *M. laevis* seeds on immobilized D-galactose in the presence of 8 M-urea affords a fully active lectin practically devoid of the 42 kDa subunit. We also present data showing that the 26 kDa subunit is devoid of cysteine residues, that the 28 kDa subunit contains two cysteine residues engaged in S-S bonds with the 46 kDa subunit, and that the latter has, in addition, two intramolecular cystine residues. Gel filtration on Sephadex G-150 in 8 M-urea/0.2 M-D-galactose of the lectin, affinity-purified in the presence of urea, afforded a pure 26 kDa subunit which exhibited both anti-A and anti-N activity, as well as high specificity for *N*-acetyl-D-galactosamine. In addition to demonstrating that the lectin is unusually stable and retains its carbohydrate-binding activity in 8 M-urea, our findings also show that the activity for different blood groups resides in the same subunit.

INTRODUCTION

We have previously described the isolation, by affinity chromatography, of a lectin from *Moluccella laevis* seeds (MLL) [1] which has several unusual features of interest, from both a functional and molecular point of view. As originally reported [2,3], it is one of the very few blood-type-N-specific lectins, and the only one which is specific for erythrocytes belonging to two different blood-group systems, since it also agglutinates preferentially type-A erythrocytes. It is inhibited strongly by *N*-acetyl-D-galactosamine at very low concentrations (0.02–0.03 mM; 300–500 times less than D-galactose) [1], in contrast with the well-studied N-specific lectin of *Vicia graminea*, which is not inhibited by monosaccharides [4]. MLL exhibits also Tn-specificity, since it reacts strongly with glycoproteins carrying multiple GalNAc-Ser/Thr groups (e.g. asialo-ovine submaxillary mucin and asialo-agalactoglycophorins) and agglutinates Tn (O^{MM}) erythrocytes [5].

The affinity-purified lectin consists of a mixture of two isolectins, a major one of molecular mass 130 kDa and a minor one of molecular mass 250 kDa [1]. Both are made up of three subunits: a 67 kDa heterodimer of two covalently S-S-linked polypeptides of 28 and 46 kDa and two non-covalently linked moieties of 26 and 42 kDa, the latter migrating after reduction with an apparent molecular mass of 46 kDa. We are not aware of any other lectin with a similar composition and arrangement of subunits [6]. An intriguing question that the above studies left

unanswered is in which subunit(s) the carbohydrate-binding site(s) of the lectin resides.

In the present communication we provide further evidence on the structure of the lectin, demonstrate that it retains its sugar-binding properties under strongly dissociating conditions (8 M-urea) and show that affinity chromatography in the presence of urea yields an active lectin species practically devoid of the 42 kDa subunit. We also describe the isolation of the 26 kDa subunit in an active form, possessing anti-A as well as anti-N activity, both of which are inhibited by micromolar concentrations of *N*-acetyl-D-galactosamine, thus proving that the latter subunit has a carbohydrate-binding site (or sites); whether the 67 kDa subunit also binds sugars requires further study.

EXPERIMENTAL

Materials

Seeds of *Moluccella laevis* ('bells of Ireland') were purchased from Weller Flowerseed Company (Guadalupe, CA, U.S.A.). Galactose-derivatized Sepharose was synthesized as described previously [1]. Affi-Gel 501 (an organomercurial resin) and Bio-Gel P-6 were from Bio-Rad (Richmond, CA, U.S.A.), Sephadex G-150 and Sepharose 4B were from Pharmacia (Uppsala, Sweden). Sequencing-grade urea was from United States Biochemical Corp. (Cleveland, OH, U.S.A.), and all other materials were of the highest purity available.

Abbreviations used: MLL, *Moluccella laevis* lectin; U-MLL, *M. laevis* lectin purified in the presence of 8 M-urea; DTT, dithiothreitol; PBS, 0.05 M-sodium phosphate buffer/0.15 M-NaCl, pH 7.4; TB, Tris/HCl buffer, pH 6.5, containing 100 mM-NaCl and 10 mM-EDTA.

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Haemagglutination

Typed human A^{MM} and O^{NN} erythrocytes were obtained from the Blood Bank of Sheba Hospital (Tel Hashomer, Israel) and used to assay the anti-A and anti-N haemagglutinating activities of the lectin respectively by the double-dilution technique in microtitre plates [1] in the absence or presence of inhibitory sugars; scoring was after 1 h at room temperature. A unit of activity is defined as the lowest concentration of lectin giving visible agglutination.

Covalent chromatography

Chromatography on Affi-Gel 501 was performed as described by Alperin *et al.* [7]. The lectin (~2 mg) was dissolved in 20 μ l of 20% SDS (SDS/protein ratio 2:1, w/w) in 50 mM-Tris/HCl buffer, pH 6.5, containing 100 mM-NaCl and 10 mM-EDTA (TB) and unfolded by heating at 100 °C for 5 min, in either the presence or absence of 10 mM-dithiothreitol (DTT). The sample was then diluted with TB to a final concentration of 0.1% SDS and loaded on to a column (1.6 cm $\times$ 15 cm) of Bio-Gel P-6 equilibrated with TB/0.1% SDS; all the following steps were carried out in the same buffer. To minimize the time required for the gel-filtration step, and thus to decrease the possibility of reformation of S-S bonds, the column was pre-calibrated so that the material emerging in the void volume could be immediately applied to a column (2.5 ml) of Affi-Gel 501. After 2 h recirculation, the Affi-Gel column was washed with 1 ml aliquots of TB/0.1% SDS until the A_{280} of the effluent reached background values. Elution was with 1 ml portions of the same buffer containing 10 mM-DTT (each of which was left in contact with the column for 30 min) until background A_{280} was reached. Fractions containing protein were pooled and treated with 9 vol. of ice-cold methanol; after 12 h at -20 °C, the precipitate was collected by centrifugation at 18 000 rev./min (39 000 *g*) for 1 h at -18 °C in a refrigerated Sorvall centrifuge (rotor SS-34) and the pelleted proteins were dried under nitrogen.

E.l.i.s.a.

Various concentrations of lectin, in the absence or presence of inhibitors, were applied to asialoglycophorin A-coated wells of an e.l.i.s.a. plate, and binding was determined with rabbit anti-(MLL IgG) and goat anti-(rabbit IgG) antibodies conjugated with alkaline phosphatase as described previously [5]. Glycophorin A_M and glycophorin A_N were prepared as described in [8].

Gel electrophoresis

SDS/PAGE was carried out on 10% gels in the discontinuous system described by Laemmli [9]. The samples were heated for 5 min at 100 °C in sample buffer, either with or without 5% β -mercaptoethanol, and the gels stained with Coomassie Brilliant Blue R-250.

Protein determination

Protein was determined by the bicinchoninic acid ('BCA') reagent (Pierce, Rockford, IL, U.S.A.), with BSA as standard. A_{280} readings were used only for monitoring column effluents.

Determination of cysteine

The 26 kDa and 67 kDa bands obtained on SDS/PAGE of unreduced MLL [1] were electroeluted from the gel [10] at a constant current of 20 mA overnight in an apparatus made at the Weizmann Institute workshop, and the proteins were precipitated with cold methanol as described for the fractions from covalent chromatography. The material from the latter band was reduced with 5% mercaptoethanol and electrophoresed again, affording two bands (28 kDa and 46 kDa), each of which was electroeluted

and processed as above. Cysteine was determined as cysteic acid on a Dionex 5-500 amino acid analyser, after hydrolysis in dimethyl sulphoxide/conc. HCl (1:1, v/v) under vacuum at 110 °C for 22 h.

RESULTS AND DISCUSSION

Examination of the stability of MLL under different dissociating conditions revealed that it retains its anti-A + N activity and carbohydrate specificity after exposure at room temperature to 8 M-urea/0.2 M-D-galactose in 0.05 M-phosphate buffer/0.15 M-NaCl, pH 7.4 (PBS) for prolonged periods (up to 72 h tested), followed by dialysis against PBS. Affinity purification of the lectin in the presence of urea was therefore attempted. For this purpose, the crude lectin {dialysed (NH₄)₂SO₄ precipitate, cf. [1]}, prepared from 10 g of seeds, was loaded on to a column (1.6 cm $\times$ 10 cm) of the galactose-Sepharose affinity gel at room temperature, the column was washed with PBS followed by 8 M-urea in PBS, and the lectin eluted with 0.2 M-D-galactose/8 M-urea in PBS (Fig. 1). No haemagglutinating activity for erythrocytes of either blood type A or N was found in the dialysed and freeze-dried material from the urea wash at a concentration of 0.5 mg/ml. The material eluted by D-galactose/urea (U-MLL; 1.5–2 mg after dialysis and freeze-drying) had a specific anti-A activity of 12.5–50 units/mg and an

Fig. 1. Affinity chromatography of *Moluccella laevis* lectin in the presence of 8 M-urea

Crude lectin-preparation [1], obtained from 10 g of seeds, was applied to a column (1.6 cm $\times$ 10 cm) of galactose-derivatized Sepharose 4B at room temperature. The column was sequentially eluted with PBS, 8 M-urea in PBS (arrow a) and 8 M-urea/0.2 M-D-galactose in PBS (arrow b). The peaks were pooled, dialysed against water and freeze-dried; samples (35 μ g of protein) were submitted to SDS/PAGE (insets). The electrophoretic pattern of unreduced (lanes 1 and 3) and reduced (lanes 2 and 4) material from the 8 M-urea (lanes 1 and 2) and 8 M-urea/0.2 M-D-galactose (lanes 3 and 4) eluates are shown. The positions of molecular-mass (*M*) standards are marked (phosphorylase *b*, 94 kDa; BSA, 67 kDa; ovalbumin, 45 kDa; α -chymotrypsinogen, 24 kDa; soybean trypsin inhibitor, 20.1 kDa; β -lactalbumin, 14.4 kDa).

Table 1. Effect of different glycoproteins and *N*-acetyl-D-galactosamine on the binding of U-MLL and the 26 kDa subunit to asialoglycophorinsAbbreviations: GPA_M, glycophorin A from O^{MM} erythrocytes; GPA_N, glycophorin A from O^{NN} erythrocytes.

Compound	Concentration (μg/ml) giving 50 % inhibition		
	U-MLL	26 kDa	MLL*
GPA _M			
Untreated	3800	2800	3500
Asialo-	5	25	7.1
Asialoagalacto-	0.025	0.035	0.025
GPA _N			
Untreated	90	450	200
Asialo-	2.4	7.2	2.3
Ovine submaxillary mucin			
Untreated	0.07	0.06	0.10
Asialo-	0.002	0.003	0.003
GalNAc	4	1	2

* Data from Duk *et al.* [5]. **Fig. 2. SDS/PAGE of fractions obtained by covalent chromatography of reduced MLL on Affi-Gel 501 under denaturing conditions**

Lane 1, bound and DTT-eluted fraction (20 μg of protein); lane 2, unbound fraction (10 μg); lane 3, mixture of unbound (10 μg) and bound (20 μg) fractions, lane 4, reduced U-MLL (40 μg). The molecular-mass standards were as in Fig. 1.

anti-N activity of 100 units/mg. The haemagglutination was inhibited by the same concentrations of D-galactose (5–10 mM) and *N*-acetyl-D-galactosamine (0.02–0.03 mM) as for MLL [1]. Furthermore, U-MLL bound to asialoglycophorin A (as measured by e.l.i.s.a.) similarly to MLL (results not shown). The concentrations of different glycoproteins and of *N*-acetyl-D-galactosamine required to inhibit this binding by 50 % are given in Table 1. U-MLL has essentially the same activity and specificity as the MLL affinity-purified in the absence of urea [1], although the yield is lower. The fact that elution of the lectin required the addition of galactose demonstrates that its carbohydrate-binding activity is maintained in the presence of 8 M-urea. On SDS/PAGE, U-MLL showed two major bands, corresponding to the 26 and 67 kDa subunits of MLL, with only traces of the 42 kDa band (Fig. 1). H.p.l.c. gel filtration on a TSK SW3000G column revealed a major component with a molecular mass of 86 kDa. Thus the 42 kDa subunit appears not to be essential for lectin activity.

To establish the status of the thiols of U-MLL, it was unfolded in SDS, reduced with DTT and fractionated by covalent chromatography on an organomercurial gel. SDS/PAGE of the resultant fractions showed that the 26 kDa subunit was not retained on

Fig. 3. Gel filtration of U-MLL on Sephadex G-150 in 8 M-urea/0.2 M-D-galactose

About 3 mg of U-MLL was dissolved in 0.3 ml of PBS containing 8 M-urea and 0.2 M-D-galactose. After 16 h at room temperature the sample was applied to a column (1.8 cm × 65 cm) of Sephadex G-150 in equilibrium with the same buffer. Elution was monitored by the absorbance at 280 nm. About 20 μg of protein from dialysed and freeze-dried peak I and peak II was analysed by SDS/PAGE (inset, left and right gels respectively). The molecular-mass standards were as in Fig. 1.

the column, whereas the 28 kDa and 46 kDa polypeptides (resulting from the reduction of the 67 kDa subunit) were bound and could be eluted with DTT (Fig. 2); a similar pattern was obtained with MLL (result not shown). The total recovery of protein was above 90 % in each of three individual experiments. When the SDS-unfolded protein was applied to the same column without prior reduction with DTT, all the protein passed through the column unchanged, suggesting that the lectin does not contain any free SH groups. Analysis of the individual subunits, electroeluted from SDS/PAGE, showed that the 26 kDa subunit was devoid of cysteine, whereas the 28 and 46 kDa subunits contained about two and six cysteine residues respectively. These data are in agreement with the results from the organomercurial chromatography and demonstrate that the failure of the 26 kDa subunit to bind to the column is not the result of the reformation of S–S bonds during the gel-filtration step. The 28 kDa and 46 kDa moieties appear to be linked through two intermolecular

cysteine residues, the latter polypeptide carrying two additional, intramolecular, cysteine residues.

To separate the 67 and 26 kDa subunits of U-MLL, the lectin was submitted to gel filtration on Sephadex G-150 in 8 M-urea/0.2 M-D-galactose, after preincubation in the same solvent for 16 h at room temperature. Three peaks were obtained (Fig. 3). Peak I (1.1 mg of protein; 35 % of the amount applied) was enriched in the 67 kDa polypeptide and also contained small proportions of the 26 kDa and 42 kDa moieties, whereas peak II (0.5 mg; 15 % of the protein applied) contained solely the 26 kDa subunit; peak III contained only traces of protein and consisted largely of a dialysable dye, originally attached to the lectin and removed only by gel filtration under the dissociating conditions. Peak I had a high anti-A, up to 200 units/mg, and low anti-N, activity, typically 2–4 units/mg; in this it differs from MLL (and U-MLL), which exhibit a preference for N cells (N/A = 2–4). The concentrations of *N*-acetyl-D-galactosamine and D-galactose required to inhibit the activity of peak I were, however, comparable with those required for the inhibition of MLL and U-MLL, indicating that its monosaccharide specificity remained unchanged. A simple explanation for the high anti-A activity of peak I cannot be given at this time. It may result from differences in the proportions of the various subunits and their assembly, and may be related to the fact that interactions of lectins with high-molecular-mass ligands can be markedly affected by the physical state of the lectin [11,12].

The 26 kDa subunit eluted in peak II agglutinated A^{MM} erythrocytes with a specific activity of 10–20 units/mg and O^{NN} erythrocytes with a specific activity of 40–80 units/mg; its sensitivity to inhibition by D-galactose and *N*-acetyl-D-galactosamine was similar to that of MLL. The 26 kDa subunit bound to asialoglycophorin A (as measured by e.l.i.s.a.) with a 5–10-fold lower affinity than did U-MLL (results not shown). The concentrations of different glycoproteins and of *N*-acetyl-D-galactosamine required to inhibit this binding by 50 % (Table 1) are similar to those found for MLL and U-MLL.

The fact that the 26 kDa subunit possess haemagglutinating activity suggests that it has at least two binding sites, or that in aqueous solution it exists as an oligomer. Attempts to estimate

the molecular mass of the isolated subunit by gel filtration in PBS, whether on a Superose or a silica column, were unsuccessful, since the protein was retarded, apparently by hydrophobic interactions with the matrix.

Our data shown that the 26 kDa subunit is exceptionally stable, since it retains activity despite the harsh treatment it has been subjected to. The results also provide direct proof that the anti-A and anti-N activities, both of which are mediated by the *N*-acetyl-D-galactosamine-binding site(s) of the lectin, reside not only in the same molecule, as originally suggested [2], but in a single polypeptide chain.

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