Detection, isolation and characterization of multiple lectins from the haemolymph of the cockroach *Blaberus discoidalis*

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Three agglutinins (lectins), designated BDL1, BDL2 and BDL3, were identified in the haemolymph of the cockroach *Blaberus discoidalis* by erythrocyte cross-adsorption and sugar inhibition tests. With the use of $(NH_4)_2SO_4$ fractionation, anion-exchange and affinity chromatography, BDL1 and BDL2 have been purified to homogeneity, and BDL3 has been partially purified to three bands on SDS/PAGE. BDL1 has a molecular-mass estimate of 390 kDa by gel filtration and approx. 158 kDa by SDS/PAGE under non-reducing conditions, further reduced to subunits of 36 kDa under reducing conditions. BDL2 has a molecular mass of approx. 140 kDa and is composed of subunits of 67 kDa which can be further reduced to identical subunits of 23 kDa. Isoelectric focusing in agarose gels revealed that BDL1 and BDL2 both focused as single bands at pH 6.0 and pH 5.2 respectively. The purified forms of BDL1 and BDL2 were stained by the periodic acid/Schiff's reagent showing that both lectins are glycoproteins. In addition, BDL1 was deglycosylated by endo-β-*N*-acetylglucosaminidase H. Immunological tests showed that these three lectins are not structurally related. All three lectins bind galactose but have different specificities for binding other sugars and for a range of vertebrate erythrocytes. BDL1 is specifically inhibited by D-(+)-glucose, D-(+)-mannose and *N*-acetyl-D-mannosamine, but not by *N*-acetyl-D-glucosamine, and BDL2 is inhibited by *N*-acetyl-D-glucosamine, but not by D-(+)-glucose, D-(+)-mannose or *N*-acetyl-D-mannosamine. BDL3 is strongly inhibited by *N*-acetyl-D-galactosamine, but not by any of the other above-mentioned sugars. Erythrocyte specificities showed that BDL1 is more specific for rabbit than mouse erythrocytes, whereas BDL2 and BDL3 are more specific for mouse than rabbit erythrocytes. The haemagglutinating activities of both the serum and isolated lectins are Ca^{2+}-dependent. Localization of BDL1 and BDL2 with fluorescein isothiocyanate-labelled antibodies showed that both lectins are associated with the granules and other areas of the cytoplasm of all blood cell types.

INTRODUCTION

Lectins are carbohydrate-binding proteins, widely distributed within the body fluids and other tissues of many invertebrates [1–4]. Although many invertebrate lectins have been purified and characterized, their exact functions in these animals are poorly understood. However, evidence has been found that these molecules may play important roles in several physiological processes. In insects, for example, lectins are probably involved in the various stages of the immune defence reactions, such as wound repair, phagocytosis, haemolymph coagulation, encapsulation, clearance of non-self substances from circulation and killing of bacteria and parasites [5–10]. They are also implicated in growth and developmental events such as moulting, differentiation and metamorphosis [11–15].

In seeking an explanation for the functional properties of lectins, emphasis is always placed on the carbohydrate-binding specificity of these molecules. Unfortunately, the carbohydrate-protein interactions of lectins may reveal little about their functional roles. Other important clues to lectin functions can be gleaned from the presence of multiple binding sites specific for different carbohydrates, the presence of additional (other than carbohydrate) binding sites, the aggregation of different specific subunits in a single oligomer (isomers) and the occurrence of multiple lectins. In insects, isolectins and multiple binding sites of lectins have been reported [16–18]. However, the presence of multiple lectins has only been implied in a few species [19].

In the present study with the cockroach *Blaberus discoidalis* we describe, for the first time for an insect, the presence and isolation of three haemolymph lectins with very different sugar specificities and distinct antigenicities. In addition, preliminary characterization of two purified lectins is presented. These results are an essential prerequisite to functional studies with these purified molecules.

MATERIALS AND METHODS

Insects and bleeding

B. discoidalis were maintained on dried cat food in fibre-glass containers at 34 ± 2 °C and given water *ad libitum*. Adult females and males were used for all the experiments. For bleeding and collection of haemolymph, insects were anaesthetized at −20 °C for approx. 20 min, and bled in a cold-room at 4 °C, through the arthrodial membrane of a posterior limb. Haemolymph was collected into sterile Eppendorf tubes on ice, and serum was prepared by centrifuging the haemolymph clot at 10000 *g* for 5 min at room temperature. The resultant serum was pooled and stored at −20 °C. Approx. 60 ml of serum was obtained from 1200 adult insects.

Erythrocytes

Calf, chicken, horse, goat, mouse, guinea-pig and sheep blood, in Alsever's solution, were purchased from Serotec Ltd. (Oxford,

Abbreviations used: TBS/Ca^{2+}, 0.01 M Tris, 0.01 M $CaCl_2$, $7H_2O$, 0.77 M NaCl, 0.02% sodium azide, pH 7.4; HA, haemagglutination; AT-Sepharose 6B, acid-treated Sepharose 6B; IEF, isoelectric focusing; TTBS, 20 mM Tris, 0.9% NaCl, 0.1% Tween 20, pH 7.4; PAS, periodic acid/Schiff; TBS, 10 mM Tris, 0.77 M NaCl, pH 7.4; Endo H, endo-β-*N*-acetylglucosaminidase H.

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U.K.). Human AB blood, in citrate/dextrose, was obtained from Singleton Hospital, Swansea. Rabbit blood was collected aseptically into 3.8% trisodium citrate. All blood samples were stored at 4 °C, and used within 3 weeks, except for the mouse blood which was tested within 1 week. Erythrocytes were washed three times before use in Tris-buffered saline plus calcium (TBS/Ca^{2+}; 0.01 M Tris, 0.01 M $CaCl_2$,$6H_2O$, 0.77 M NaCl, 0.02% sodium azide, pH 7.4) and resuspended at a 2% (v/v) concentration in the same buffer containing 2% final concentration BSA (Sigma, Poole, Dorset, U.K.).

Haemagglutination (HA) assays

HA activities of whole serum and the chromatographic fractions were tested against the above range of vertebrate erythrocytes. For routine testing, however, only human AB or rabbit erythrocytes were used as indicator cells. The HA assays were performed in 96 V-bottomed well microtitre plates [L.I.P. (Equipment and Services) Ltd., Shipley, Yorks., U.K.]. Serum or isolated lectin preparations (25 μl) were serially diluted 2-fold in TBS/Ca^{2+}, pH 7.4, and an equal volume of erythrocyte suspension was added to each well. The plates were shaken gently and incubated for 60 min at room temperature. The HA titre was defined as the reciprocal of the highest dilution showing visual agglutination of the test erythrocytes.

Cross-adsorption assays

These experiments were carried out to test for the presence of multiple lectins in the whole serum. These were performed as described by Millar and Ratcliffe [20] with modification. Human AB and rabbit erythrocytes were washed, as above, and used for adsorption with whole serum or lectin-enriched samples (see below). Portions (100 μl) of the latter were mixed with equal volumes of the washed erythrocyte solutions for 1 h at room temperature with occasional shaking. The mixtures were then centrifuged, and the supernatants removed and adsorbed against erythrocytes for a second and third time under the same conditions. A 25 μl sample from each supernatant was tested for HA to monitor any change in agglutinating activity.

The lectin-enriched samples were prepared by passing 3 ml of whole serum through an acid-treated Sepharose 6B (AT-Sepharose 6B) column (1.5 cm × 15 cm, prepared as below), equilibrated with TBS/Ca^{2+}, pH 7.4. After extensive washing with 200 ml of TBS/Ca^{2+}, pH 7.4, at a flow rate of 10 ml/h to remove the unbound protein from the column, the bound proteins were eluted with 25 ml of 0.2 M D-(+)-galactose in TBS/Ca^{2+}, pH 7.4. Eluates (designated lectin-enriched fractions) were dialysed against TBS/Ca^{2+}, pH 7.4, to remove the sugar, and concentrated with poly(ethylene glycol) until titres of 8192 against rabbit and 256 against human AB erythrocytes were obtained. With this lectin-enrichment technique, minor lectins can be concentrated to detectable levels.

HA inhibition assays

The sugar specificities of the lectins in whole serum or after purification were investigated by competitive binding using the following sugars and glycoproteins: D-(+)-galactose, α-lactose, D-(+)-glucose, D-(+)-mannose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, α-D-(+)-fucose and α-L-(−)-fucose, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, methyl-α-D-mannopyranoside, methyl-β-D-mannopyranoside, laminarin (a β-1,3-glucan), fetuin and mucin. All of these were purchased from Sigma except for lactose and glucose which were obtained from BDH (Poole, Dorset, U.K.). Stock solutions of sugars and glycosubstances were prepared in TBS/Ca^{2+}, pH 7.4, at 0.2 M and 1% concentration respectively and stored at −20 °C until use.

For each inhibition test, serial 2-fold dilutions of 25 μl portions of the inhibitors in TBS/Ca^{2+}, pH 7.4, were performed in microtitre plates. Subsequently, 25 μl of whole serum or isolated lectin preparations adjusted to give a titre of 1:16, was added to each well, mixed by shaking and incubated for 30 min at room temperature. Then 50 μl of 2% human AB or rabbit erythrocyte suspension was added and the minimum concentration of inhibitors required to block agglutination was determined microscopically after 1 h.

$(NH_4)_2SO_4$ fractionation

Insect serum (6 ml) was diluted with an equal volume of 0.02 M Tris buffer containing 10 mM Ca^{2+}, pH 8.2, and the lectins were fractionated by $(NH_4)_2SO_4$ at a concentration between 30 and 50%. The resulting precipitates were dissolved in 15 ml of 0.01 M Tris/HCl containing 10 mM Ca^{2+}, pH 8.2, and subsequently dialysed overnight with three changes against 400 ml of the same buffer.

Isolation of lectins with chromatographic gels

Ion-exchange chromatography

DEAE-cellulose (20 g; Sigma) was washed in 0.5 M NaOH for 10 min, rinsed in distilled water, followed by 0.8 M HCl, and finally in 0.5 M NaOH, in sequence, in order to remove contaminants. The washed matrix was thoroughly rinsed free of NaOH with distilled water and mixed with a quantity of starting buffer (0.01 M Tris buffer containing 0.01 M Ca^{2+}, pH 8.2) sufficient to produce a thin suspension. The treated matrix was then packed in a column (20 cm × 1.0 cm) and thoroughly washed with the starting buffer. A sample (approx. 20 ml) from the $(NH_4)_2SO_4$ fractionation was centrifuged to remove insoluble materials and applied to the DEAE-cellulose column at a flow rate of 10 ml/h. The column was then washed with the starting buffer until the baseline was achieved and then elution was carried out with a linear gradient of 0–250 mM NaCl in 200 ml of the starting buffer. Fractions (3 ml) from the throughflow or the separated peaks (peaks I and II) were collected, tested for HA activity and active peaks pooled for affinity chromatography.

Affinity chromatography

Two affinity media were used. AT-Sepharose 6B was prepared by treating Sepharose 6B (Pharmacia, Uppsala, Sweden) with 0.2 M HCl at 50 °C for 2 h [21], and *N*-acetyl-D-galactosamine–Sepharose 6B (GalNAc–Sepharose 6B) was made up by conjugating the sugar to epoxy-activated Sepharose 6B (Sigma) according to the manufacturer's instructions. Both affinity gels were packed in columns (20 cm × 1.5 cm) and equilibrated with the starting buffer (Tris buffer containing 0.01 M Ca^{2+}, pH 8.2). For the purification of *B. discoidalis* lectin 1 (BDL1), the throughflow preparation (19 ml) from the DEAE-cellulose column was first incubated with an equal volume of 0.2 M *N*-acetyl-D-glucosamine in the starting buffer overnight at 4 °C and then slowly (10 ml/h) loaded on an AT-Sepharose 6B column. The column was then washed with 100 ml of starting buffer at pH 8.2 containing 0.5 M NaCl, then 100 ml of TBS/

Ca^{2+}, pH 7.4, containing 1 M NaCl, and finally with 100 ml of TBS/Ca^{2+}, pH 7.4, buffer before elution of bound protein with 25 ml of 0.3 M D-(+)-glucose in TBS/Ca^{2+}, pH 7.4, buffer. The purification of *B. discoidalis* lectin 2 (BDL2) and the partial purification of lectin 3 (BDL3) was achieved by passing pooled peak II fractions from the DEAE-cellulose column through the GalNAc-Sepharose 6B column, and peak I fractions through the AT-Sepharose 6B column after the same washing procedures as for the BDL1 purification. Elution was carried out with 25 ml of 0.2 M lactose for BDL2, and 25 ml of 0.2 M D-(+)-glucose followed by 25 ml of 0.2 M D-(+)-galactose for BDL3, in TBS/Ca^{2+}, pH 7.4, buffer.

Molecular-mass estimation

Molecular-mass estimations were carried out by h.p.l.c. on a Zorbax GF 250 column (Du Pont, Wilmington, DE, U.S.A.) with 0.01 M Tris buffer containing 0.01 M Ca^{2+} at pH 7.4 as the mobile phase. The column was calibrated with high- and low-molecular-mass gel-filtration calibration kits (Pharmacia). The molecular mass of subunits of purified lectins was estimated by electrophoresis (see below).

Protein determination

Protein determination was carried out by the method of Bradford [22] with BSA (Sigma) as a standard.

Electrophoresis

SDS/PAGE was carried out by the method of Laemmli [23]. Samples were adjusted to 0.5–1 μg of protein and denatured with 1% SDS containing 2% 2-mercaptoethanol (BDH) by boiling for 3 min. Treated samples were centrifuged (10000 *g*, 5 min) before being loaded on to the gels. Electrophoresis (Mini Protean II System, Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts., U.K.) was carried out at a constant voltage of 200 V for approx. 1 h. Gels were then fixed for 45 min in 40% (v/v) methanol containing 10% (v/v) acetic acid and stained with silver nitrate using Bio-Rad staining kits. For determination of the molecular mass, the gels were calibrated with both high- and low-molecular-mass marker proteins (Pharmacia). Native PAGE was performed by the method of Maurer [24] in 7.5% polyacrylamide gels (LKB 2001 Vertical Electrophoresis System). Gels were run at a constant current of 25 mA/gel for approx. 3.5 h and the protein bands visualized using the silver staining method, as above.

Isoelectric focusing (IEF)

IEF was performed with an LKB Multiphor II flat bed electrophoresis apparatus at 10 °C, using a 1% agarose IEF (Pharmacia) plate gel containing 12% sorbitol and 6.3% carrier Pharmalyte (pH 3–10, Pharmacia), according to the manufacturer's instructions. Proteins (10 μg in 2 μl of distilled water) were directly applied to the gel plate using a sample applicator mask (Pharmacia) and focused at a constant power of 15 W for 2 h. The gel was fixed immediately after the run was completed in 5% sulphosalicylic acid containing 10% trichloroacetic acid for 30 min and stained with 0.2% Coomassie Blue R250 in the destaining solution (30% ethanol containing 10% acetic acid). The protein bands were visualized after destaining. For pI determination, an IEF marker kit (Sigma) containing amyloglucosidase (pI 3.6), trypsin inhibitor (pI 4.6), β-lactoglobulin (pI 5.1), carbonic anhydrase II (bovine, pI 5.9), carbonic anhydrase I (human, pI 6.6), myoglobin (pI 6.8, 7.2), L-lactate dehydrogenase (pI 8.3, 8.4, 8.6), trypsinogen (pI 9.3) and Methyl Red (marker dye, pI 3.8) was used for calibration.

Immunization, immunodiffusion and Western blots

Antisera against the isolated lectins, BDL1 and BDL2, were prepared in female New Zealand White rabbits (approx. 4 kg) as described by Richards et al. [18]. Briefly, a 0.6 ml lectin sample (approx. 80 μg of protein per injection) emulsified with 0.4 ml of Freund's complete adjuvant (Sigma) was injected intramuscularly into the thigh. Injections were given initially three times at 2-day intervals, followed by three more injections 7 days later. Bleeding was carried out 1 week after the final injection either via the ear veins or by cardiac puncture. The freshly drawn blood was left at room temperature for 1 h to allow clot formation, and then at 4 °C for 12 h to allow contraction of the clot. Serum was separated from the clots by centrifugation at 1500 *g* for 30 min at 4 °C and serum samples were stored at −70 °C until required.

The reactivity of the antisera was determined by Ouchterlony double gel diffusion in 1% purified agar (Code L28, Oxoid Ltd., Basingstoke, Hants., U.K.) prepared in borate-buffered saline (0.025 M borax, 0.1 M boric acid, 0.075 M NaCl, pH 8.0) [25]. Sample wells (3 mm in diameter) were punched in the gels with an LKB template (Pharmacia LKB) and filled with 10 μl of antigen or antisera samples. Tests were carried out with either antigens (whole serum, BDL1, BDL2 and fractions containing BDL3) in the centre wells and 2-fold-diluted antisera against BDL1 or BDL2 in the outer wells for determining specificity and titres of antibodies, or antigens (whole serum) in the centre wells and different antisera in the outer wells for detection of cross-reactivity of antibodies. The gels were then incubated at 37 °C for 24–48 h to develop the precipitation lines. Results were photographed using a Polaroid camera under darkfield illumination.

For Western blots, SDS/PAGE was performed as above. The electrophoretic transfer of proteins from the gel to 0.45 μm Hybond C-Super nitrocellulose membranes (Amersham International, Slough, Berks., U.K.) was based on a modified method of Towbin et al. [26] using a Bio-Rad Trans-Blot cell with 25 mM Tris, 192 mM glycine, pH 8.3, as blotting buffer. After blotting, the nitrocellulose membranes were immersed in Tris/Tween-buffered saline (TTBS; 20 mM Tris, 0.9% NaCl, 0.1% Tween 20, pH 7.4) containing 5% milk powder (Marvel, Birmingham, U.K.) for 1 h before incubation with the rabbit polyclonal antiserum solution (1:500 dilution of antiserum in TTBS containing 1% BSA) for 4 h. After extensive washing with TTBS, membranes were overlaid with goat anti-rabbit IgG conjugated with peroxidase (Sigma; 1:2000 dilution in TTBS containing 1% BSA) for 2 h. The immunogenic protein bands were visualized using 4-chloro-1-naphthol/H_2O_2 (both from Sigma) as a chromogenic substrate, as described by Hawkes et al. [27].

Staining for carbohydrates and deglycosylation of purified BDL1 and BDL2

The carbohydrate content of purified BDL1 and BDL2 was detected using the periodic acid/Schiff (PAS) technique [28]. SDS/PAGE of purified BDL1 and BDL2 (10 μg of protein each) was carried out on a 10% slab gel, as above. After electrophoresis, the gel was fixed in 7.5% acetic acid for 1 h, rinsed twice with

distilled water, oxidized in 0.2% periodic acid for 45 min, rinsed in tap water for 1 min, and stained with Schiff's reagent for another 45 min. Finally, the gel was washed in running tap water for 5 min and destained with 10% acetic acid.

Deglycosylation of the purified BDL1 and BDL2 (2 μg of protein each) was carried out by incubation of the purified lectins with 0.5 unit of endo-β-*N*-acetylglucosaminidase H (Endo H; Boehringer, Mannheim, Germany) for both 12 and 24 h, in 0.1 M acetate buffer, pH 5.2, in the presence of 1% SDS and 2% 2-mercaptoethanol. The resulting digested products were analysed in Western blots, as described above.

Bivalent cation requirement for lectin activities

Tests for requirement of bivalent cations for lectin activities were performed as described previously [18]. Portions of diluted serum (titre 64; 100 μl each) or purified/isolated lectin preparations were dialysed against 200 ml of Tris-buffered saline (TBS, 10 mM Tris containing 0.77 M NaCl at pH 7.4) either with or without 0.01 M Ca^{2+}, 0.01 M Mg^{2+} or 0.01 M Mn^{2+}, or with 0.01 M EDTA at pH 7.4, for 24 h with three changes of buffer. HA assays were then performed using rabbit or human erythrocytes washed in the corresponding dialysing buffer. Attempts to recover any lost HA activity in the samples without bivalent cations was carried out by reintroducing Ca^{2+} by dialysis against TBS/Ca^{2+}, pH 7.4, buffer.

Effects of reducing agents and detergents on lectin activities

Serum (titre 4096; 100 μl) or purified lectin preparations were dialysed overnight against 200 ml of 2.5% 2-mercaptoethanol, 6 M urea or 1% SDS in TBS/Ca^{2+}, pH 7.4, buffer or against TBS/Ca^{2+}, pH 7.4, alone as a control for 24 h with three changes of buffer. HA tests were carried out, as above, after redialysing samples overnight in TBS/Ca^{2+}, pH 7.4, to remove the free reducing agents or detergents.

Localization of BDL1 and BDL2 by immunofluorescence

Haemocyte monolayers were set up on Multispot microscope slides (C. A. Hendley Ltd.) and fixed as described by Richards et al. [29]. The fixed haemocyte monolayers were incubated with blocking agents (1% BSA + 1% horse serum, in PBS containing 100 mM glycine and 100 mM Tween 20, pH 7.4) to mask non-specific binding sites for 1 h at room temperature and, subsequently, with 1:500 dilutions of rabbit anti-BDL1 or -BDL2 sera in the blocking agent for 2 h at room temperature. The slides were then washed three times (5 min each) with TBS/Ca^{2+}, pH 7.4, before being overlayed with a 1:2000 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma) for a further 1 h at room temperature. The stained haemocytes were photographed with a Zeiss photomicroscope II using both phase-contrast and u.v. after the excess second antibody had been washed away with TBS/Ca^{2+}, pH 7.4, buffer (three times, 5 min each). Controls utilized the blocking agents alone or pre-immunized rabbit serum in place of the first antibody.

RESULTS

HA assays

The HA patterns of whole serum, purified BDL1, BDL2 and BDL3 against a range of erythrocytes were determined. BDL1 agglutinated most erythrocyte types except goat and sheep, with the highest titre of 4096 against rabbit erythrocytes, and less HA activity (titre $\leqslant$ 32) against human AB, guinea-pig, calf and chicken erythrocytes. BDL2 did not agglutinate calf, chicken, goat or sheep erythrocytes. The highest titre of 256 was against mouse erythrocytes with lower activities (titre $\leqslant$ 32) against human AB, rabbit, guinea-pig and horse erythrocytes. BDL3 had almost the same specificity for erythrocytes as BDL2, but much higher HA activity occurred against mouse erythrocytes and less against the other erythrocyte types tested.

Cross-adsorption experiments

As shown in Figure 1, there was no indication of the presence of multiple lectins using the whole serum for cross-adsorption. Thus removal of HA activity with both human AB or rabbit erythrocytes by adsorption three times left little or no activity against the other blood type. This is probably due to the minor

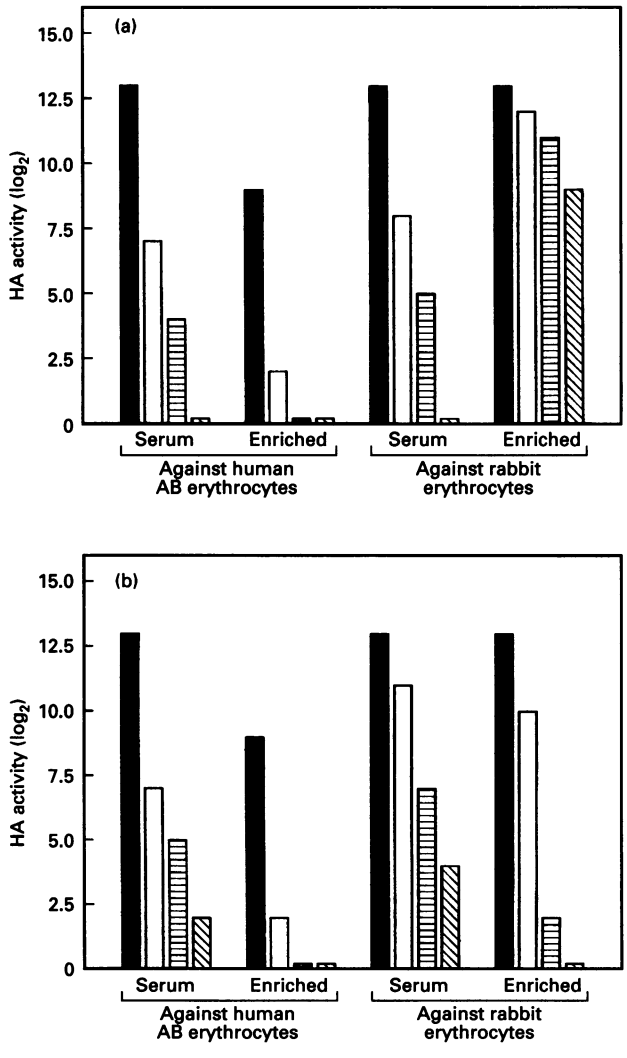

Figure 1 Cross-adsorption tests of *B. discoidalis* whole serum and lectin-enriched fraction against human AB and rabbit erythrocytes

(**a**) Human AB erythrocytes were used to adsorb whole serum or a lectin-enriched fraction three times and then tested against either human AB or rabbit erythrocytes. (**b**) Conditions as in (**a**) except rabbit erythrocytes were used for adsorption. ■, Unadsorbed control; □, adsorbed once; ▤, adsorbed twice; ▧, adsorbed three times.

Table 1 HA inhibition tests with *B. discoidalis* whole serum, and isolated BDL1, BDL2 and BDL3 fractions

Whole serum, BDL1, BDL2 or partially purified BDL3 were adjusted in concentration to give an agglutination titre of 1:16 against a 2% erythrocyte suspension. For each test, 2-fold serial dilutions of 25 μl portions of inhibitors in TBS/Ca^{2+} were made in microtitre plates and subsequently an equal volume of serum or lectin preparation was added. Either 2% human AB erythrocytes (for BDL3 lectin) or 2% rabbit erythrocytes (for serum, BDL1 and BDL2) (50 μl in each case) was added, mixed thoroughly, incubated at room temperature for 60 min and the minimum concentration of inhibitors required to block agglutination determined by microscopic examination of the wells. Data presented are from one representative experiment repeated three times. Values > 200 mM sugars or 1% fetuin, mucin and laminarin indicate that no inhibition of agglutination was recorded. Non-standard carbohydrate abbreviations: Me-α-D-Gal and Me-β-D-Gal, methyl α-D- and β-D-galactoside; Me-α-D-Man and Me-β-D-Man, methyl α-D- and β-D-Mannoside.

Sugars or glycosubstances	Minimum concentration of sugars or glycosubstances required for complete inhibition (mM or %)			
	Serum	BDL1	BDL2	BDL3
GalNAc	50	50	0.78	0.78
GlcNAc	100	> 200	25	> 200
ManNAc	50	50	> 200	> 200
D(+)Gal	50	100	3.13	0.78
D(+)Glc	50	100	> 200	> 200
D(+)Man	50	6.25	> 200	> 200
Lactose	50	50	3.13	1.56
D(+)Fuc	100	50	1.56	3.13
L(−)Fuc	100	> 200	> 200	> 200
Me-α-D-Gal	12.5	12.5	12.5	nd
Me-β-D-Gal	50	25	12.5	nd
Me-α-D-Man	> 200	> 200	> 200	nd
Me-β-D-Man	> 200	> 200	> 200	nd
Laminarin	1%	> 1%	> 1%	> 1%
Mucin	3.125%	0.0039%	0.25%	0.0039%
Fetuin	3.125%	0.00156%	> 1%	0.0039%

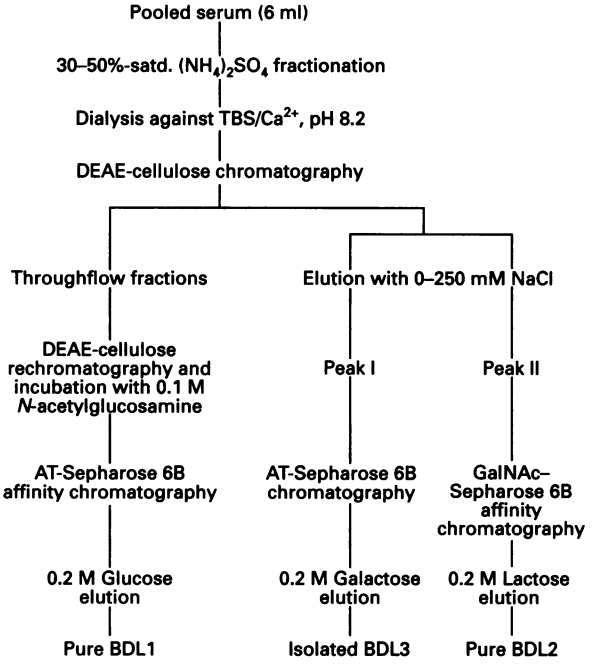

Figure 2 Flow chart outlining the steps used to isolate the serum lectins of *B. discoidalis*

lectin activities being masked by the dominant lectins in whole serum. This assumption was proved by cross-adsorption experiments using a lectin-enriched sample. Adsorption tests on the lectin-enriched sample using human AB erythrocytes reduced HA activity to zero after two adsorptions against human AB, whereas against rabbit erythrocytes HA activity remained at a remarkably high level even after three adsorptions with human AB cells (Figure 1a). However, adsorption of the lectin-enriched sample by rabbit erythrocytes removed lectin activity against both human and rabbit erythrocytes (Figure 1b), indicating that the multiple lectins present recognize identical determinants on the rabbit blood cells.

HA inhibition assay

The HA inhibition of sugars and glycosubstances for whole serum, BDL1, BDL2 and BDL3 was similar, but some key differences were recorded (Table 1). All three lectins were inhibited by *N*-acetyl-D-galactosamine, D-(+)-galactose, D-lactose and mucin. None of the lectins were inhibited by α-L-(−)-fucose (> 200 mM) or laminarin (> 1%). BDL1 was inhibited more by D-(+)-mannose, *N*-acetyl-D-mannosamine, D-(+)-glucose and fetuin than were BDL2 and BDL3, but not by *N*-acetyl-D-glucosamine. BDL2, unlike BDL1, was inhibited by *N*-acetyl-β-D-glucosamine and not by D-(+)-mannose, *N*-acetyl-D-mannosamine, D-(+)-glucose and fetuin. Unlike BDL1, but like BDL2, BDL3 was not inhibited by *N*-acetyl-D-mannosamine, D-(+)-glucose and D-(+)-mannose, and, unlike BDL2, not by *N*-acetyl-D-glucosamine. Both BDL1 and BDL2 were inhibited to a similar extent by the α and β forms of methyl-D-galactopyranoside suggesting an absence of anomeric specificity for these methyl sugars.

Isolation of *Blaberus* lectins

The protocol for the isolation of the *B. discoidalis* lectins is presented in Figure 2. This method involved $(NH_4)_2SO_4$ fractionation, DEAE-cellulose chromatography and affinity chromatography.

$(NH_4)_2SO_4$ fractionation (30–50% cut) typically recovered

Table 2 Summary of purification of *B. discoidalis* lectins

Data were pooled from ten separate experiments.

Step	Total volume (ml)	Total protein (mg)	10^{-3} × Total activity (units)	Specific activity (units/mg)	Fold	Yield (%)
Serum*	60	2220	39080	17600	1	100
$(NH_4)_2SO_4$*	200	1200	32770	27300	1.5‡	83‡
DEAE-cellulose						
Throughflow*	380	399	31130	78000	1§	100§
Peak II*	120	60	307	5100	1§	100§
Peak I†	89	53	228	4300	1§	100§
Affinity chromatography						
BDL1*	3.0	1.9	15730	8322000	107‖	51‖
BDL2*	2.3	1.8	23.6	13200	26‖	8‖
BDL3†	3.1	1.5	31.7	20900	5‖	14‖

* HA activity against rabbit erythrocytes.
† HA activity against human AB erythrocytes.
‡ Fold and yield calculated for $(NH_4)_2SO_4$ fractionation.
§ Fold and yield calculated for individual lectins.
‖ Fold and yield calculated for affinity chromatography.

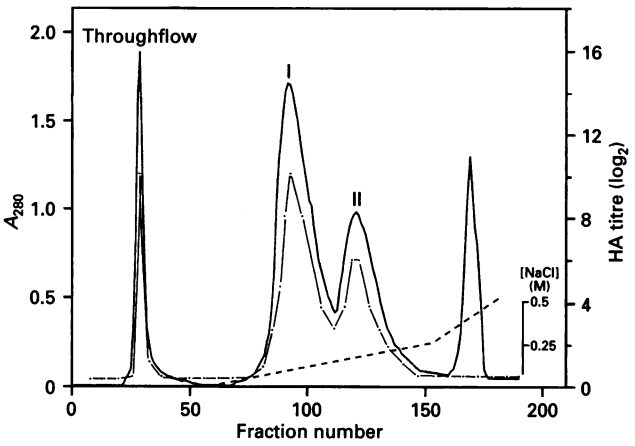

Figure 3 DEAE-cellulose column chromatography of *B. discoidalis* haemolymph lectins

A sample (30–50% cut) from $(NH_4)_2SO_4$ fractionation was centrifuged at 10000 ***g*** for 10 min. The clear supernatant (20 ml) was applied to a DEAE-cellulose column (20 cm × 1.0 cm) at a flow rate of 10 ml/h. The column was washed with starting buffer (0.01 M Tris buffer containing 0.01 M Ca^{2+}, pH 8.2) until the absorbance reached baseline. The adsorbed proteins were eluted with a 200 ml linear gradient of 0–500 mM NaCl. Fractions of 3 ml were collected and assayed for lectin activity. ——, A_{280}; –·–·, HA activity; – – – –, NaCl gradient.

83% of HA activity from the serum with up to 1.5-fold purification (Table 2). After DEAE-cellulose chromatography, HA activity was found in the throughflow and fractions constituting two eluted peaks I and II (Figure 3). The active throughflow fractions were pooled, and subjected to a second run on the same DEAE-cellulose column under identical conditions. Active fractions (38 ml) in the throughflow of the second run were collected and used for further purification of BDL1. The active fractions in peak I (12 ml) and peak II (8.9 ml) from the first run of the DEAE-cellulose column were pooled separately and used for BDL3 and BDL2 purification respectively (Figures 2 and 3). This DEAE-cellulose chromatography stage was the key step for the separation of the three lectins.

Before affinity chromatography, the throughflow fractions from the DEAE-cellulose chromatography step were incubated with 0.1 M *N*-acetyl-D-glucosamine in order to eliminate any possible binding of the BDL2 lectin to the affinity column. For affinity chromatography, the BDL1 preparation from the throughflow of the above DEAE-cellulose step was loaded on an AT-Sepharose 6B column. After extensive washing, the bound BDL1 was eluted with 0.2 M D-glucose. High HA activity was detected in the peak fractions (Figure 4a) after extensive dialysis and concentration. One further lectin, BDL2, was purified from peak II fractions from the DEAE-cellulose step by loading on a conjugated GalNAc–Sepharose 6B column. The column was washed with the same buffers used for the AT-Sepharose 6B column and the bound lectin was eluted with 0.2 M D-lactose in TBS/Ca^{2+}, pH 7.4. Lectin activity was found in the peak fractions (Figure 4b) after dialysis and concentration, as above. Attempts to purify the final lectin, BDL3, were made by passing the active fractions from peak I of the DEAE-cellulose chromatography step through an AT-Sepharose 6B column. The column was washed under the same conditions as for the BDL1 purification. Elution of the bound lectin was carried out with 0.2 M D-(+)-glucose, followed by 0.2 M D-(+)-galactose. Although HA activity was detected in the peak of galactose elution (Figure 4c), the result was unsatisfactory, as three bands remained in the electrophoretic gels (see below). A summary of the purification of BDL1, BDL2 and BDL3 is shown in Table 2.

Homogeneity and molecular-mass determination of the purified lectins

During purification, each step was monitored by SDS/PAGE and the final isolated lectins by both SDS/PAGE and native PAGE. The purity of BDL1 and BDL2 was confirmed by native PAGE in which the former appeared as a single band at the top of the gel indicating a low charge density, whereas the latter formed a single diffuse band which entered some way into the gel indicating a higher charge density at pH 8.3 (Figure 5a). In the

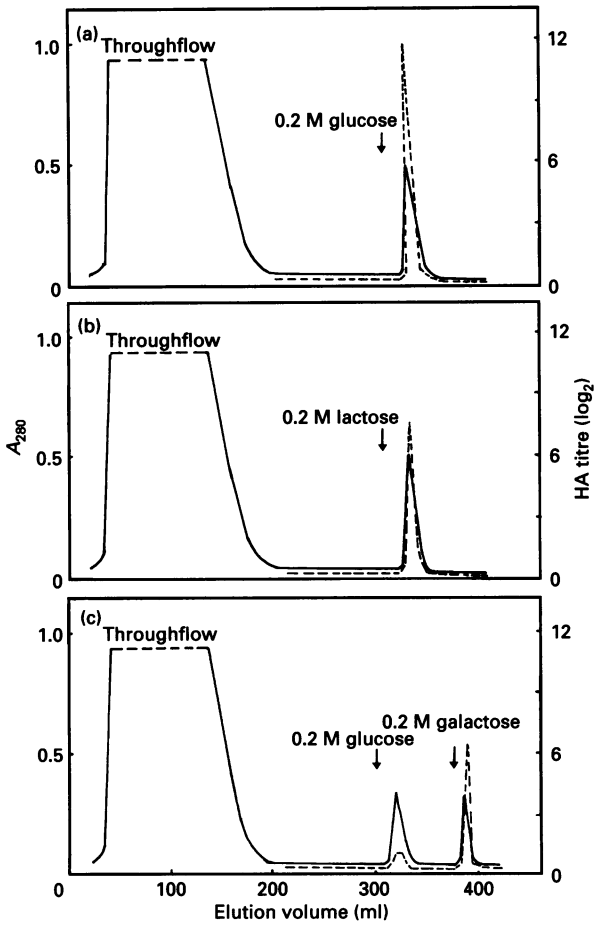

Figure 4 Affinity chromatography of *B. discoidalis* haemolymph lectins

(**a**) BDL1 purification. Throughflow fractions from two consecutive runs on a DEAE-cellulose column were first incubated with an equal volume of 0.2 M *N*-acetyl-D-glucosamine in the starting buffer (TBS/Ca^{2+}, pH 8.2) and slowly (10 ml/h) applied to an AT-Sepharose 6B column (20 cm × 1.5 cm) equilibrated with the starting buffer. The column was washed subsequently with 100 ml of starting buffer containing 0.5 M NaCl, 100 ml of TBS/Ca^{2+}, pH 7.4, containing 1 M NaCl and 100 ml of TBS/Ca^{2+}, pH 7.4. The bound protein was eluted with 25 ml of 0.2 M D-(+)-glucose in TBS/Ca^{2+} buffer, pH 7.4. (**b**) BDL2 purification. Active fractions from peak II of DEAE-cellulose chromatography were passed through a GalNAc–Sepharose column (10 cm × 1.5 cm). The column was washed as for BDL1 purification and the bound protein was eluted with 0.2 M D-lactose in TBS/Ca^{2+}, pH 7.4. (**c**) Partial purification of BDL3. Active fractions from peak I of DEAE-cellulose chromatography were passed through an AT-Sepharose 6B column (20 cm × 1.5 cm). The column was washed as for BDL1 purification and the bound proteins were eluted with 0.2 M D-(+)-glucose followed by 0.2 M D-(+)-galactose in TBS/Ca^{2+}, pH 7.4. ——, A_{280}; – – –, HA activity.

SDS/polyacrylamide gels, under non-reducing conditions, both purified BDL1 and BDL2 showed one band as intact subunits with molecular masses of 158 kDa and 67 kDa respectively (Figure 5b). Under reducing conditions, these subunits dissociated into single bands on SDS/PAGE of 36 kDa for BDL1 and 23 kDa for BDL2 (Figure 5c). The confirmation of the homogeneity of purified BDL1 and BDL2 was also made by IEF agarose electrophoresis and gel filtration on a Zorbax GF 250 h.p.l.c. column. In the agarose IEF gel, both BDL1 and BDL2 appeared as single bands with pI values corresponding to 6.0 and 5.2 respectively (Figure 5d). With gel filtration, a symmetrical peak was observed for both lectins, and the molecular masses were estimated by calibrated columns with known molecular markers to be 390 kDa for BDL1 and 140 kDa for BDL2 (Figure 6). Immunodiffusion tests showed a single clear precipitation line

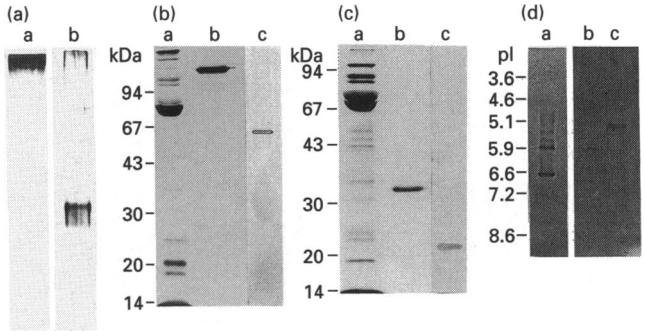

Figure 5 Electrophoresis and IEF of purified *B. discoidalis* lectins, BDL1 and BDL2

(**a**) Native PAGE of BDL1 (lane a) and BDL2 (lane b). Purified lectins BDL1 and BDL2 (2 μg each) were loaded on 7.5% polyacrylamide gels at pH 8.3 and run at a constant current of 25 mA per gel for 3.5 h. Gels were stained with silver nitrate. (**b**, **c**) SDS/PAGE of purified lectins and serum from *B. discoidalis*. Samples of serum (20 μg) and purified lectins (0.5–1 μg) were denatured with 1% SDS sample buffer under non-reducing conditions and with 1% SDS sample buffer plus 2% 2-mercaptoethanol under reducing conditions and heated for 3 min before being loaded on 10% SDS/polyacrylamide gels. (**b**) Non-reducing conditions. (**c**) Reducing conditions. Lane a, whole serum; lane b, BDL1; lane c, BDL2. (**d**) IEF of purified BDL1 and BDL2. IEF agarose gel of serum (lane a), BDL1 (lane b) and BDL2 (lane c).

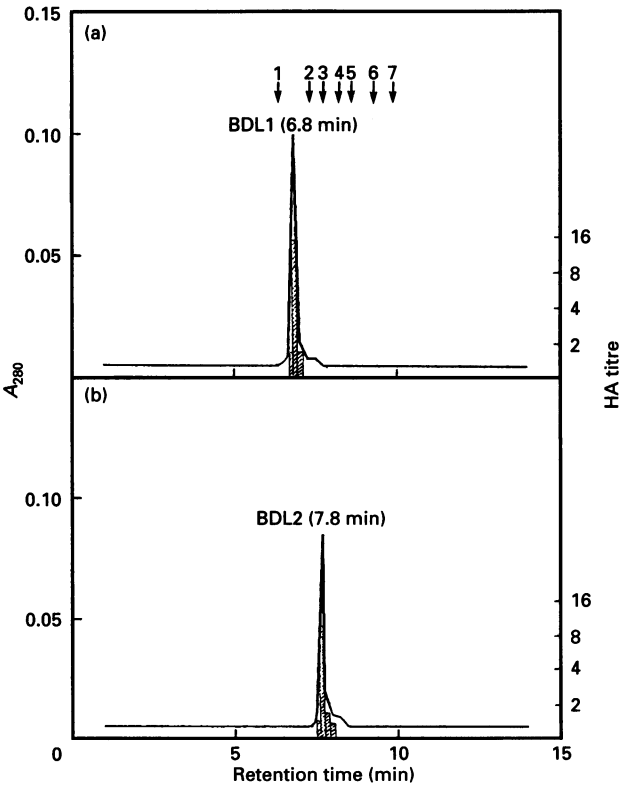

Figure 6 Molecular-mass determination of purified *B. discoidalis* BDL1 (a) and BDL2 (b) by gel filtration on a Zorbax GF-250 h.p.l.c. column

Typical chromatograms showing retention times and purity of BDL1 and BDL2 lectin preparations. Arrows show retention times of molecular-mass standards. 1, urease (545 kDa; hexamer); 2, urease (272 kDa; trimer); 3, BSA (132 kDa; dimer); 4, BSA (66 kDa); 5, chicken egg albumin (45 kDa); 6, carbonic anhydrase (29 kDa); 7, α-lactalbumin (14.2 kDa). ——, A_{280}; ▨, HA titre.

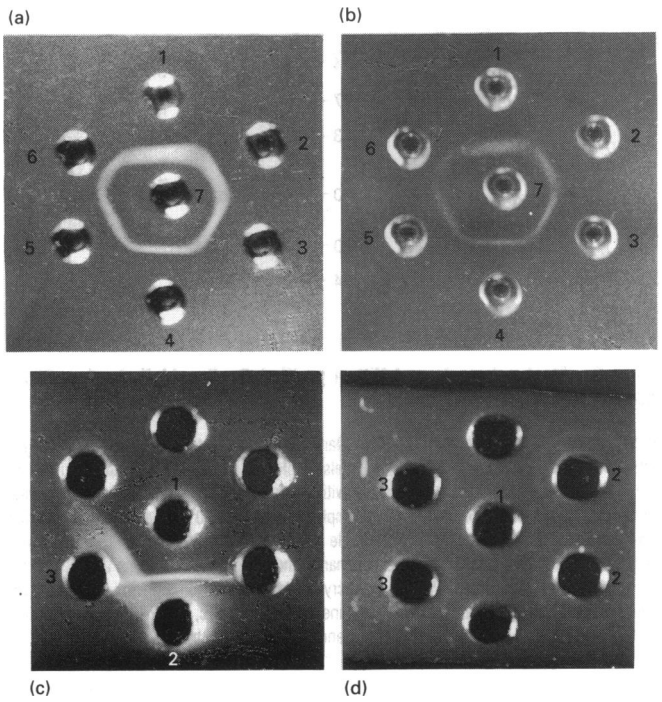

Figure 7 Immunodiffusion analysis of purified BDL1, BDL2 and partially purified BDL3

Wells were filled with 10 μl of antigen or antibody solutions as indicated. Precipitation lines were developed at 37 °C for 24–48 h and results were photographed under darkfield illumination. (**a**) The centre well (7) contains whole serum and the outer wells (1–6) contain anti-BDL1 serum diluted 2-fold. (**b**) Conditions as in (**a**) except that dilutions of anti-BDL2 serum were used in the outer wells. (**c**) Centre well (1) contains whole serum and outer wells (2, 3) contain anti-BDL1 and anti-BDL2 sera respectively. (**d**) Centre well (1) contains partially purified BDL3 preparation and outer wells contain anti-BDL1 serum (2) and anti-BDL2 serum (3).

in gels for both BDL1 (Figure 7a) and BDL2 (Figure 7b), also indicating the purity of the lectins against which the antisera were raised. Figure 7(c) also shows the lack of cross-reactivity between anti-BDL1 and anti-BDL2 sera. Antisera to BDL1 and BDL2 did not cross-react with any components in the partially purified preparation containing BDL3 (Figure 7d). Western-blot analyses demonstrated no cross-reactivity of BDL1 or BDL2 antibodies with any other proteins including BDL3 (Figure 8). The purification of BDL3 was incomplete and revealed three bands on SDS/PAGE (results not shown).

Staining for carbohydrates and deglycosylation of purified BDL1 and BDL2

Using the PAS technique, purified BDL1 and BDL2 were positively stained at protein levels of 10 μg/lane, indicating that both purified lectins were glycoproteins. Deglycosylation of BDL1 with Endo H showed apparent changes in mobility of the subunits on SDS/PAGE under reducing conditions. The BDL1 subunits shifted from the 36 kDa position to 32 kDa after enzyme treatment for 12 and 24 h (Figure 9a), confirming that the native BDL1 is a glycoprotein and that the oligosaccharide chain is removed by Endo H. As Endo H is specific for cleavage of asparagine-linked oligosaccharide with high mannose content, then BDL1 probably contains a high concentration of this sugar.

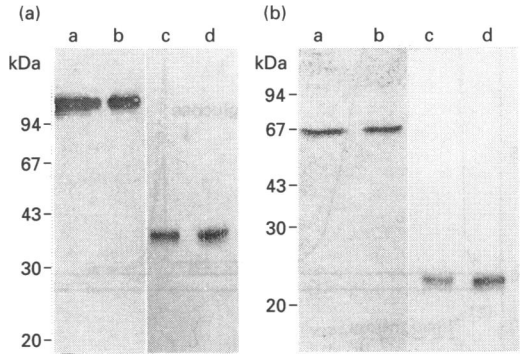

Figure 8 Western-blot analysis of *B. discoidalis* serum and purified BDL1 and BDL2

SDS/PAGE was carried out as described in the Materials and methods section and serum (20 μg) and purified BDL1 (**a**) and BDL2 (**b**) (1 μg each sample) lectins were run under both reducing and non-reducing conditions and blotted on nitrocellulose filters. The protein bands were then monitored with anti-BDL1 (**a**) and anti-BDL2 (**b**) sera. Proteins in lanes A and B were run under non-reducing conditions and those in lanes C and D were run under reducing conditions. Lanes A and D contained purified lectins and lanes B and C contained whole serum.

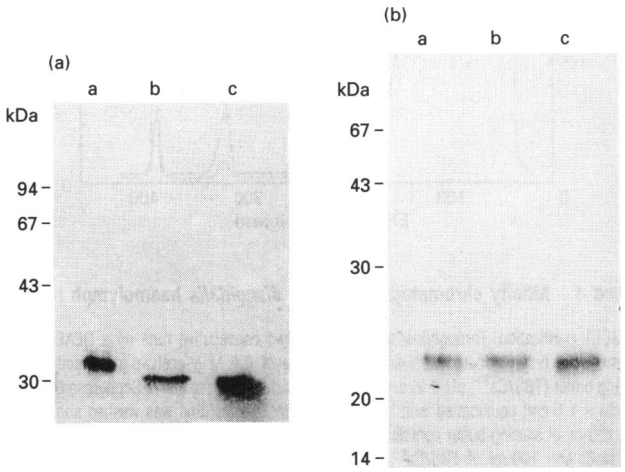

Figure 9 Deglycosylation of purified BDL1 and BDL2 by treatment with Endo H

Purified lectins (2 μg of each sample) were incubated without (lane A) and with 0.5 unit of Endo H for 12 (lane B) or 24 h (lane C) in 0.1 M acetate buffer, pH 5.2, in the presence of 1% SDS and 2% 2-mercaptoethanol. The resulting products (0.5–1 μg) were subjected to SDS/PAGE and Western blotting as in Figure 8. (**a**) BDL1 treatments; (**b**) BDL2 treatments.

No effect was observed when BDL2 was treated with Endo H (Figure 9b).

Bivalent cation requirement for HA activity

HA activities for BDL1, BDL2 and BDL3 were found to be Ca^{2+}-dependent. Removing Ca^{2+} by dialysing the lectin solutions against either TBS alone or TBS with 0.01 M EDTA resulted in a complete loss of activity of all three lectins and a 94% reduction in the HA activity of whole serum (results not shown). However, the activity of all the lectins and whole serum could be restored after redialysis against normal TBS/Ca^{2+}, pH 7.4. In addition, the HA activity of the serum and BDL1 could be partially maintained during dialysis against TBS by the addition

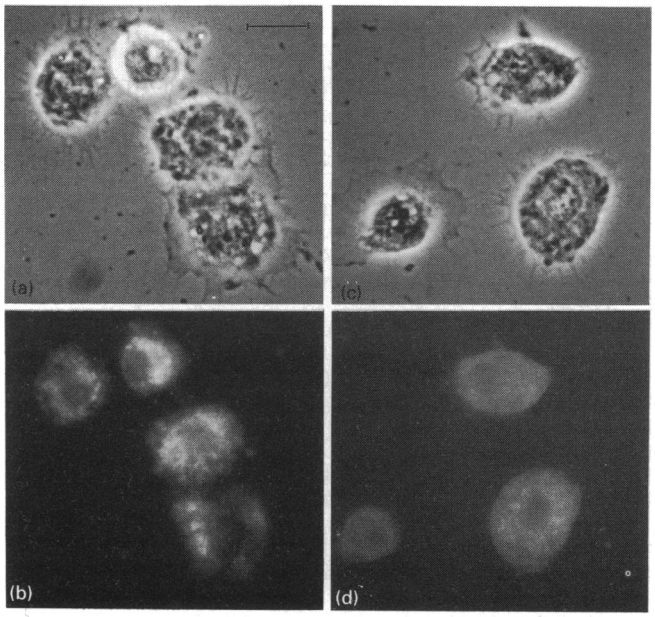

Figure 10 Distribution of BDL1 (a, b) and BDL2 (c, d) lectins in *B. discoidalis* haemocytes

Distribution of BDL1 (**a**) phase contrast and (**b**) immunofluorescence of same field as in (**a**) showing strong staining of the cytoplasmic granules. Distribution of BDL2 (**c**) phase contrast and (**d**) immunofluorescence of same field as in (**c**). Cells incubated with preimmune sera showed a low level of background fluorescence. Scale bar = 10 μm.

of 0.01 M Mg^{2+}, and HA activity of the serum to a lesser extent by the addition of 0.01 M Mn^{2+}. For BDL2 and BDL3, only Ca^{2+} could maintain HA activity.

Effects of reducing agents and detergents

The HA activity of the isolated lectin preparations was completely lost after treatment with 1% SDS, 2.5% 2-mercaptoethanol or 6 M urea. The HA activity of whole serum, however, although inhibited by these substances (urea > 2-mercaptoethanol > SDS) was not completely lost.

Localization of purified lectins in the blood cells (haemocytes)

As shown in Figure 10, all of the blood cells stained positively for both BDL1 and BDL2. The granules were often stained more strongly than other cell components (Figure 10b).

DISCUSSION

Using a combination of $(NH_4)_2SO_4$ fractionation, ion-exchange and affinity chromatography, two lectins, BDL1 and BDL2, have been purified to homogeneity as judged by electrophoresis, gel filtration, immunological analysis and differences in pI in IEF agarose gels. The third lectin, BDL3, was only partially purified, but separated from the other lectins, as judged by non-cross-reaction of the BDL3 preparations with either anti-BDL1 or anti-BDL2 antibodies and by sugar inhibition tests.

The purified lectins, BDL1 and BDL2, are different, not only in structure, but also in sugar-binding specificities, erythrocyte agglutination, bivalent cation requirement and agglutinating efficiency. For comparative purposes, Table 3 summarizes the properties of these three lectins. Physicochemical analysis of *B. discoidalis* lectins showed their remarkable differences in both native and subunit molecular masses. BDL1 has a molecular mass estimated as approx. 390 kDa, and is composed of subunits of approx. 158 kDa which can be further dissociated to 36 kDa subunits. In contrast, BDL2 has a molecular mass of 140 kDa, is composed of subunits of approx. 67 kDa, which can be further dissociated to 23 kDa subunits. Both BDL1 and BDL2 are glycoproteins, although only BDL1 can be deglycosylated by an endoglycosidase, Endo H, and thus has a high mannose content on the oligosaccharide chain. The sugar-binding specificities of the purified lectins are also very different. BDL1 is specifically inhibited by D-(+)-mannose, *N*-acetyl-D-mannosamine and D-(+)-glucose, but not by *N*-acetyl-D-glucosamine, whereas BDL2 is inhibited by *N*-acetyl-D-glucosamine.

According to Mäkelä [30,31], monosaccharides that react with lectins can be divided into four groups on the basis of their configuration at C-3 and C-4 of the pyranose ring. Thus lectins

Table 3 Properties of *B. discoidalis* lectins

ND, not determined.

Property	BDL1	BDL2	BDL3
Molecular mass (kDa)			
SDS/PAGE (reduced)	36	23	
SDS/PAGE (non-reduced)	158	67	ND
Gel filtration (native)	390	140	
pI	6.0	5.2	ND
Monosaccharide inhibitors	Man, ManNAc, Glc, lactose, Gal, GalNAc	GlcNAc, lactose, Gal, GalNAc	Lactose, Gal, GalNAc
Erythrocyte specificity	Rabbit > mouse > horse	Mouse > rabbit > guinea-pig	Mouse > human AB > rabbit
HA efficiency: specific activity (units/mg of protein)	8322000	13200	20900
Bivalent cation requirement	$Ca^{2+} > Mg^{2+}$	Ca^{2+}	Ca^{2+}
Glycoprotein	Yes	Yes	ND
Deglycosylation by Endo H	Yes	No	ND
Blood-cell-associated	Yes	Yes	ND

that bind to different sugars can also be categorized. For example, group I are L-fucose-specific, group II are *N*-acetyl-D-galactosamine/galactose-specific, group III are mannose/glucose-specific and group IV are idose, gulose, L-glucose and L-xylose-specific. As yet, no lectin has been discovered that binds group-IV sugars. The *B. discoidalis* lectins, BDL1, BDL2 and BDL3, all fall into different groups. BDL1, which reacts with glucose and mannose, belongs to group III, whereas BDL3, which binds to *N*-acetyl-D-galactosamine/galactose, would be classified as group II. BDL2 could be classified as a separate group, an *N*-acetylglucosamine-specific group, as defined by Golstein and Poretz [32]. It is extremely interesting, and no doubt of significance, that the *B. discoidalis* multiple lectins are not just confined to one carbohydrate-binding group. Thus these lectins have a basic difference from the multiple lectins found in the other invertebrates, such as *Halocynthia roretzi*, in which a galactose-specific lectin and an *N*-acetylgalactosamine-specific lectin are present [33,34]. Both of these lectins would be in group II according to Mäkelä's classification. Similarly, DCL-I and DCL-II lectins from the tunicate, *Didemnum candidum* are both galactose-binding lectins [35,36]. Therefore the diverse specificities of the *B. discoidalis* multiple lectins may be related to the physiological functions of these molecules based on lectin–carbohydrate interactions. In addition, as far as we are aware, BDL1 is the first mannose-binding lectin found in an invertebrate.

The biological importance of multiple lectins is not well understood. Vasta [37], however, proposed that the occurrence of multiple lectins in the same animal would expand diversity in the recognition capabilities of that individual. Undoubtedly, diversification in physicochemical properties may possibly reflect diversification in functions. Indeed, in addition to the complex carbohydrate–protein interactions that arise from multiple lectins, there are possibly other binding sites, such as those that interact with the components of the prophenoloxidase cascade, since it has been shown that both BDL1 and BDL2 can significantly enhance activation of prophenoloxidase in haemocyte lysates from *B. discoidalis* [38]. Furthermore, the enhancement is dose-dependent and the activity cannot be abrogated by sugar inhibition [38]. This is similar to the case of the plant lectin, concanavalin A, which has an additional auxin-binding site [39], and to a mannose-binding lectin which activates the classical pathway of complement in a vertebrate [40,41]. This latter example is extremely relevant as it illustrates clearly the activation of an enzyme cascade, the complement system, involved early on in immune reactivity in vertebrates. The prophenoloxidase system in arthropods is also an enzyme cascade probably involved in immune recognition and is, like the complement system, activated by a mannose-binding lectin, BDL1 [38]. Additional work is clearly required to investigate the role of the *Blaberus* lectins in immune reactivity of this insect.

This work was supported by a studentship from the Sino–British Friendship Scholarship Scheme, the Royal Society and the Science and Engineering Research Council (grant nos. GR/F 17421 and GR/G 40224). The technical assistance of Mr. I. Tew and Mr. L. O'Brien is also gratefully acknowledged.

Received 9 December 1992/3 March 1993; accepted 17 March 1993

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