A study of novel lectins and their involvement in the activation of the prophenoloxidase system in *Blaberus discoidalis*

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Endogenous and exogenous lectins have been found to activate the prophenoloxidase (proPO) system of the cockroach, *Blaberus discoidalis*, to the same extent as laminarin, a previously known microbial activator of proPO. The lectins can also further enhance this laminarin activation of the proPO system. Non-lectin proteins did not display any activation properties. The time course of proPO activation was studied after reconstitution of the reaction system using purified lectins, a trypsin-like enzyme, a trypsin inhibitor and partially purified lectin-binding proteins from the cockroach haemolymph. Lectin activation of the proPO

system is probably not mediated by the lectin sugar-binding sites, as specific inhibitory sugars failed to abrogate the enhanced effect. The results suggest that alternative binding site(s) on the lectins may be involved in the proPO activation process. Evidence also suggests that several different lectins are involved in the regulation of the proPO system through separate receptors or binding molecules on the haemocytes, and that they exert their effects early in the sequence of events leading to conversion of proPO into its active form, possibly via regulation of serine proteases and protease inhibitors.

INTRODUCTION

In invertebrates, important immune events in eliminating non-self include encapsulation and nodule formation [1,2], which sequester foreign matter too numerous or too large to be dealt with by phagocytosis alone. Usually, melanization of the resulting capsules occurs [1], in which the key enzyme in the oxidation of phenols, ultimately yielding melanin, is phenoloxidase (PO). This enzyme exists as a zymogen, prophenoloxidase (proPO), in most invertebrates studied [2,3], and is activated by a specific enzyme [4–6] which itself is activated at the end of a chain of serine proteases [3,7–9]. The whole system is initiated by micro-organisms and their surface components, including lipopolysaccharides, peptidoglycan and β -1,3-glucans [10–13], probably by interaction with specific receptors in the plasma or at the haemocyte surface [14–16]. The proPO system is further thought to have a role in immunity, as parasitoids can inhibit host PO [17,18] and an activated proPO system is able to enhance both phagocytosis *in vitro* [19,20] and encapsulation *in vivo* [21]. Furthermore, PO inhibitors such as phenylthiourea can also inhibit the encapsulation response [22,23].

However, the proPO system is not the sole mediator of the arthropod defences. Other factors are released from haemocytes when the proPO system is activated [21,24], possibly including various lectins [8]. Lectins can agglutinate foreign matter and thus render it more suitable for uptake. Studies in insects have also shown that lectins can mediate attachment between haemocytes and non-self particles [25], cause lysis [26], increase phagocytosis [27] of foreign particles and activate the coagulation system [28]. In vertebrate and plant systems also lectins are important non-self-recognition molecules and are involved in the attachment of bacteria to macrophages [29], activation of the complement system [30] and legume–*Rhizobium* associations [31].

The distribution of lectins and the proPO system between the cellular and humoral compartments in insects is a matter of debate but in some species, for instance *Leucophaea maderae*, *Galleria mellonella* and *Blaberus discoidalis*, they are associated with the haemocytes [32–36]. In the blood cells, biochemical interaction may therefore occur between these two non-self-recognition systems, although little is known of such events in insect immune defences. The presence of lectin-binding sites on melanocytes [37] and the activation of proPO by a lectin in plants [38] may indicate that such interactions occur widely. In the present study, we report, for the first time, a link between lectins and the proPO system of arthropods using the cockroach, *B. discoidalis*. Potent activation of the cockroach immune system can be achieved with commercially available laminarin, a β -1,3-glucan. The lectins used to activate the *B. discoidalis* proPO were purified from the whole serum of *B. discoidalis* and designated BDL1, BDL2 and GSL [36,39]. BDL1 is mannose/glucose-specific, BDL2 is inhibited by *N*-acetylgalactosamine and *N*-acetylglucosamine, and GSL is a β -1,3-glucan-specific lectin [14]. In addition, a comparison has been made of the activation of *B. discoidalis* proPO by plant lectins with similar specificities to the purified cockroach lectins.

MATERIALS AND METHODS

Insects and preparation of serum

West Indian leaf cockroaches, *B. discoidalis*, were reared at $34 \pm 2^\circ\text{C}$ and fed on dried cat food and water *ad libitum*. Adults of both sexes were used for all the experiments. Serum was prepared from groups of 50 cockroaches by first chilling the insects at -20°C for about 20 min. The insects were then punctured with a 21G needle through the arthrodial membrane of a posterior limb and haemolymph was withdrawn with a sterile Pasteur pipette in a cold-room at 4°C . The haemolymph

Abbreviations used: BDL, *Blaberus discoidalis* lectin; Con A, concanavalin A; GSL, β -1,3-glucan-specific lectin; HLS, haemocyte lysate supernatant; PO, phenoloxidase; proPO, prophenoloxidase; TBS/Ca²⁺, Tris-buffered saline plus calcium; Tbd, trypsin-like enzyme from *B. discoidalis*; Tlbd, trypsin inhibitor from *B. discoidalis*.

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was centrifuged (10000 *g*; 4 °C; 5 min) and the serum divided into aliquots and stored at -20 °C.

Preparation of haemocyte lysate supernatant (HLS)

An HLS was prepared from four insects as a source of the proPO system, following the procedure of Leonard et al. [40]. The cockroaches were chilled at -20 °C for 20 min before being injected with and bled into 0.4 ml of ice-cold anticoagulant (10 mM EDTA, 100 mM glucose, 62 mM NaCl, 30 mM trisodium citrate, 26 mM citric acid, pH 4.6; 370 mOsm). The haemolymph was centrifuged at 1000 *g* for 5 min at 4 °C and plasma was removed before the cell pellet was washed with 10 mM sodium cacodylate/20 mM CaCl₂, pH 7 (cac buffer-20) containing 0.25 M sucrose. The cells were then homogenized in cac buffer-20, the cell debris was removed by centrifugation (1500 *g*; 30 min; 4 °C) and the supernatant designated as the HLS.

Haemagglutination assay

Assays for agglutinating activity and sugar inhibition were carried out with serially diluted *B. discoidalis* serum in 96-well V-bottomed microtitre plates (Nunc Life Technologies, Uxbridge, Middx., U.K.) as described by Chen et al. [36]. Lectin activity is expressed as the reciprocal of the highest dilution showing visual agglutination against rabbit erythrocytes, except for GSL which was tested against baker's yeast, *Saccharomyces cerevisiae*. The specific activities of the lectins used in the experiments are: BDL1, 3280000 units/mg; BDL2, 51200 units/mg; GSL, 8000 units/mg; concanavalin A (Con A), 164000 units/mg; and *Triticum vulgaris* lectin (WGA) 328000 units/mg. The following sugars were used as inhibitors as these have been shown previously to be specific for the *Blaberus* lectins [36]: D-(+)-galactose, lactose, D-(+)-glucose, D-(+)-mannose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine and laminarin (all from Sigma Chemical Co., Poole, Dorset, U.K.).

Isolation of lectins and other proteins

At least four lectins have previously been detected in the serum of *B. discoidalis* [39]; three of these, namely BDL1 (a D-glucose/D-mannose-specific lectin), BDL2 (an *N*-acetylglucosamine-specific lectin) and GSL (a β -1,3-glucan-specific lectin), were purified to homogeneity from 1200 adult insects as previously described [36,40]. These purified lectins were used in all the following experiments.

A trypsin-like enzyme (Tbd) and a trypsin inhibitor (Tibd) were purified from the serum of *B. discoidalis* by gel filtration, DEAE-cellulose chromatography and affinity chromatography. One millilitre of serum was first loaded on a Sepharose 6B (Pharmacia LKB, Uppsala, Sweden) column (1.5 cm $\times$ 80 cm) equilibrated and eluted with 0.01 M Tris/HCl, pH 8/10 mM Ca²⁺. Fractions with trypsin-like enzyme and inhibitor activities, detected by the assay described below (see under 'Assay for protease and protease inhibitor activity'), were pooled and then loaded separately on a DEAE-cellulose column (1.5 cm $\times$ 20 cm) equilibrated with 0.01 M Tris/HCl, pH 8/10 mM Ca²⁺. The bound proteins were eluted with a 0–250 mM NaCl gradient in the equilibration buffer. Finally, fractions with trypsin and trypsin inhibitor activity were passed through affinity columns made by immobilizing soyabean trypsin inhibitor (type I-S; Sigma) or trypsin [type XIII; Tos-Phe-CH₂Cl (TPCK)-treated; from bovine pancreas; Sigma] on CNBr-activated Sepharose 6B (8 ml each) according to the manufacturer's instructions. The Tbd and Tibd were eluted with 0.1 M glycine, pH 3, from trypsin

inhibitor-Sepharose 6B and trypsin-Sepharose 6B respectively after the columns had been washed with 1 M NaCl in TBS/Ca²⁺. Eluates of Tbd or Tibd were collected into 1 M TBS/Ca²⁺ to neutralize pH to 7. The purified Tbd and Tibd were checked for purity by SDS/PAGE or HPLC gel filtration on a Zorbax column, as described previously [36].

Receptors or binding molecules for two lectins, Con A and GSL, in the HLS were isolated by affinity chromatography using immobilized Con A (10 mg; Sigma) and purified GSL (5 mg) from *B. discoidalis* respectively on 3 ml of CNBr-activated Sepharose 6B (Sigma), according to the manufacturer's instructions. Briefly, two 5 ml aliquots of HLS (5 mg/ml) were each loaded on either Con A-Sepharose 6B or GSL-Sepharose 6B columns equilibrated with TBS/Ca²⁺, pH 8. The binding proteins were then eluted from each column with 0.1 M acetate buffer, pH 4, containing 0.5 M NaCl after thorough washing of the columns with TBS/Ca²⁺ plus 1 M NaCl. Eluates of Con A-binding or GSL-binding molecules were dialysed against TBS/Ca²⁺, pH 7, and the purity of the proteins was determined by electrophoresis.

Antibodies against the *Blaberus* lectins, BDL1, BDL2 and GSL, were raised in rabbits and purified separately on a Protein A column (1 ml) as described previously [36,39]. The purity of these antibodies was checked by running samples on SDS/polyacrylamide gels and by HPLC gel filtration on a Zorbax column. Antibody against Con A was purchased from Sigma.

Plant lectins from *Bandeiraea simplicifolia* (BS-I), *Lens culinaris* (Lch), *Phaseolus vulgaris* (PHA-L) and *Tetragonolobus purpureas* (TPA), as well as WGA and Con A, were purchased from Sigma and made up in cac buffer-20 at the concentration indicated in the Results section. For controls, other purified proteins, namely lysozyme, aldolase and thyroglobulin, were similarly obtained and tested in cac buffer-20 within the range 0.5–200 μ g/ml.

Electrophoresis and Western blotting

SDS/PAGE was carried out by the method of Laemmli [41]. Samples were adjusted to 1 μ g of protein and denatured with 1 % SDS containing 2 % 2-mercaptoethanol (BDH, Poole, Dorset, U.K.) by boiling for 3 min. Electrophoresis (Mini Protean II System, Bio-Rad Laboratories, Hemel Hempstead, Herts, U.K.) was carried out at a constant voltage of 200 V for 50 min. Gels were stained with silver nitrate using Bio-Rad staining kits. Western blotting was carried out by transblotting proteins from SDS/polyacrylamide gels on to nitrocellulose membranes (Amersham International, Slough, Bucks., U.K.), the protein bands being first probed by Con A, BDL1 or GSL, then monitored by anti-lectin antibodies as described previously [36].

Partial purification of proPO

HLS (1 ml; 3 mg/ml), prepared as described above but finally homogenized in 1 % Ampholyte (Pharmacia LKB) solution instead of cac buffer-20, was first loaded for 30 min on a preparative isoelectric focusing apparatus (MinipHor; Rainin Instrument Co.) to separate proPO from the putative serine proteases. The proPO fractions were then identified by measuring PO activity towards L-dopa using commercial trypsin (bovine pancreas; Sigma) as an activator (see below) and finally passed through a Blue Sepharose CL-6B column (Sigma; 20 cm $\times$ 1 cm) equilibrated at room temperature with cac buffer-20 and eluted in 10 ml of the same buffer [14]. The throughflow fractions collected contained proPO free of activating proteases, as detected by the method described below.

Assay for proPO activation by lectins

The assay was carried out by a modified method of Gregorio and Ratcliffe [42] at 30 °C or room temperature in 96-well flat-bottomed microtitre plates (Nunc). Incubation mixtures consisted of 25 μ l of HLS plus 25 μ l of lectin, non-lectin protein (lysozyme, aldolase or thyroglobulin), cac buffer-20 and/or laminarin (a β -1,3-glucan; 1 mg/ml in cac buffer-20). After 1 h at 30 °C, 25 μ l of L-dopa (3 mg/ml in distilled water) was added for a further 30 min to 10 h and the absorbance at 492 nm measured using an ELISA plate reader (MikroTEK Laborsysteme G.m.b.H.) at 30 min intervals during this time.

Assay for protease and protease inhibitor activity

This assay was carried out by the method of Söderhäll [43] with modifications. HLS (25 μ l) was incubated with or without 25 μ l of purified lectins and/or 25 μ l of laminarin for 1 h at 30 °C, as above, for the proPO assay. Then 25 μ l of chromogenic substrate (S-2160, S-2586, S-2484, S-2222, S-2444, S-2302, S-2238, S-2351, S-2288 or S-2266; Kabi Vitrum, Mölndal, Sweden) was added for 30 min at 30 °C. Results were recorded by measuring absorbance at 405 nm at intervals between 30 min and 10 h.

Protease inhibitor activity was assayed by the method of Ramesh et al. [44]. The assay mixture contained, in 1 ml, 0.1 M Tris/HCl, pH 8, 1 mM S-2444, 2 μ g of bovine trypsin, 10 mM Ca^{2+} and 50 μ l of protease inhibitor fractions from each purification step. A reaction mixture that lacked the protease inhibitor served as a control. After incubation of the assay tube at 30 °C for 30 min, the reaction was terminated by the addition of 0.5 ml of 50 % acetic acid and the liberated *p*-nitroaniline was measured spectrophotometrically at 405 nm. Percentage inhibition (%I) was calculated using the formula:

$$\%I = [1 - (A_1/A_0)] \times 100$$

where A_1 and A_0 represent absorbance in the presence and absence of the inhibitor respectively.

A microassay for protease inhibitor activity was performed in a microtitre plate. Bovine trypsin (25 μ l; 0.2 μ g; Sigma) was first incubated with 25 μ l of fractions containing protease inhibitors for 30 min at 30 °C and then 25 μ l of 2 mM chromogenic substrate, S-2444, was added for another 1 h. The reaction was stopped by adding 25 μ l of 50 % acetic acid. Results were obtained by measuring liberated *p*-nitroaniline (A_{405}). Controls were prepared without trypsin inhibitor.

Mechanism of proPO activation by lectins

In order to verify the mechanism of proPO activation by lectins, a series of experiments was undertaken as follows.

Lectin interaction with proPO

These experiments were carried out in a microtitre plate with 25 μ l of HLS or partially purified proPO fraction, free from serine proteases and inhibitors, treated with 25 μ l of lectin, including BDL1 (50 μ g/ml), BDL2 (100 μ g/ml), GSL (80 μ g/ml) and Con A (100 μ g/ml), with or without laminarin (1.0 mg/ml), for 30 min at 30 °C. Any PO activity was recorded after the addition of 25 μ l of L-dopa by measuring A_{492} on a plate reader. A control was prepared with isolated proPO plus bovine trypsin.

Activation of proPO by combined lectins

Assays with a mixture of lectins were carried out in a microtitre plate in a final volume of 100 μ l of cac buffer-20 containing 25 μ l of HLS, 25 μ l of laminarin and a combination of two

lectins (25 μ l of each). After incubation for 1 h at room temperature, 25 μ l of L-dopa was added and PO activity was measured 3 h later. The lectins used in the experiments were BDL1 (50 μ g/ml), BDL2 (100 μ g/ml), GSL (80 μ g/ml) and Con A (100 μ g/ml). Possible interactions between individual lectins were investigated by Western blotting using Con A and anti-(Con A) antibody as probes.

Study of activation sites of lectins

These experiments were performed with various lectin sugar inhibitors and specific antibodies to the *Blaberus* lectins, BDL1, BDL2 and GSL, or to the plant lectin, Con A, to block any activation sites on the lectins that might be involved in interaction with proPO. Individual sugar solutions (25 μ l; 0.2 M in cac buffer-20) or antibodies (25 μ l; 1 mg/ml) were preincubated with 25 μ l of lectin for 30 min, followed by incubation of the treated lectin with 25 μ l of HLS for another 1 h at room temperature. PO activity was measured by the addition of 25 μ l of L-dopa after incubation for a further 3 h at 30 °C. Controls lacked sugar inhibitors or antibodies.

Activation of proPO regulated by serine proteases and inhibitors

These experiments were performed in a final volume of 100 μ l consisting of 25 μ l of partially purified proPO or HLS with 25 μ l of purified *Blaberus* trypsin-like enzyme (Tbd; 50 μ g/ml) or 25 μ l of bovine pancreas trypsin (Tbpt; 50 μ g/ml) or purified *Blaberus* trypsin inhibitor (Tibd; 50 μ g/ml) or other trypsin inhibitors, e.g., soyabean trypsin inhibitor (type I-S), chicken egg ovomucoid (type III-O) and ovinhibitor (type IV-O) (all at 50 μ g/ml). PO activity was measured by the addition of 25 μ l of L-dopa after incubation for 3 h at 30 °C. Controls lacked either trypsin or trypsin inhibitor. Possible interactions between Tbd, Tibd and lectins were investigated by Western blotting using Con A, BDL1 or GSL and their antibodies as probes.

Receptor- or lectin-binding molecules interacting with proPO

HLS (25 μ l) was incubated for 1 h at 30 °C with 25 μ l of isolated lectin-binding protein, i.e. Con A-binding protein (80 μ g/ml) or GSL-binding protein (100 μ g/ml) or the following combinations: lectin-binding protein + 25 μ l of laminarin (1 mg/ml); lectin-binding protein + 25 μ l of GSL (80 μ g/ml) or Con A (100 μ g/ml); lectin-binding protein + 25 μ l of laminarin + 25 μ l of GSL or Con A. PO activity was recorded after the addition of 25 μ l of L-dopa (3 mg/ml) 3 h later using the proPO activity assay above.

Protein determination

Protein was measured by using the Coomassie Blue dye-binding method of Bradford [45] with BSA (Sigma) as a standard.

Statistics

Unless otherwise indicated, results are given as means $\pm$ S.E.M. ($n > 4$). An analysis of variance was performed on all data sets using statistical software (InStat; GraphPad Software, San Diego, CA, U.S.A.).

RESULTS

Activation and enhancement of proPO system by lectins

Table 1 shows that endogenous lectins by themselves can activate proPO in HLS to a level similar to that caused by laminarin

Table 1 Enhancement of *B. discoidalis* proPO activity in the presence of endogenous and exogenous lectins and proteins in both the presence and absence of laminarin

All lectins were used at concentrations to achieve maximal activation. HLS, 2.0 mg/ml protein; laminarin, 1.0 mg/ml; BDL1, 50 μ g/ml; BDL2, 100 μ g/ml; GSL, 80 μ g/ml; Con A, 35 μ g/ml; lysozyme, 0.5 μ g/ml; aldolase, 200 μ g/ml; thyroglobulin, 200 μ g/ml. The substrate used to detect PO activity was L-dopa and the appearance of dopachrome was measured at 492 nm. For details of the assay see the Materials and methods section. Data were analysed with a two-tailed *t* test. **P* < 0.05 relative to control 1; ***P* < 0.05 relative to control 2.

Incubation mixture	PO activity ($\Delta A_{492\text{nm}}$ /10 min)
HLS + cac buffer (control 1)	0.04 $\pm$ 0.01
HLS + laminarin (control 2)	0.11 $\pm$ 0.02*
HLS + BDL1	0.10 $\pm$ 0.02*
HLS + BDL2	0.10 $\pm$ 0.01*
HLS + GSL	0.12 $\pm$ 0.02*
HLS + Con A	0.09 $\pm$ 0.02*
HLS + BDL1 + laminarin	0.18 $\pm$ 0.03**
HLS + BDL2 + laminarin	0.23 $\pm$ 0.03**
HLS + GSL + laminarin	0.21 $\pm$ 0.03**
HLS + Con A + laminarin	0.16 $\pm$ 0.04**
HLS + lysozyme	0.03 $\pm$ 0.02
HLS + aldolase	0.03 $\pm$ 0.01
HLS + thyroglobulin	0.04 $\pm$ 0.01
HLS + lysozyme + laminarin	0.11 $\pm$ 0.02
HLS + aldolase + laminarin	0.10 $\pm$ 0.02
HLS + thyroglobulin + laminarin	0.11 $\pm$ 0.02

alone. Lectin and laminarin in combination caused significantly enhanced activity compared with their effects when used alone. The exogenous lectin, Con A, also had a similar action to BDL1, BDL2 and GSL, as did the other plant lectins, BS-I, LcH, PHA-L, TPA and WGA (results not shown). Activation of proPO was not detected using non-lectin proteins such as lysozyme, aldolase and thyroglobulin (Table 1).

The time course of proPO activation in HLS was also examined using different lectins at various concentrations in the presence of laminarin. Significant activation of proPO was observed with different concentrations of the lectins, BDL1, BDL2 and GSL, but Con A activated proPO significantly only at higher concentrations (Figure 1). In each case, there was a dose-related response which increased over the 10 h period of observation, except for the highest dose of Con A which reached saturation at 7 h, and GSL which lost dose-dependency after 7 h. The quantity of BDL1, BDL2, GSL and Con A required to attain maximum activation after 3 h in the presence of laminarin was 50, 16, 13 and 2.5% respectively of that required to attain maximum activation in its absence.

Mechanism of proPO system activation by lectins

Effects of lectins on partially purified proPO and on serine protease activity

In order to confirm the involvement of serine proteases in activation of proPO by lectins, a partially purified preparation of proPO was made by preparative isoelectric focusing followed by Blue Sepharose CL-6B chromatography. The first four through-flow fractions contained proPO free from activating proteases (Figure 2). In the absence of additional factors, no activation of the partially purified proPO occurred. The proPO was, however, activated by bovine pancreas trypsin (Tbpt) and when lectins

Figure 1 Time course of proPO activation by laminarin and purified *B. discoidalis* lectins

Lectins (25 μ l) at the concentrations (μ g/ml) indicated were incubated with 25 μ l of HLS (2 mg/ml) and 25 μ l of laminarin (1 mg/ml) for 1 h at room temperature; 25 μ l of L-dopa (3 mg/ml) was then added and incubation continued for another 30 min. PO activity was recorded at intervals of 30 min by using a microtitre plate reader. Controls lacked lectins. (a) BDL1; (b) BDL2; (c) GSL; (d) Con A. $\circ$, Laminarin + lectin; $\blacklozenge$, laminarin; $\blacktriangle$, background.

were added, only very limited activation of proPO occurred (results not shown).

The ability of the *Blaberus* lectins, BDL1, BDL2 and GSL, and Con A to enhance protease activity of the HLS was

PO activity																							
Protease activity	s-2160														+/	+	++	+/					
	s-2586						+	+/			+	++	++	++	++	++	++	++	+	+/			
	s-2484																+++	+/					
	s-2222									+/	-	+	+	++	++	++	++	+	+	+/			
	s-2444						+								+	+	+	+/					
	s-2302						+	+/					+	+	+	+/							
	s-2238										+/	-	++	++	++	++	+/						
	s-2351										+/	-	+	+			+	+/					
	s-2288											+	++	++	++	++	++	+/					
	s-2266							+/	-	+	++	++	++	++	++	++	++	++	+	+/			
Fraction no.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		

Figure 2 Partial purification of proPO and separation of serine proteases from *B. discoidalis* haemolymph

Fractions (25 μ l) from a partially purified proPO preparation made by preparative isoelectric focusing and gel filtration on a Blue Sepharose CL-6B column (see the Materials and methods section) were incubated with 0.2 μ g of bovine trypsin in 100 μ l of cac buffer-20 at room temperature for 1 h. The colour (PO activity) was developed for up to 10 h in the dark by addition of 25 μ l of L-dopa (3 mg/ml). The reaction is shown as PO activity. Serine protease activities were also detected by 25 μ l of isoelectric focusing fractions incubated with 25 μ l of various chromogenic substrates (all at 2 mM) in a volume of 100 μ l of cac buffer-20. The liberated *p*-nitroaniline was measured at 405 nm 3 h later and the activity was converted into active index: —, negative ($A_{405} < 0.05$); +, moderate. ($A_{405} = 0.05-0.1$); ++, strong ($A_{405} > 0.1$).

Table 2 Effect of lectins and/or laminarin on protease activity in *B. discoidalis* HLS

Protease activity was determined by measuring the change in A_{405} caused by cleavage of the chromogenic substrate S-2444 (< Glu-Gly-Arg-*p*-nitroaniline HCl). For details of the assay see the Materials and methods section. Data were analysed with the two-tailed *t* test where * denotes results significantly ($P < 0.05$) higher than control. HLS, 2 mg/ml; laminarin, 1 mg/ml.

Incubation mixture	Protease activity ($\Delta A_{405}/10$ min)
HLS + cac buffer (control)	0.11 $\pm$ 0.01
HLS + laminarin	0.17 $\pm$ 0.02*
HLS + BDL1**	0.18 $\pm$ 0.03*
HLS + Con A†	0.18 $\pm$ 0.02*
HLS + BDL1‡ + laminarin	0.20 $\pm$ 0.02*
HLS + Con A§ + laminarin	0.19 $\pm$ 0.03*

** 7 mg/ml.

† 35 mg/ml.

‡ 3.5 mg/ml.

§ 0.9 mg/ml.

subsequently examined. The substrate employed was S-2444 (< Glu-Gly-Arg-*p*-nitroaniline HCl) as this was one of the chromogenic peptides shown to be hydrolysed by activated HLS in *Blaberus* (Figure 2). Protease activity was enhanced in a similar way to PO activity by both the lectins and laminarin, although there was no further increase with laminarin and lectins in combination (Table 2). Figure 2 also indicates that multiple serine proteases are present in the HLS as detected by different specific chromogenic substrates. The time course of protease activity in HLS is similar to that of proPO activation in terms of its time-dependency. Only proteases specific for substrates S-2222, S-2444, S-2238, S-2288, S-2586 and S-2266 showed a response to the laminarin and lectin activation (Figure 3).

Figure 3 Time course of serine protease activation by laminarin and lectins

HLS (25 μ l; 5 mg/ml) was incubated with 25 μ l of laminarin (1 mg/ml) + 25 μ l of GSL (80 μ g/ml) for 1 h at 30 °C, then 25 μ l of chromogenic substrate (2 mM) was added for another 30 min. Results were recorded by measuring A_{405} for up to 2 h. Only six of ten substrates used are shown here, i.e. those hydrolysed by the proteases in response to laminarin + GSL activation. The other substrates tested that were hydrolysed were the same as in Figure 2. ▲, Background; ○, S-2266; ○, S-2586; □, S-2288; ◇, S-2238; ▽, S-2444; △, S-2222.

Effect of lectins in combination on proPO activation

Lectins tested in combination, but concentrated to avoid a dilution effect, showed different effects on proPO activation. Most lectin combinations, for example Con A + BDL1, Con A + GSL and BDL1 + GSL, inhibited laminarin activation of proPO compared with each lectin alone. For example, after 3 h incubation with HLS and laminarin, Con A + GSL gave an absorbance of 0.114 ± 0.01 , whereas Con A and GSL alone gave readings of 0.085 ± 0.02 and 0.360 ± 0.02 respectively. In contrast, BDL2 combined with any of the above lectins had a different effect, with the same enhancement of proPO activation as that obtained with the single lectin (results not shown). Western blotting shows that Con A interacts with BDL1 and GSL, but not with BDL2 (Figure 4).

Study of activation sites of lectins

Antibodies against the lectins BDL1, BDL2 and GSL in PO assays with either HLS or HLS + lectins destroyed their proPO activating and agglutinating functions for the specific lectins only (results not shown). Sugar inhibition tests in which the lectins BDL1, BDL2 and Con A or the HLS were preincubated for 1 h with an equal volume of sugar(s) specific for the test lectin showed no inhibitory effect of the PO-enhancing activity of these lectins, although preincubating the lectin with its specific sugar inhibited its agglutinating properties [36]. However, with GSL, methyl α -D-mannopyranoside and D-mannose significantly inhibited proPO activation but not agglutination by GSL; other sugars also reduced PO activity but not significantly (Figure 5).

Purification of a trypsin-like enzyme and a trypsin inhibitor and their roles in proPO activation

As serine proteases and protease inhibitors have been shown to be important in the proPO cascade [46], an attempt was made to

Figure 4 Western-blot analysis of interactions between Con A and insect lectins

SDS/PAGE was carried out as described in the Materials and methods section; serum (10 μ g) and purified BDL1, BDL2, GSL and human C1q (1 μ g each sample) were run under reducing conditions and blotted on a nitrocellulose filter. The blot was first incubated with 20 μ g/ml Con A in the blocking solution containing 1% BSA and 1% goat serum for 1 h and then the lectin interaction was monitored by anti-(Con A) antibody. Lane a, serum; lane b, BDL1; lane c, BDL2; lane d, GSL; lane e, human C1q. Arrows indicate positions of BDL1, GSL and C1q subunits A, B and C.

Figure 5 Effects of sugars on the activation of proPO by GSL

Individual sugar solutions (25 μ l; 0.2 M) were first preincubated with 25 μ l of GSL (80 μ g/ml) for 30 min and then mixed with 25 μ l of HLS (2 mg/ml) for 1 h at room temperature. PO activity was recorded as A_{492} by the addition of 25 μ l of L-dopa (3 mg/ml) after incubation for a further 3 h at 30 °C. The control contained no added sugar inhibitors, and the absorbance by laminarin plus HLS alone is also shown. MMP, Methyl α -D-mannopyranoside. * P < 0.05; *** P < 0.001.

purify any serine proteases and protease inhibitors present in the haemolymph. With the use of gel filtration on Sepharose 6B, anion exchange on DEAE-cellulose and affinity chromatography, a trypsin-like enzyme and a trypsin inhibitor were purified to homogeneity (Figure 6). On SDS/PAGE, both *Blaberus* trypsin (Tbd) and trypsin inhibitor (Tibd) appeared as single bands with molecular masses of 28 and 34 kDa respectively, under either reducing or non-reducing conditions (Figure 7). The purified Tbd and Tibd were tested for their effects on the activation of partially purified proPO and proPO from HLS. Figure 8 shows that Tbd alone significantly activated proPO from both sources,

Figure 6 Purification of a trypsin-like enzyme and a trypsin inhibitor from the haemolymph of *B. discoidalis*

(a) Serum (1 ml; 30 mg/ml) was applied to a Sepharose 6B column (90 cm $\times$ 1.5 cm) and eluted at a flow rate of 18 ml/h with TBS/ Ca^{2+} , pH 8. The fractions were collected and assayed for protease (■) and protease inhibitor (○) activities. ●, A_{280} (b) The active protease fractions from the above gel-filtration column were first pooled and loaded on a DEAE-cellulose column (15 cm $\times$ 1.0 cm); active proteases were then eluted with a gradient of 0–250 mM NaCl in TBS/ Ca^{2+} , pH 8 (not shown). Finally, the active protease fractions from the DEAE-cellulose column were loaded on a soyabean trypsin inhibitor–Sepharose 6B column (8 ml), and the bound *Blaberus* trypsin (Tbd) was eluted with 0.1 M glycine/HCl, pH 3.5, after extensive washing of the column with 1 M NaCl in 0.01 M Tris buffer plus 0.01 M Ca^{2+} . Eluates were collected in 1 M Tris buffer with 0.01 M Ca^{2+} . ■, Protease activity; ●, A_{280} . (c) Fractions with the inhibitor from the Sepharose 6B column (a) were pooled and loaded on a bovine trypsin–Sepharose 6B column (10 ml). The bound *Blaberus* trypsin inhibitor (Tibd) was eluted and collected as in (b) for Tibd. ○, Protease inhibitor activity; ●, A_{280} .

as also did bovine trypsin (Tbpt), but Tibd and a trypsin inhibitor (type III-O) almost completely inhibited proPO activation compared with that obtained by either laminarin plus GSL or laminarin plus Con A. The time course of proPO activation by Tbd was similar to those of proPO activation by laminarin plus lectins (results not shown). No direct interaction

Figure 7 SDS/PAGE of the trypsin-like enzyme and the trypsin inhibitor from *B. discoidalis*

HLS (5 mg/ml) and purified *Blaberus* trypsin (Tbd; 2 µg) and trypsin inhibitor (TIbd; 2 µg) were denatured with 1% SDS and 2% 2-mercaptoethanol by boiling for 3 min before loading on an SDS/12% polyacrylamide gel. The gel was stained with silver nitrate. Lane a, HLS; lane b, Tbd; lane c, TIbd.

was found between Tbd or TIbd and the lectins, Con A, BDL1 and GSL, when checked by Western blotting.

Purification of lectin-binding molecules from HLS and their roles in proPO activation

Preliminary experiments showed that lectins do not act directly on proPO but through earlier stages of the proPO cascade via activation of serine proteases (see Table 2). An attempt was made to identify the haemocyte-associated molecules that interact with the lectins and laminarin. Using affinity columns made by immobilizing the lectins, GSL and Con A, on Sepharose 6B, two major proteins with molecular masses of about 18 and 28 kDa were identified in HLS that bound to GSL and Con A respectively (Figure 9). These proteins have consequently been defined as GSL-binding and Con A-binding proteins. The role of these proteins in activation of proPO in HLS was investigated. As shown in Figure 10, although both the GSL-binding protein and Con A-binding protein alone activated proPO, they exhibited different effects when in combination with lectins or laminarin. Thus the GSL-binding protein inhibited proPO activation with either the GSL lectin alone or with the lectin plus laminarin (Figure 10a), whereas, in contrast, the Con A-binding protein significantly enhanced laminarin, Con A, and Con A lectin plus laminarin activation of proPO (Figure 10b).

DISCUSSION

The purified serum lectins from *B. discoidalis*, BDL1, BDL2 and GSL, which are specific for D-glucose/D-mannose, N-acetylglucosamine/N-acetylgalactosamine and β -1,3-glucans respectively, are all able to activate haemolymph proPO. They can also enhance laminarin activation of proPO. This activity is not restricted to endogenous lectins as all plant lectins tested also activated proPO similarly. Activation of proPO, however, appears to be lectin-specific as all non-lectin proteins examined failed to produce this effect. Proteins capable of enhancing β -1,3-glucan activation of proPO have previously been purified [14–16] but no agglutinating activity has yet been ascribed to them. Other lectins from both insect and crayfish that are also specific for microbial wall components have been purified but not yet tested for proPO activation [35,47].

Figure 8 Effects of the trypsin-like enzyme and the trypsin inhibitor on proPO activation

HLS (25 µl) was incubated with equal volumes of laminarin (La), *Blaberus* trypsin (Tbd, 50 µg/ml), *Blaberus* trypsin inhibitor (TIbd, 50 µg/ml), bovine trypsin (Tbpt, 50 µg/ml), trypsin inhibitor (type III-O; TI-O; 50 µg/ml), and the lectins GSL (80 µg/ml) (a) or Con A (100 µg/ml) (b), in various combinations as indicated, in a 100 µl volume of cac buffer-20 for 1 h, and then 25 µl of L-dopa (3 mg/ml) in distilled water was added and incubation continued for another 3 h. PO activity was measured at 492 nm.

ProPO activation by lectins does not appear to rely on specific sugar-binding events, as this process could not be inhibited by the specific sugars for the lectins, BDL1, BDL2 and Con A. This implies that the PO-enhancing effect is independent of the sugar-binding domains of the lectins. Söderhäll et al. [38] have also demonstrated that a purified lectin from *Daucus carota* activates carrot proPO in the presence of CaCl₂ and, again, this effect is not abrogated by the presence of specific inhibitory sugars. Exogenous lectins, however, had no effect in their system. Söderhäll and co-workers also found that the agglutinating activity of carrot cell lectin was partially inhibited at a Ca²⁺ concentration at which PO activation by the lectin was maximal, implying two separate functions on the same molecule. In mammals also a mannose-binding protein found to activate both the classical and alternative pathways of the complement system has additional binding sites that interact with other components, such as the C1r₂C1s₂ complex, and thus may be able to mimic the role of C1q [48,49]. Furthermore, *Limulus* factor C, a lipopolysaccharide-binding protein that initiates coagulation, is also

Figure 9 Isolation of lectin-binding molecules from the HLS of *B. discoidalis*

HLS (5 μ l; 5 mg/ml) was loaded on to either GSL-Sepharose 6B (a) or Con A-Sepharose 6B (b) columns equilibrated with TBS/Ca²⁺, pH 8. The binding proteins were eluted with 0.1 M acetate buffer, pH 4, containing 0.5 M NaCl, after thorough washing of the columns with 1 M NaCl in the starting buffer. (c) The isolated lectin-binding proteins were subjected to SDS/PAGE on a 10% gel; and gel was then stained with silver nitrate. Lane a, GSL-binding proteins; lane b, Con A-binding proteins.

known to have several domains that include separate lectin-like and serine protease-like areas [32]. In actual fact, our latest results suggest that BDL1 contains a second active site which is interactive with other molecules (C. Chen, R. P. Newton and N. A. Ratcliffe, unpublished work). The inhibitory effects of methyl α -D-mannopyranoside and D-mannose on GSL activation of proPO may not be due to blockage of the sugar-binding sites, as these two sugars were clearly shown not to be inhibitors of agglutinating activities with this molecule [39]. Further experiments on the effects of combinations of lectins on the enhancement of proPO activation with laminarin showed significant reductions in PO activity. These effects are known to be due to lectin interactions with each other, resulting in neutralization of their activation potential, as both BDL1 and

Figure 10 Effects of lectin-binding molecules from HLS on proPO activation

HLS (25 μ l) was incubated with 25 μ l of isolated GSL-binding protein (GBP) (a) or Con A-binding protein (CBP) (b) plus 25 μ l of laminarin (La), GSL or Con A for 1 h at 30 °C. Then 25 μ l of L-dopa was added and PO activity recorded at 492 nm after a further 3 h incubation.

GSL are glycoproteins containing polysaccharides with a high concentration of mannose which may serve as binding sites for mannose-specific lectins such as Con A or BDL1 [39]. It is likely that, *in vivo*, multiple lectins interact with each other and form a network to exert a complex effect as, for example, on proPO activation as well as in other lectin functions [39].

Lectins can also enhance protease activity in the *B. discoidalis* HLS under the same conditions in which the proPO system is activated, although no further activity was observed with the combination of laminarin and lectins when tested with the S-2444 substrate. This may be explained by the presence of multiple proteases and protease inhibitors and the broad specificity of the substrate used. Furthermore, in a partially purified proPO preparation, in which the activating protease(s) had been removed, no lectin-mediated activation or enhancement of laminarin activation of the proPO system occurred. These results indicate that the activity of the lectin is not associated directly with the proPO itself but probably through the regulation of serine proteases and protease inhibitors, as suggested in this study by the effects of purified *Blaberus* trypsin-like enzyme (Tbd) and trypsin inhibitor (TIbd) and PO activities, or at some other initial stage in the enzyme cascade, although no direct interactions between Tbd, TIbd and purified lectins were found.

The early stage may be at the haemocyte membrane itself with the lectins interacting with an associated glycoprotein, as it has already been shown that there are *N*-acetylglucosamine, galactose and mannose residues on *Blaberus* haemocytes capable of acting as receptors for endogenous and exogenous lectins [50]. Confirmation of this concept was provided by isolation of the lectin-binding molecules from the HLS. Duvic and Söderhäll [51] have also demonstrated that a membrane receptor present in haemocytes of the crayfish, *Pacifastacus leniusculus*, specifically binds the complex of a β -1,3-glucan with the β -1,3-glucan-binding protein and thus is involved in various immune activities. As, in the present study, different binding proteins were found by using Con-A-Sepharose 6B or GSL-Sepharose 6B affinity chromatography, this might infer that the receptors for lectins and various activators, such as laminarin, exist as separate entities on the haemocytes involved in the proPO system. These results support the findings of Yoshida et al. [52] with *Bombyx mori* that different receptor entities for β -1,3-glucan and peptidoglycan exist within the proPO-activating system of insects.

Experiments with the lectin receptors of binding molecules also suggest that the involvement of lectin in activation of proPO may be complex and variable. For example, the GSL-binding molecule showed no proPO-enhancing effects when tested with the GSL lectin or in combination with GSL plus laminarin, but acted more like an inhibitor. Thus the GSL-binding protein interacted with GSL and subsequently interfered with its activation of the proPO system. In contrast, the Con A-binding protein in the HLS enhanced Con A and laminarin activation of proPO. This shows that the regulation of the proPO system is governed by the nature of both the lectin and the binding receptor on/in the haemocytes. The role of the lectin-binding proteins or receptors of this insect needs further investigation.

In conclusion, the most significant finding of the present paper is that the concept [53] of lectins and proPO acting separately as specific and non-specific immune molecules respectively is clearly incorrect. Evidence presented here suggests that they may not be mutually exclusive but part of a single interacting system possibly involving lectin-binding molecules at the haemocyte membrane. In this context, it may be significant that, in the mammalian system, there are lectin-binding sites on human melanocytes [37] and that an acute-phase protein that is a mannose-specific lectin from rabbit and human serum has been found to activate complement [54,55], a cascade with which the proPO system has been considered to be analogous [38,56,57].

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REFERENCES

- Salt, G. (1970) Cambridge Monographs in Experimental Biology. Cambridge University Press London
- Ratcliffe, N. A., Rowley, A. F., Fitzgerald, S. W. and Rhodes, C. P. (1985) *Int. Rev. Cytol.* **9**, 183–350
- Söderhäll, K. and Smith, V. J. (1986) in *Hemocytic and Humoral Immunity in Arthropods* (Gupta, A. P., ed.), pp. 251–285, John Wiley and Sons, New York
- Dohke, K. (1973) *Arch. Biochem. Biophys.* **157**, 203–209
- Aspan, A., Sturtevant, J., Smith, V. J. and Söderhäll, K. (1990) *Insect Biochem.* **20**, 709–718.
- Aspan, A. and Söderhäll, K. (1991) *Insect Biochem.* **21**, 363–374
- Yoshida, H. and Ashida, M. (1986) *Insect Biochem.* **16**, 539–545
- Söderhäll, K. and Smith, V. J. (1986) in *Immunity in Invertebrates* (Brehélin, M., ed.), pp. 208–220, Springer-Verlag, Berlin
- Saul, S. J. and Sugumaran, M. (1987) *Arch. Insect Biochem. Physiol.* **5**, 1–11
- Söderhäll, K. and Unestam, T. (1979) *Can. J. Microbiol.* **25**, 406–414
- Ashida, M., Ishizaki, Y. and Iwahana, H. (1983) *Biochem. Biophys. Res. Commun.* **113**, 562–568
- Pye, A. E. (1974) *Nature (London)* **251**, 610–613
- Brookman, J. L., Ratcliffe, N. A. and Rowley, A. F. (1989) *Insect Biochem.* **19**, 47–57
- Söderhäll, K., Rögner, W., Söderhäll, I., Newton, R. P. and Ratcliffe, N. A. (1988) *Insect Biochem.* **18**, 323–330
- Ochiai, M. and Ashida, M. (1988) *J. Biol. Chem.* **263**, 12056–12062
- Duvic, B. and Söderhäll, K. (1990) *J. Biol. Chem.* **265**, 9327–9332
- Stoltz, D. B. and Cook, D. I. (1983) *Experimentia* **39**, 1022–1024
- Beckage, N. E., Metcalf, J. S., Nesbit, D. J., Schleiffer, K. W., Zetlan, S. R. and de Buron, I. (1990) *Insect Biochem.* **20**, 285–294
- Söderhäll, K., Smith, V. J. and Johansson, M. W. (1986) *Cell Tissue Res.* **245**, 43–49
- Brookman, J. L., Ratcliffe, N. A. and Rowley, A. F. (1988) *J. Insect Physiol.* **34**, 337–345
- Söderhäll, K., Vey, A. and Ramstedt, M. (1984) *Dev. Comp. Immunol.* **8**, 23–29
- Nappi, A. J. (1973) *Parasitology* **66**, 23–32
- Brewer, F. D. and Vinson, S. B. (1971) *J. Invertebr. Pathol.* **18**, 287–289
- Dularay, B. and Lackie, A. M. (1985) *Insect Biochem.* **15**, 827–834
- Richards, E. H. and Ratcliffe, N. A. (1990) *Dev. Comp. Immunol.* **14**, 269–282
- Komano, H. and Natori, S. (1985) *Dev. Comp. Immunol.* **9**, 31–40
- Pendland, J. C., Heath, M. A. and Boucias, D. G. (1988) *J. Insect Physiol.* **34**, 533–540
- Minnick, M. F., Rupp, R. A. and Spence, K. D. (1986) *Biochem. Biophys. Res. Commun.* **137**, 729–735
- Tobias, P. S., Soldau, K. and Ulevitch, R. J. (1986) *J. Exp. Med.* **164**, 777–793
- Ikedo, K., Sannoh, T., Kawasaki, T. and Yamashina, I. (1987) *J. Biol. Chem.* **262**, 7451–7454
- Dazzo, R. B. and Truchet, G. L. (1983) *J. Membr. Biol.* **73**, 1–16
- Muta, T., Miyata, T., Misumi, Y. et al. (1991) *J. Biol. Chem.* **266**, 6554–6561
- Schmit, A. R., Rowley, A. F. and Ratcliffe, N. A. (1977) *J. Invertebr. Pathol.* **29**, 232–234
- Amirante, G. A. and Mazzalai, F. G. (1978) *Dev. Comp. Immunol.* **2**, 735–740
- Matha, V., Grubhoffer, L., Weyda, F. and Hermanova, L. (1990) *Cytobios* **64**, 35–42
- Chen, C., Ratcliffe, N. A. and Rowley, A. F. (1993) *Biochem. J.* **294**, 181–190
- Nau, P. and Schaumburglever, G. (1990) *Arch. Dermatol. Res.* **282**, 520–525
- Söderhäll, I., Bergenstråhle, A. and Söderhäll, K. (1990) *Plant Physiol.* **93**, 657–661
- Chen, C. (1993) Ph.D. thesis, University of Wales
- Leonard, C., Söderhäll, K. and Ratcliffe, N. A. (1985) *Insect Biochem.* **15**, 803–810
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Gregorio, E. and Ratcliffe, N. A. (1991) *Parasite Immunol.* **13**, 551–564
- Söderhäll, K. (1983) *Comp. Biochem. Physiol. B* **74**, 221–224
- Ramesh, N., Sugumaran, M. and Mole, J. E. (1988) *J. Biol. Chem.* **263**, 11523–11527
- Bradford, M. M. (1976) *Anal. Chem.* **72**, 248–254
- Sugumaran, M. and Kanost, M. R. (1993) in *Parasites and Pathogens of Insects*, Vol. 1. Parasites (Beckage, N. E., Thompson, S. N. and Federici, B. A., eds.), pp. 317–342, Academic Press, San Diego
- Jomori, T., Kubo, T. and Natori, S. (1990) *Eur. J. Biochem.* **190**, 201–206
- Ohta, M., Okada, M., Yamashina, I. and Kawasaki, T. (1990) *J. Biol. Chem.* **265**, 1980–1984
- Lu, J., Thiel, S., Wiedemann, H., Timpl, R. and Reid, K. B. (1990) *J. Immunol.* **144**, 2287–2294
- Richards, E. H., Ratcliffe, N. A. and Renwanz, L. (1989) *Cell Tissue Res.* **257**, 445–454
- Duvic, B. and Söderhäll, K. (1992) *Eur. J. Biochem.* **207**, 223–228
- Yoshida, H., Ochiai, M. and Ashida, M. (1986) *Biochem. Biophys. Res. Commun.* **141**, 1177–1184
- Drif, L. and Brehélin, M. (1989) *J. Insect Physiol.* **35**, 729–736
- Kawasaki, N., Kawasaki, T. and Yamashina, I. (1989) *J. Biochem. (Tokyo)* **106**, 483–489
- Ikedo, K., Sannoh, T., Kawasaki, N., Kawasaki, T. and Yamashina, I. (1987) *J. Biol. Chem.* **262**, 7451–7454
- Söderhäll, K. and Smith, V. J. (1984) in *Infection Processes of Fungi* (Aist, J. and Roberts, D. W., eds.), pp. 160–167, Rockefeller Foundation Study, Bellagio
- Ashida, M. and Söderhäll, K. (1984) *Comp. Biochem. Physiol. B* **77**, 21–26