Immulectin-2, a Lipopolysaccharide-Specific Lectin from an Insect, *Manduca sexta*, is Induced in Response to Gram-Negative Bacteria

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SUMMARY

A lipopolysaccharide-specific lectin, immulectin-2, was isolated from plasma of the tobacco hornworm, Manduca sexta. Immulectin-2 has specificity for xylose, glucose, lipopolysaccharide and mannan. A cDNA clone encoding immulectin-2 was isolated from an Escherichia coli-induced M. sexta larval fat body cDNA library. The cDNA is 1253 bp long, with an open reading frame of 981 bp, encoding a 327-residue polypeptide. Immulectin-2 is a member of the C-type lectin superfamily. It consists of two carbohydrate recognition domains, which is similar to the organization of M. sexta immulectin-1. Immulectin-2 was present at a constitutively low level in plasma of control larvae, and increased 3-4 fold after injection of Gram-negative bacteria or lipopolysaccharide. Immulectin-2 mRNA was detected in fat body of control larvae, and its level increased dramatically after injection of E. coli. The concentration of immulectin-2 in plasma did not change significantly after injection of Gram-positive bacteria or yeast, even though its mRNA level was increased by these treatments. Compared to immulectin-1, immulectin-2 has a more restricted specificity for binding to Gram-negative bacteria. Immulectin-2 at low physiological concentrations agglutinated E. coli in a calcium-dependent manner. It also bound to immobilized lipopolysaccharide from E. coli. Binding of immulectin-2 to lipopolysaccharide stimulated phenol oxidase activation in plasma. The properties of immulectin-2 are consistent with its function as a pattern recognition receptor for detection and defense against Gram-negative bacterial infection in M. sexta.
INTRODUCTION

Insects have a rapid and effective system for defense against microbial infections, which shares many characteristics with the innate immune system of vertebrates (1-3). Proteins that specifically bind to microbial components play an important role in nonself recognition and clearance of invading microbes. Such proteins are known as pattern recognition receptors, because they bind to certain molecular patterns present in the array of carbohydrate components on the surface of microorganisms (4). These microbial patterns include lipopolysaccharide (LPS) and peptidoglycan from bacterial cell walls, and β-1, 3-glucan from fungal cell walls.

Due to their ability to bind to terminal sugars on glycoproteins and glycolipids, lectins are primary candidates for pattern recognition receptors in innate immunity. Animal C-type lectins (calcium-dependent lectins) have been reported to be important in pathogen recognition and cellular interactions (5). Collectins, a subgroup of the C-type lectin superfamily, play a key role in the first line of defense against infection (6, 7). Collectins contain a carbohydrate recognition domain (CRD) connected to a collagen-like domain (8). The most extensively studied collectin is the serum mannose-binding protein (MBP). MBP can activate the complement system through a recently discovered pathway – the lectin pathway (9). Activation of the complement system by MBP is associated with C1r/C1s-like proteases (10, 11). MBP also functions directly as an opsonin to increase the efficiency of phagocytosis of bacteria (12, 13).

Recently, C-type lectins have been isolated from a few insect species. These C-type lectins function in insect innate immune system by participating in hemocyte nodule formation (14, 15), activating prophenol oxidase in hemolymph (16), and opsonization (17). Among these insect lectins is a group of C-type lectins that contain two tandem CRDs. Lectins of this new type include immulectin-1 from the tobacco hornworm, Manduca sexta (16), and LPS-binding lectins from the silkworm, Bombyx mori (15) and the fall webworm, Hyphantria cunea (18, 19). We report here an LPS-specific C-type lectin, immulectin-2 from M. sexta, which contains two CRDs and binds to Gram-negative bacteria and to LPS, and stimulates phenol oxidase activation in hemolymph. The synthesis of M. sexta immulectin-2 (IML-2) is induced in fat body after
injection of Gram-negative bacteria or LPS.
EXPERIMENTAL PROCEDURES

Insects and Plasma Samples – M. sexta eggs were initially obtained from Carolina Biological Supply, and larvae were reared using artificial diet as described by Dunn and Drake (20). Larvae in the second or third day of the fifth instar were injected with *Micrococcus lysodeikticus* (Sigma) (150 µg per larva), lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (Sigma) (20 µg per larva), formalin-killed *E. coli* strain XL1-blue, *Pseudomonas aeruginosa* ATCC27853, *Serratia marcescens* strain (obtained from James Urban, Division of Biology, Kansas State University) (all bacteria at 10⁸ cells per larva), or *Saccharomyces cerevisiae* (10⁷ cells per larva) suspended in 50 µl water, or with 50 µl saline (0.85% NaCl) as a control. Hemolymph was collected using a 27-gauge needle at several time intervals post-injection. Hemocytes were removed by centrifugation at 5,000 g for 5 min, and plasma samples were stored at -20°C.

Purification of Immulectin-2 (IML-2) and Production of Antibodies – Plasma (200 ml) collected 24 h post-injection of *E. coli* was dialyzed against 4 L of 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM CaCl₂ for 24 h at 4°C. After removal of a precipitate by centrifugation for 10 min at 10,000 g, dialyzed plasma was loaded onto an equilibrated mannan-agarose column (Sigma) (1.6 x 5.0 cm) at a flow rate of 0.5 ml/min. The column was washed with the starting Tris buffer containing 2 mM CaCl₂ until A₂₈₀ of the eluant was less then 0.01. The bound protein was then eluted with 5 mM EDTA in the starting Tris buffer lacking CaCl₂. Protein fractions were analyzed by SDS-PAGE. Purified IML-2 (600 µg) was used as antigen for the production of polyclonal rabbit antiserum (Cocalico Biologicals, Inc.).

Protein Analysis – Purified IML-2 (2.0 µg) was denatured in 20 mM phosphate buffer, pH 7.2, 1% SDS, 2% (v/v) 2-mercaptoethanol by heating at 100°C for 3 min. The denatured protein was then incubated with 1 U of N-glycosidase F or 1 mU of O-glycosidase (Boehringer Mannheim) in 50 µl of 50 mM phosphate buffer, pH 7.2, 0.1% SDS, 0.5% (v/v) Nonidet P-40 and 0.5% (v/v) 2-mercaptoethanol for 24 h at 37°C. Treated and untreated protein samples were then analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue.
The mass of IML-2 purified from plasma was determined by matrix-assisted laser desorption ionization (MALDI) mass spectrometry at Keck facility, Yale University. IML-2 was also subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane (0.2 µm, Bio-Rad). The protein bands were visualized by staining with Amido Black. IML-2 bands were cut out, and amino-terminal sequences were determined by automated Edman degradation using an Applied Biosystems Model 473A Protein Sequencing System at the Biotechnology Core Facility of Kansas State University.

Analysis of IML-2 by HPLC Gel Filtration Chromatography – Twenty-five µg of purified IML-2 was analyzed by gel filtration HPLC (Bio-Sil SEC 250, 300 mm x 7.8 mm, Bio-Rad). The column was eluted with 50 mM sodium phosphate, pH 6.8, 150 mM NaCl at 1.0 ml/min. Protein peaks detected by A_280 were collected and dried (SpeedVac, Savant). Samples of proteins from the peaks were analyzed by immunoblotting, using rabbit anti-IML-2 antiserum. A molecular mass standard curve was generated by plotting log of the mass of a set of standards (thyroglobulin, 670 kDa; IgG, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; and vitamin B12, 1.35 kDa, Bio-Rad) against retention time.

Isolation of cDNA Clones – An M. sexta larval fat body cDNA library (24 h after injection of E. coli) in λ ZAPII (Stratagene) was screened using antiserum to IML-2 by the method of Ausubel et al. (21). Positive clones were purified to homogeneity and subcloned by in vivo excision of pBluescript phagemids. The nucleotide sequences of the cDNA clones were determined from double-stranded plasmid DNA templates by the dideoxynucleotide method using an automated DNA Sequencer (Iowa State University, DNA sequencing facility). The cDNAs were sequenced using subcloned restriction fragments and oligonucleotide primers derived from previously determined sequences.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) – Because the longest cDNA clone (pM13) was incomplete at the 5’ end, rapid amplification of cDNA ends (RACE) was used to obtain the 5’ end sequence of IML-2. RACE reactions were performed as described by Frohman (22). Briefly, 5 µg of total RNA from E. coli-induced larval fat body was reverse
transcribed to cDNAs by M-MuLV reverse transcriptase using oligo (dT) as primer. The cDNAs were then tailed with dCTP using terminal transferase.

For nested polymerase chain reactions (PCRs), the reaction was set up as follows: 5 µl of 10 x PCR buffer A (Fisher), 3 µl of 10 mM dNTPs, 3 µl of 25 mM MgCl₂, 25 pmole of each primer, 1 µl of cDNA pools or first round PCR product, 1 unit of Taq DNA polymerase (Fisher), and water to bring the total volume to 50 µl. For the first round PCR, the tailed cDNA pools were used as template, and a sequence specific primer PMR5 (5’ GAT GGA TCC CAT TTG TGA GGT 3’) and Anchor primer (5’ CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG 3’) (Gibco BRL) were used. For the second round PCR, the first round PCR product was used as template, and a sequence specific primer PMR7 (5’ AAC GGA TCC CTC AAG ATG GCA 3’) and Universal Amplification Primer (UAP) (5’ CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT AC 3’) (Gibco BRL) were used as primers. PCR reactions were performed as follows: denaturing for 30 seconds at 94°C, annealing for 40 seconds at 50°C (first round) or 58°C (second round) and extension for 40 seconds at 72°C for a total of 40 cycles.

A PCR product of 280 bp was obtained from nested PCR reactions and purified by low melting point agarose gel electrophoresis. The purified PCR product was cloned into plasmid vector pGEMR-T (Promega). Plasmid DNA containing the insert was prepared, and the insert was sequenced as described above.

**Computer Analysis of Sequence Data** – Sequence analysis was performed using the GCG Sequence Analysis Software Package version 7.3.1 (23) and IBI Pustell programs.

**Immunoblot Analysis** – Plasma samples collected from the fifth instar larvae injected with bacteria, yeast or saline as described above, were analyzed by SDS-PAGE by the method of Laemmli (24), and IML-2 was identified by immunoblotting. Four µl cell-free plasma from each larva at different time intervals of post-injection was separated on 12% SDS-PAGE, and proteins were transferred to nitrocellulose membrane. The membrane was blocked with 5% dry skim milk and then incubated with rabbit antiserum to IML-2 (1:2000 dilution). Antibody
binding was visualized by a color reaction catalyzed by alkaline phosphatase conjugated to goat anti-rabbit IgG (Bio-Rad). The IML-2 band intensities were measured using Kodak Digital Science 1D gel analysis software, and the amount of IML-2 in plasma was estimated using known concentrations of purified IML-2 as standards. For each group, plasma from four individual larvae was analyzed.

**Northern Analysis** – Total RNA from fat body or hemocytes collected 24 h after injection of saline (0.85% NaCl), *M. lysodeikticus*, *S. cerevisiae*, or *E. coli* (strain XL1 blue) was prepared by the method described previously (16). RNA samples (20 µg) were separated by agarose gel electrophoresis in the presence of formaldehyde (25), transferred to a positively-charged nylon membrane (GeneScreen Plus, Dupont), and probed with IML-2 cDNA or *M. sexta* ribosomal protein S3 (rpS3) cDNA (26) labeled by random primer extension with α-32P-dCTP.

**Hemagglutination Assay** Trypsinized and glutaraldehyde treated erythrocytes from human bloods group B or O were purchased from Sigma. All other erythrocytes were glutaraldehyde treated and were also from Sigma. These erythrocytes were trypsinized as described by Haq et al. (27), and suspended in Tris-buffered saline (TBS) (25 mM Tris-HCl, 137 mM NaCl and 3 mM KCl, pH 7.0) as a 10% suspension.

For hemagglutination assay, erythrocytes were prepared as a 2% suspension in TBS. IML-2 was serially diluted twofold with 25 µl of TBS containing 5 mM CaCl2 in wells of a microtiter V-shape plate. Then 25 µl of 2% erythrocytes were added and mixed well. The plate was incubated for 1 h at 37°C. Agglutinated erythrocytes formed a diffuse mat, while unagglutinated erythrocytes formed a clear red dot at the bottom of the well.

To test carbohydrate specificity for IML-2, the hemagglutination assay was conducted by mixing IML-2 (1.0 µg/ml in TBS containing 5 mM CaCl2) with serial dilutions of various carbohydrates at room temperature for 30 min. Horse erythrocytes (2%) were then added and the plate was incubated at 37°C for 1 h before scoring for agglutination.

**Agglutination of Bacteria and Yeast by IML-2** – Fluorescein isothiocyanate (FITC)-labeled *Staphylococcus aureus*, *E. coli*, and *S. cerevisiae* (Molecular Probes) were suspended in
TBS and used for the agglutination assay. IML-2 purified from plasma of E. coli-injected larvae was used in the assay performed as described previously (16). To test whether the agglutination of E. coli requires calcium, FITC-labeled E. coli was incubated with IML-2 (final concentration of 10 µg/ml) in TBS containing 1 mM EDTA and the assay was performed as described in Yu et al. (16).

**Binding of IML-2 to Immobilized LPS** – Wells of a flat bottom 96-well assay plate (Costar, Fisher) were coated with LPS from E. coli 0111:B4 (Sigma) by a method modified from Tobias et al. (28) and Koizumi et al. (14). Briefly, LPS was suspended at 40 µg/ml in water and sonicated for 3 × 15 s, and 50 µl (2 µg) of LPS suspension was added to each well. The plate was then incubated at room temperature until the water evaporated completely. The plates were heated at 60°C for 30 min, and then blocked with 200 µl/well of 1 mg/ml BSA in Tris buffer (TB) (50 mM Tris-HCl, 50 mM NaCl, pH 8.0) for 2 h at 37°C. The plates were then rinsed four times with 200 µl/well of TB. IML-2 diluted with TB containing 5 mM CaCl₂ and 0.1 mg/ml BSA was added at 50 µl/well, and binding was allowed to occur for 3 h at room temperature. The plates were then rinsed four times with 200 µl/well of TB, and rabbit anti-IML-2 antiserum (diluted 1000-fold with TB containing 0.1 mg/ml BSA) was added at 100 µl/well. After incubation for 2 h at 37°C, the wells were rinsed four times with 200 µl/well of TB. Alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad) diluted 3000-fold with TB containing 0.1 mg/ml BSA was added at 100 µl/well and incubated for 2 h at 37°C. The wells were washed again as described above, and 50 µl/well of 1 mg/ml p-nitro-phenyl phosphate (prepared in 10 mM diethanolamine, 0.5 mM MgCl₂) was added and incubated at room temperature for 20 min. Absorbance at 405 nm of each well was determined using a microtiter plate reader (Bio-Tek Instrument, Inc.).

**Activation of Plasma Prophenol Oxidase** – LPS from E. coli 0111:B4 (Sigma), mannan (Sigma), or IML-2 purified from plasma of E. coli-injected larvae, all in TBS, were added separately or in combination to 40 µl of cell free hemolymph collected from naive fifth instar day 2 larvae. Total volume was adjusted to 50 µl with TBS, and the mixture was incubated at room
temperature. At various times after mixing, 5 µl aliquots were removed and added to 0.7 ml of 2 mM dopamine in 50 mM sodium phosphate, pH 6.5 for measurement of phenol oxidase activity (16). Absorbance at 470 nm was measured over 6 min.
RESULTS

Purification and Properties of Immulectin-2 – When plasma collected from *M. sexta* larvae 24 h after injection of *E. coli* was passed through a mannan-agarose column, a protein which bound to mannan could be eluted with EDTA. This protein, designated immulectin-2 (IML-2), was more than 90% pure after this one step affinity purification. Approximately 1.5 mg of purified IML-2 was recovered from 100 ml of plasma. IML-2 also bound to immobilized glucose (data not shown). Purified IML-2 appeared as two closely spaced bands at approximately 37 kDa (IML-2a) and 38.5 kDa (IML-2b) in analysis by SDS-PAGE (Fig. 1, lane 2). The masses of IML-2a and IML-2b determined by mass spectrometry (MALDI-TOF) were 35,381 and 36,240 Da, respectively, indicating that SDS-PAGE analysis slightly overestimates the mass of the IML-2 isoforms. The amino-terminal sequences of the proteins recovered from these two bands were determined by Edman degradation. Twenty-four residues were determined from IML-2a, while 10 residues were obtained from IML-2b. Both amino-terminal sequences were identical and perfectly matched the deduced amino acid sequence from an IML-2 cDNA clone described below (Fig. 2).

When purified IML-2 was analyzed by gel filtration HPLC, a major peak eluted at 10.87 min, which was just after the ovalbumin standard (10.24 min) (Fig. 3). A small peak eluting at 9.78 min and a peak eluting at the column’s void volume (6.50 min) were also observed. Fractions from all three peaks were identified as IML-2 by immunoblotting (data not shown). Using a standard curve generated by plotting log molecular mass of a set of standard proteins versus retention times, the calculated masses of the three IML-2 peaks are: 39.5 kDa (10.87 min), 90 kDa (9.78 min), and > 670 kDa (6.50 min). These results suggest that IML-2 is present mainly in monomeric form in solution, with lower amounts of dimers and oligomers. Fractions in a broad trough between the dimer and oligomer peaks also contained IML-2, indicating that IML-2 oligomers with different numbers of subunits may exist in solution.

cDNA Cloning and Sequence Analysis – Purified IML-2 from plasma was used as an antigen for producing a rabbit polyclonal antiserum. We used this antiserum as a probe to screen
an *E. coli*-induced *M. sexta* larval fat body cDNA library. From 1.2 x 10^5 lambda phage screened, we obtained two positive clones, pM13 and pM18, which encoded IML-2. These two clones were identical in sequence. Clone pM13 had an insert of 1183 bp, which contained the complete 3’ end with a poly(A) tail, but it was not complete at the 5’ end. To obtain the full length sequence, 5’ rapid amplification of cDNA ends (RACE) was performed, and a fragment extending 70 bp farther at the 5’ end was cloned. The full length sequence of IML-2 cDNA was 1253 bp long, with an open reading frame of 981 bp, encoding a 327-residue polypeptide (Fig. 2).

The deduced amino acid sequence of IML-2 contains a 19-residue secretion signal peptide, confirmed by Edman degradation of the mature protein (Fig. 2). The calculated mass of the mature protein is 35,203 Da, which is less than the masses of IML-2a and IML-2b determined by mass spectrometry (35,381 and 36,240 Da). A potential N-linked glycosylation site is present in the IML-2 sequence at Asn-253. Treatment of IML-2 with N-glycosidase F, which cleaves at N-linked glycosylation sites, resulted in two slightly more separated protein bands, with apparent molecular masses of 36 and 34 kDa (Fig. 1, lane 3). This result suggests that both IML-2 isoforms are N-glycosylated. Treatment of IML-2 with O-glycosidase did not change the mobility of the two protein bands (data not shown), indicating that IML-2 has no O-linked glycosylation.

Analysis of the amino acid sequence deduced from the cDNA indicated that IML-2 is a member of the C-type lectin superfamily. It contains two C-type carbohydrate recognition domains (CRDs), an amino-terminal domain, CRD1 (residues 1-136), and a carboxyl-terminal domain, CRD2 (residues 137-288). This feature of IML-2 is similar to another *M. sexta* lectin, immulectin (now designated IML-1) (16), and to lectins from other two insect species: LPS-binding proteins from the silkworm, *Bombyx mori* (15) and the fall webworm, *Hyphantria cunea* (18). Figure 4 shows an alignment of these four insect C-type lectins with tandem CRD structure. IML-2 shows 55% identity to *B. mori* LPS-binding protein (BmLBP) and 47% to Hdd15, but only 27% to *M. sexta* IML-1. In comparisons with vertebrate C-type lectins, CRD1
of IML-2 was most similar (26% identity) to rat macrophage asialoglycoprotein-binding protein (accession number P49301), while CRD2 was most similar (25% identity) to rat CD23 (accession number S34198), an IgE receptor.

*Induced Expression of IML-2 after Injection of Bacteria or Yeast* — IML-2 was present constitutively at a low level in plasma of naive larvae, with an average of 18.5 ± 8.5 µg/ml (SD) (measured from 36 larvae, with a range of 3.8 µg/ml to 36.5 µg/ml). After injection of *E. coli*, the concentration of both IML-2a and IML-2b in plasma consistently dropped within 2 h post-injection, but then increased to the original level at 6 h and continued to increase up to 48 h post-injection (Fig. 5). The level of IML-2a in hemolymph of naive larvae was significantly lower than that of IML-2b. The concentration of both IML-2a and IML-2b increased after injection of *E. coli*, but the ratio of their concentrations changed, with a greater relative increase in IML-2a (Fig. 5B). Northern analysis also showed that IML-2 mRNA was present at low level in fat body of control larvae (injected with saline), and was induced to much higher level after injection of *E. coli* (Fig. 6). IML-2 mRNA level was also increased after injection of Gram-positive bacteria (*M. lysodeikticus*) or yeast (*S. cerevisiae*). IML-2 mRNA was not detected in hemocytes of either control larvae or larvae injected with yeast or bacteria (Fig. 6). Injection of saline, *M. lysodeikticus* (Gram-positive), or *S. cerevisiae* (yeast) did not significantly change the concentration of IML-2 in plasma 24 h post-injection (Fig. 7). However, after injection of three different Gram-negative bacteria (*E. coli*, *P. aeruginosa* and *S. marcescens*) or LPS from *E. coli*, IML-2 concentration increased 3-4 fold in plasma 24 h post-injection.

*Ligand Binding Specificity of IML-2* — A hemagglutination assay was performed in order to test the ligand binding specificity of IML-2. IML-2 agglutinated horse erythrocytes most effectively, followed by human group A and B erythrocytes (Table 1). We used inhibition of agglutination of horse erythrocytes to identify carbohydrates that bind to IML-2. Xylose and glucose inhibited agglutination by IML-2 (Table 2). Among polysaccharides tested, LPS and mannan inhibited the agglutinating activity of IML-2.

*Agglutination of E. coli by IML-2* — To test whether IML-2 can bind to the surface of
microorganisms, we performed an agglutination assay using bacteria or yeast. When *E. coli* was incubated with IML-2 at a concentration higher than 0.5 µg/ml, large aggregates of bacteria were observed (Fig. 8A), and the size of the aggregates increased with greater IML-2 concentration. When EDTA was added to the mixture to chelate calcium, IML-2 did not aggregate *E. coli* even at 10 µg/ml. However, when calcium was added to overcome the effect of EDTA, large aggregates of *E. coli* were observed again (Fig. 8B). When either *S. aureus* (Gram-positive bacteria) or *S. cerevisiae* (yeast) were incubated with IML-2 even at 10 µg/ml, no obvious aggregates were observed (data not shown). These results indicate that IML-2 recognizes surface molecules of Gram-negative bacteria (*E. coli*), but not those of Gram-positive bacteria (*S. aureus*) or yeast (*S. cerevisiae*).

**Binding of IML-2 to LPS** - A candidate ligand for IML-2 is LPS, a polysaccharide specific to the surface of Gram-negative bacteria. To measure binding of IML-2 to LPS, we performed an ELISA assay. IML-2 at different concentrations was added to wells of a microtiter plate coated with LPS from *E. coli* strain 0111:B4. After an incubation period and washing, the bound IML-2 was detected using antiserum to IML-2. As increasing amounts of IML-2 were added, more IML-2 bound to immobilized LPS (Fig. 9). Binding of IML-2 to LPS was saturable, reaching a maximum at 15-20 µg/ml of IML-2. Non-linear regression analysis of the binding data showed that binding of IML-2 to LPS fits a two-site binding model, with a high affinity site (Kd1 = 0.3 µg/ml) and a lower affinity site (Kd2 = 7.6 µg/ml). This result is consistent with the two CRD organization of IML-2 (Fig. 2 and 4) and suggests that both CRDs can bind to LPS.

**Activation of Prophenol Oxidase** – Exposure of insect hemolymph to microbial components such as LPS, β-1, 3-glucan, or peptidoglycan results in activation of prophenol oxidase (29). To test whether binding of LPS by IML-2 may be involved in the prophenol oxidase activation system, purified IML-2, alone or in combination with LPS or mannan, was added to diluted *M. sexta* plasma, and phenol oxidase activity was measured after various incubation times (Fig. 10). Addition of IML-2 alone, or IML-2/mannan complex to plasma did
not activate prophenol oxidase. However, addition of IML-2 combined with LPS resulted in significant activation of prophenol oxidase within 10 min, and phenol oxidase activity continued to increase up to 50 min (Fig. 10). When LPS was added to plasma in the absence of IML-2 (replaced with bovine serum albumin as a control), phenol oxidase activity did not significantly increase until 45-50 min, and the activity remained lower than that of plasma incubated with IML-2/LPS complex. These results suggest a role for IML-2 as a pattern recognition receptor in activation of prophenol oxidase. Upon binding to LPS, IML-2 appears to trigger the protease cascade that activates phenol oxidase as in immune reaction to Gram-negative bacterial infection.
DISCUSSION

C-type lectins are important in the innate immune system of mammals, because they can recognize pathogens and directly function as effectors to neutralize or clear those pathogens (5). Recently, C-type lectins have been isolated from a few invertebrate species, which lack adaptive immunity and depend solely on innate immune responses (2, 3). Because insects and other invertebrates lack antibodies or clonal selection of lymphocytes, molecules such as lectins that can recognize infectious pathogens and stimulate protective responses are essential components of their immune systems (30). We have isolated from plasma of the tobacco hornworm, *M. sexta*, a C-type lectin, immulectin-2 (IML-2), which binds to Gram-negative bacteria and stimulates phenol oxidase activation. IML-2 contains two carbohydrate recognition domains (CRDs), which is similar to the organization of immulectin-1, another C-type lectin from *M. sexta* (16), and C-type lectins from two other lepidopteran insect species, *B. mori* (15) and *H. cunea* (18). *M. sexta* IML-2 is 55% identical in sequence to *B. mori* lipopolysaccharide-binding protein, 47% identical to *H. cunea* lectin, and only 27% identical to *M. sexta* IML-1. These insect C-type lectins, which all bind to bacterial LPS (14, 16, 19) and are made up of two CRDs, form a distinct group, differing from most animal C-type lectins that contain a single CRD. It is known that single C-type CRDs have weak affinity for carbohydrates and that multivalent interactions of mammalian C-type lectin oligomers are responsible for their specific binding at the cell surface of pathogens (5). The insect lectins with tandem CRDs may have increased binding affinity to carbohydrates on the surface of pathogens. Formation of oligomers of the lectins, as observed with IML-2, should further increase their strength of binding to polysaccharides on microbial surfaces.

Although all C-type lectin CRDs have sequence similarity, including conserved hydrophobic residues and four invariant cysteine residues, they can be divided into two types, a “short form” approximately 115 residues long, and a “long form” approximately 130 residues long, which includes two additional disulfide-bonded cysteine residues at the amino-terminus (31, 32). In the insect two-domain lectins, including IML-2, the amino-terminal CRD1 is the
short-form, while the carboxyl-terminal CRD2 is the long form, with two additional cysteines near its amino-terminus (Cys-144 and Cys-160 in IML-2) (Fig. 2 and 4).

The crystal structure of a CRD from rat mannose binding protein-A has demonstrated that the binding site for mannose involves one of two bound calcium ions, along with key amino acid residues that interact with the sugar by hydrogen bonding to the equatorial 3-OH and 4-OH groups of mannose (33). The natures of the amino acid residues that interact with the 3-OH group (Glu-185 and Asn 187 in mannose binding protein) are important in determining the binding specificity of C-type CRDs. Lectins such as mannose binding protein-A, which have Glu and Asn at these positions typically interact with mannose or glucose (or other sugars with similar adjacent equatorial hydroxyls). CRDs that have Gln and Asp at the same positions bind galactose (or similar sugars with an axial 3-OH and equatorial 4-OH) (31-33). In CRD2 of IML-2, these two residues are Glu-250 and Asn-252 (Fig. 4), which would be predicted to lead to binding of mannose or glucose. CRD1 of IML-2 has Glu-99 and Gly-101 at these two positions, which would also be consistent with a binding site for glucose or mannose. These predictions based on the sequence of IML-2 are consistent with its binding properties, since it bound to immobilized mannan and glucose. However, it did not bind to an immobilized mannose column, suggesting that its affinity for mannose as a monosaccharide is low, but that multiple interactions with mannan increase the strength of binding. Agglutination of horse erythrocytes by IML-2 was inhibited most efficiently by the monosaccharides xylose and glucose and was inhibited poorly or not at all by mannose and galactose. Xylose is a pentose, whose 2-, 3-, and 4-hydroxyl groups have the same configurations as those in glucose. Mannose differs from glucose only at the configuration of 2-OH, while galactose differs from glucose at the 4-OH. These results suggest that the 2-, 3-, and 4-hydroxyl groups of monosaccharides may participate in the binding to CRDs of IML-2. Determination of the three dimensional structure of IML-2 will be needed to provide direct evidence for carbohydrate-binding mechanisms by its CRDs.

IML-2 has a more restricted ligand binding specificity than IML-1. IML-2 agglutinated
only Gram-negative bacteria, whereas IML-1 agglutinated Gram-positive and Gram-negative bacteria, and yeast (16). The two critical residues for ligand binding specificity in CRD2 of both IML-1 and IML-2 are Glu and Asn (Fig. 4), with predicted specificity for glucose or mannose as discussed above. However, these critical residues differ in CRD1 of the two IMLs. In IML-1, they are Gln and Arg; while in IML-2, they are Glu and Gly. Perhaps this difference leads to a broader ligand binding specificity for IML-1.

Lipopolysaccharide from \textit{E. coli} was the most efficient inhibitor of erythrocyte agglutination by IML-2, and IML-2 caused aggregation of \textit{E. coli} but not Gram-positive bacteria or yeast. These results point toward LPS as a ligand for IML-2 and a function in recognition of Gram-negative bacteria. An assay using immobilized LPS demonstrated that IML-2 binds to LPS in a concentration-dependent manner. These results are similar to those found with insect proteins related to IML-2. \textit{B. mori} LPS-binding protein and the individual recombinant CRDs of \textit{H. cunea} lectin have been shown to bind to bacterial LPS (14, 19).

IML-2 was present in plasma as two isoforms with molecular masses of 35,381 (IML-2a) and 36,240 Da (IML-2b). These two isoforms had identical amino-terminal sequences. Both were larger than the calculated mass (35,203 Da) deduced from amino acid sequence of an IML-2 cDNA, suggesting that they are post-translationally modified, perhaps by glycosylation. In the deduced amino acid sequence from an IML-2 cDNA, there is a potential N-glycosylation site at Asn-253 (Fig. 2). Treatment of IML-2 with N-glycosidase F, which cleaves at N-linked glycosylation sites, resulted in the mobility shift of both IML-2 isoforms. One possible explanation for the relationship of the IML-2 isoforms is that IML-2a is derived from IML-2b by a truncation at the carboxyl-terminus. A second explanation is that IML-2a and IML-2b are very similar products of two related genes. We cannot rule out either possibility with the data available at this time.

IML-2 concentration in plasma decreased significantly within the first hours after injection of \textit{E. coli}, which may indicate that its binding to bacteria during the early stage of an infection removes a portion of the protein from circulation. This protein is replaced by the
induced synthesis of IML-2 by Gram-negative bacteria. Injection of bacteria or yeast into *M. sexta* larvae led to increased levels of IML-2 mRNA in fat body, the tissue responsible for synthesis of most insect plasma proteins. Similar induced gene expression has been observed with a number of other defense response genes in *M. sexta* and in other insect species (2, 3), including *M. sexta* IML-1 and C-type lectins from *B. mori* (15) and *H. cunea* (18). However, IML-2 concentration in plasma did not change significantly 24 h after injection of *M. lysodeikticus* or *S. cerevisiae*, whereas it increased 3-4 fold after injection of Gram-negative bacteria (*E. coli*, *P. aeruginosa*, *S. marcescens*) or bacterial LPS (Fig. 7). This surprising result is not easily explained, but it may indicate that translation of IML-2 is positively regulated by the presence of LPS. This aspect of the regulation of IML-2 gene expression requires further study.

C-type lectins have evolved to function in immune recognition in a wide range of animal species, including mammals and insects. Individual insect species probably contain several lectins, including C-type lectins of different specificities, for detecting a variety of pathogens. Recognition of microorganisms by these lectins may trigger different immune responses. Wilson *et al.* (34) concluded that multiple endogenous serum lectins in the cockroach, *Blaberus discoidalis*, play important roles in insect innate immunity. Multiple lectins have also been isolated from three other insect species, the American cockroach *Periplaneta americana* (35-38), the West Indian leaf cockroach *B. discoidalis* (34, 39, 40), and the silkworm *B. mori* (14, 15, 41-43). In *M. sexta*, we have identified a family of C-type lectins with different specificities. In addition to IML-1 (16) and IML-2, we have cloned cDNAs for two other C-type lectins with specificity for N-acetylgalactosamine/glucose (Yu, Scholz, Zhu and Kanost, unpublished results). These two C-type lectins, like IML-1 and IML-2, also have two-CRD domain structure. Such families of C-type and other lectins, along with other recognition receptors such as β-1,3-glucan recognition protein (39, 41, 44) and peptidoglycan recognition protein (42, 43, Zhu and Kanost, unpublished results) appear to have an important role in the innate immune system in insects. Upon binding to pathogens, these proteins trigger immune responses including phagocytosis, aggregation of pathogens which are trapped in hemocyte nodules, and activation of
M. sexta IML-2 appears to function as a pattern recognition protein specific for Gram-negative bacteria, through its interaction with LPS. IML-2 combined with LPS caused rapid activation of phenol oxidase in plasma. The much slower phenol oxidase activation observed when LPS alone was added to plasma may be due to the low concentration of IML-2 in hemolymph of naive insects. In M. sexta, as in other insects, phenol oxidase is activated by a specific proteolytic cleavage of its zymogen, prophenol oxidase, a defensive response that is amplified by a serine proteinase cascade reminiscent of the complement system (47). Active phenol oxidase can oxidize plasma phenols to diphenols and can convert diphenols to quinones. The reactive quinones may themselves be toxic to microorganisms, and they can function as precursors for melanotic encapsulation of pathogens and parasites for protection of the insect host. Future work is needed to learn how pattern recognition proteins such as IML-2 interact with other molecules to trigger the proteinase cascade that leads to phenol oxidase activation.
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The abbreviations used are: CRD, carbohydrate recognition domain; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HPLC, high performance liquid chromatography; IML, immulectin; LPS, lipopolysaccharide; MBP, mannose-binding protein; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction; SDS, sodium dodecyl sulfate; TB, Tris buffer; TBS, Tris-buffered saline.

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Figure Legends

**Fig. 1. Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis of IML-2.** IML-2 was purified from plasma of *E. coli*-injected larvae by affinity chromatography, using mannan-agarose column. Purified IML-2 (2 µg) was then treated with or without N-glycosidase F. The samples were analyzed by 12% SDS-PAGE and stained with Coomassie Brilliant Blue. A solid arrow points to isoform IML-2a, and an open arrow points to IML-2b. (lane 1: 1 µl plasma; lane 2: 2 µg purified IML-2; lane 3: 2 µg IML-2 treated with N-glycosidase F).

**Fig. 2. Nucleotide and Deduced Amino Acid Sequences of IML-2.** The deduced amino acid sequence is shown below the cDNA sequence. Amino acid residues in the mature protein are assigned positive numbers, and those in the signal peptide are assigned negative numbers. A potential N-linked glycosylation site is marked with a ‘u. Cys residues that define C-type lectin short-form CRDs are marked with t, while two extra cysteine residues in the long-form CRD2 are marked with h. The amino-terminal sequence of the mature IML-2 was determined by Edman degradation. The sequence obtained from IML-2b (higher mass isoform) is double-underlined, and the sequence from IML-2a (lower mass isoform) begins at the same position but extends further (underlined). In the cDNA sequence, the polyadenylation sequence AATAAA is double-underlined.

**Fig. 3. Analysis of IML-2 by HPLC Gel Filtration Chromatography.** Twenty-five µg of IML-2 purified from plasma of *E. coli*-injected larvae was analyzed by gel filtration HPLC as described in Experimental Procedures and detected by A280. Native protein standards (Bio-Rad) were also analyzed with the column to generate a molecular mass standard curve (data not shown). The first peak (6.50 min) eluted earlier than the largest standard (thyroglobulin, 670 kDa). The apparent masses calculated for the other IML-2 peaks were 90 kDa (dimer) and 39.5 kDa (monomer), respectively.
Fig. 4. Alignment of Insect C-type Lectins Consisting of two Carbohydrate Recognition Domains (CRDs). The mature polypeptide sequences of four insect lectins are aligned. Residues conserved in three of the four lectins are marked with an * above the alignment. Positions at which the same residue is conserved in all four sequences are marked with the symbol for that amino acid above the alignment. IML-2: *M. sexta* immulectin-2; BmLBP: *Bombyx mori* LPS-binding protein; Hdd15: putative lectin from the fall webworm, *Hyphantria cunea*; IML-1: *M. sexta* immulectin-1. The two amino acid residues that are most important for the determination of ligand binding specificity (33) are marked with ‘u below the alignment. Invariant cysteine residues that define CRDs are marked with 6, while the extra two cysteines in the long-form CRD2 are marked with h.

Fig. 5. Induction of IML-2 after Injection of *E. coli*. Fifth instar day 2 larvae were injected with $10^8$ formalin-killed *E. coli* cells. Hemolymph was collected at different times post-injection. (A): The Concentration of IML-2 Increased in Plasma after Injection of *E. coli*. Cell free plasma (4 µl) was separated by SDS-PAGE (12%), and IML-2 was detected by immunoblotting. The concentration of IML-2 was measured as described in Experimental Procedures. The bar represents the standard error of the mean (n=4). (B): A typical Western Blot Result from the Data Set Shown in (A). A solid arrow points to isoform IML-2a, and an open arrow points to IML-2b.

Fig. 6. Northern Hybridization of IML-2 mRNA. Samples of total RNA (20 µg) from hemocytes or fat body of larvae injected with saline (C), yeast (*S. cerevisiae*) (Y), *M. lysodeikticus* (M) or with *E. coli* (E) were subjected to 1% agarose gel electrophoresis in the presence of 2.2 M formaldehyde. The RNA was transferred to a positively-charged nylon membrane, and probed with $^{32}$P-labeled IML-2 cDNA (panel A) or ribosomal protein S3 (rpS3) cDNA (panel B). The arrow points to the 1.3 kb IML-2 mRNA present in fat body of control larvae and induced in fat body after injection of microorganisms.
**Fig. 7. IML-2 Concentration in Plasma Increased only after Injection of Gram-Negative Bacteria.** Fifth instar day 2 larvae were injected with saline, Gram-positive bacteria (*M. lysodeikticus*), yeast (*S. cerevisiae*), LPS from *E. coli*, or Gram-negative bacteria (*E. coli, P. aeruginosa, S. marcescens*). Hemolymph was then collected at different times post-injection. Plasma (4 µl) was separated by SDS-PAGE (12%), and IML-2 was detected by immunoblotting. The concentration of IML-2 was measured as described in Experimental Procedures. The bar represents the standard error of the mean (n=4).
Fig. 8. Agglutination of *E. coli* by IML-2. (A): *Agglutination of E. coli by IML-2 is Concentration-Dependent.* Different concentrations of IML-2 purified from plasma of *E. coli*-injected larvae were incubated with FITC-labeled *E. coli* (1.0 x 10⁹ cells/ml) in TBS containing 2 mM CaCl₂. After incubation for 45 min at room temperature, cells were examined by fluorescence microscopy. (B): *Agglutination of E. coli by IML-2 is Calcium-Dependent.* IML-2 (10 µg/ml) was incubated with FITC-labeled *E. coli* (1.0 x 10⁹ cells/ml) in TBS containing 1 mM EDTA for 45 min at room temperature. Five µl of cells were removed and observed by fluorescence microscopy (Panel labeled EDTA). CaCl₂ was then added to 10 mM final concentration to the rest of cells, and the mixture was incubated for another 45 minutes. Cells were then observed by fluorescence microscopy (Panel labeled Ca²⁺).

Fig. 9. Binding of IML-2 to Immobilized LPS. IML-2 purified from plasma was prepared at different concentrations in Tris buffer containing 5 mM CaCl₂ and 0.1 mg/ml BSA. IML-2 was then added to LPS-coated microtiter plates, and the binding assay was performed as described in Experimental Procedures. Each point represents the mean of four individual measurements ± S.E. The solid line represents a nonlinear regression calculation of a two-site binding curve (R² = 0.95), and the dotted line represents the curve calculated for one-site binding (R² = 0.93).
**Fig. 10. Activation of Phenol Oxidase by IML-2.** Forty µl of cell free hemolymph collected from a naive fifth instar day 2 larva was incubated with 1.0 µg of LPS and 5 µg of BSA, 1.0 µg of LPS and 1.0 µg of IML-2, 1.0 µg of mannan and 1.0 µg of IML-2, or 1.0 µg of IML-2 alone at room temperature. At time intervals, an aliquot of hemolymph was removed for assay of phenol oxidase activity. The points represent the mean of four individual measurements (except for the points at 45 and 50 min for LPS plus BSA, which were from two individual measurements). The bar represents the standard error of the mean.
Table 1. Hemagglutinating Activity of *M. sexta* Immulectin-2 on Erythrocytes

| Erythrocytes         | Minimum agglutinating concentration of IML-2 (µg/ml) |
|----------------------|-----------------------------------------------------|
| Horse                | 0.6                                                 |
| Human Group A        | 2.5                                                 |
| Human Group B        | 2.5                                                 |
| Human Group O        | 40                                                  |
| Sheep                | 20                                                  |
| Rabbit               | no activity at 40 µg/ml                             |
| Bovine               | no activity at 40 µg/ml                             |
Table 2. Effects of Saccharides on Hemagglutinating Activity of *M. sexta* Immulectin-2

| Saccharides                          | Minimal inhibitory concentration (mM) |
|--------------------------------------|--------------------------------------|
| D-Xylose                             | 1                                    |
| D-Glucose                            | 10                                   |
| D-Mannose                            | NI<sup>a</sup>                       |
| D-Galactose                          | 100                                  |
| N-Acetylglucosamine                  | NI<sup>a</sup>                       |
| Maltose                              | 100                                  |
| Sucrose                              | NI<sup>a</sup>                       |
| LPS                                  | 10 µg/ml                             |
| Mannan                               | 50 µg/ml                             |
| Laminarin (β-1, 3-glucan)            | NI<sup>b</sup>                       |

<sup>a</sup> not inhibited at 100 mM
<sup>b</sup> not inhibited at 1.0 mg/ml
Immulectin-2, a lipopolysaccharide-specific lectin from an insect, *manduca sexta*, is induced in response to gram-negative bacteria

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