Characterization of a Major Peritrophic Membrane Protein, Peritrophin-44, from the Larvae of Lucilia cuprina

dNA AND DEDUCED AMINO ACID SEQUENCES*

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The peritrophic membrane is a semi-permeable chitinous matrix lining the gut of most insects and is thought to have important roles in the maintenance of insect gut structure, facilitation of digestion, and protection from invasion by microorganisms and parasites. Proteins are integral components of this matrix, although the structures and functions of these proteins have not been characterized in any detail. The peritrophic membrane from the larvae of the fly Lucilia cuprina, the primary agent of cutaneous myiasis in sheep, was shown to contain six major integral peritrophic membrane proteins. Two of these proteins, a 44-kDa glycoprotein (peritrophin-44) and a 48-kDa protein (peritrophin-48) together represent >70% of the total mass of the integral peritrophic membrane proteins. Peritrophin-44 was purified and its complete amino acid sequence was determined by cloning and sequencing the DNA complementary to its mRNA. The deduced amino acid sequence codes for a protein of 356 amino acids containing an amino-terminal signal sequence followed by five similar but non-identical domains, each of approximately 70 amino acids and characterized by a specific register of 6 cysteines. One of these domains was also present in the noncatalytic regions of chitinases from Brugia malayi, Manduca sexta, and Chelonus. Peritrophin-44 has a uniform distribution throughout the larval peritrophic membrane. Reverse transcriptase-polymerase chain reaction detected the expression of peritrophin-44 in all three larval instars but only trace levels in adult L. cuprina. The protein binds specifically to tri-N-acetyl chitotriose and reacetylated chitosan in vitro. It is concluded that the multiple cysteine-rich domains in peritrophin-44 are responsible for binding to chitin, the major constituent of peritrophic membrane. Peritrophin-44 probably has roles in the maintenance of peritrophic membrane structure and in the determination of the porosity of the peritrophic membrane. This report represents the first characterization of an insect peritrophic membrane protein.

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The gut of most insects is lined with a membrane called the peritrophic membrane (PM) or peritrophic matrix, which is composed of chitin, proteoglycans, and protein (1). There are two types of insect PMs. Type 1 PM is synthesized from the digestive epithelial cells that line the gut of the insect, whereas type 2 PM is synthesized as a continuous tube from a specialized organ, the cardia (or proventriculus) situated in the anterior midgut region of the insect. The functions of this semi-permeable membrane are not entirely clear but are likely to be crucial for the protection of underlying digestive epithelial cells from bacterial damage and parasite invasion, as well as facilitating the digestive process due to the membrane's ability to partition digestive enzymes and ingested food between the endo- and ecto-PM spaces (1). The PM is the first barrier in insects encountered by a number of ingested viral and protozoal organisms (1–8), some of which use blood- or tissue-feeding insects as vectors for transmission to vertebrate hosts, where these organisms cause considerable morbidity and mortality. There is evidence that the PM of insect vectors can affect the survival and virulence of these pathogenic organisms (8–11).

Proteins bound to PM have been reported to account for a considerable proportion (35–55%) of the total mass of the PM in many insects (1). A subset of these proteins is the strongly bound or integral PM proteins, which can only be released from the PM by strong denaturants (12). These latter proteins, which we have named "peritrophins," are probably of central importance for the maintenance of the structural and protective biological functions of the PM and may be involved in determining the ultrafilter character of the membrane (13). Disruption of the functions of these integral PM proteins by chemical or immunological means could lead to novel mechanisms of insect control. Indeed, it is possible to vaccinate sheep against the tissue- and blood-feeding larvae of the fly Lucilia cuprina using isolated PM proteins (12, 14). It was demonstrated that antibodies to a PM protein, peritrophin-44, inhibited the free movement of small 6 nm gold particles from the gut lumen across the PM to the underlying digestive epithelial cells. The primary mechanism of action of the vaccine on the larvae was therefore postulated to involve the antibody-mediated blockage of the pores in the PM and the subsequent starvation of the larvae (14). The larvae of the related flies Chrysomya bezziana (Old World Screwworm) and Cochliomyia hominivorax (New World Screwworm) cause a similar cutane-
ous myiasis in a wider spectrum of vertebrate hosts including man (15). We have taken advantage of the ability of L. cuprina larvae to shed their type 2 PM continuously from the hind gut and our ability to culture these larvae in vitro to allow the isolation of sufficient quantities of PM for detailed study of one of the most abundant integral PM proteins. This is the first characterization of a PM protein from any insect.

EXPERIMENTAL PROCEDURES

Laboratory chemicals were analytical grade and were generally purchased from Sigma or Ajax Chemical Company (Auburn, Australia). Nucleotides and oligonucleotides were purchased from Gibco BRL (Gaithersburg, MD) and Perkin-Elmer Cetus (Norwalk, CT). Peptide amino acid sequences obtained from peritrophin-44 were used to design degenerate oligonucleotide primers (Pharmacia Gene Assembler Plus oligonucleotide synthesizer), which were used in conjunction with the polymerase chain reaction (PCR) (24) to amplify DNA coding for a fragment of peritrophin-44 from genomic DNA. Primers were designed from the amino acid sequences of two peptides from peritrophin-44 i.e. PM30022 and PM7204. The former peptide amino acid sequence represented a region near the amino terminus of peritrophin-44 purified from TBS containing 10 mM Tris-HCl, pH 7.5/150 mM NaCl (TBS)/0.1 mM PMSF (GST-peritrophin-44) and was used to design the 5'-CCGA/CTTCCGGA/TCAT/CC/CTTGGTG/ATGTTAG/ATGTTAG/CT/CCCTC-3'. The antisense primer (5'-fold redundancy) deviated from the internal peptide sequence (GMAYNYYG) was 5'-CCGA/ATC/CTCGGA/ATGTTAG/ATGTTAG/CTGCCAT/GA/T/CC/CC-3'. Bracketed nucleotides show alternatives at a specific position. Genomic DNA was prepared from L. cuprina first instar larvae (25). The production of cDNA and the construction of a λ gt11 cDNA library from firstinst larval of L. cuprina have been described elsewhere (23).

Production of a Genomic DNA Fragment Speciﬁc for Peritrophin-44—PCR was performed on 1 µg of genomic DNA in the presence of 100 µM of each of the four dNTPs, 100 µM each of oligonucleotide primer, 100 µM each of dNTPs/µl of each of oligonucleotide primer, 1 µl of perfect match enhancer (Stratagene; 1 unit)/0.5 unit of AmpliTaq (Promega). Ampliﬁcation took place over 40 cycles, each of which consisted of denaturation for 150 s at 94°C, annealing for 150 s at 59°C, and extension for 150 s at 72°C. Appropriate controls were also included (24). Samples of the PCR reaction were analyzed on 1% agarose gels. A unique band of 860 bp was produced when either genomic DNA or cDNA was used in the PCR reaction, suggesting the absence of introns in the genomic DNA. The L. cuprina PCR product derived from genomic DNA (500 ng) was isolated and sequenced on both strands using standard procedures (23, 26).

Construction of a cDNA Clone Coding for Peritrophin-44—The cDNA library in λ gt11 was screened with the 860-bp DNA fragment ampliﬁed by PCR (described above) from genomic DNA and labeled with digoxi-}

Sequence of a Major Insect Peritrophic Membrane Protein

by centrifugation (50,000 g) followed. Solutions: water; 100 mM Tris-HCl, pH 7.5/150 mM NaCl/5 mM urea/1 mM EDTA/1 mM benzamidine. A major peak containing proteins was extracted with 40 ml of TBS containing 6 M urea and 0.1 mM PMSF. The extracellular PM was then extracted with 40 ml of TBS containing 6 M urea and 0.1 mM PMSF. The extracellular PM was then extracted with 40 ml of TBS containing 6 M urea and 0.1 mM PMSF. The extracted proteins were concentrated and subjected to gel permeation chromatography (0.5 ml/min) on a column of Superose 12 (Pharmacia Biotech Inc.) equilibrated with 50 mM Tris-HCl, pH 7.5/6 M urea/1 mM EDTA/1 mM benzamidine. A major peak containing proteins (M , 400,000–48,000) was pooled, dialyzed against 20 mM Tris-HCl, pH 8.5/40 mM urea/1 mM benzamidine/1 mM EDTA, and subjected to anion exchange chromatography on a MonoQ column (Pharmacia) equilibrated with a 50 mM Tris-HCl, pH 7.5/6 M urea/1 mM EDTA/1 mM benzamidine. A major peak containing proteins (M , 40,000–80,000) was pooled, dialyzed against 20 mM Tris-HCl, pH 8.5/40 mM urea/1 mM benzamidine/1 mM EDTA, and subjected to anion exchange chromatography on a MonoQ column (Pharmacia) equilibrated with a 50 mM Tris-HCl, pH 7.5/6 M urea/1 mM EDTA/1 mM benzamidine. A major peak containing proteins (M , 40,000–80,000) was pooled, dialyzed against 20 mM Tris-HCl, pH 8.5/40 mM urea/1 mM benzamidine/1 mM EDTA, and subjected to anion exchange chromatography on a MonoQ column (Pharmacia) equilibrated with a 50 mM Tris-HCl, pH 7.5/6 M urea/1 mM EDTA/1 mM benzamidine. A major peak containing proteins (M , 40,000–80,000) was pooled, dialyzed against 20 mM Tris-HCl, pH 8.5/40 mM urea/1 mM benzamidine/1 mM EDTA, and subjected to anion exchange chromatography on a MonoQ column (Pharmacia) equilibrated with a 50 mM Tris-HCl, pH 7.5/6 M urea/1 mM EDTA/1 mM benzamidine. A major peak containing proteins (M , 40,000–80,000) was pooled, dialyzed against 20 mM Tris-HCl, pH 8.5/40 mM urea/1 mM benzamidine/1 mM EDTA, and subjected to anion exchange chromatography on a MonoQ column (Pharmacia) equilibrated with a 50 mM Tris-HCl, pH 7.5/6 M urea/1 mM EDTA/1 mM benzamidine. A major peak containing proteins (M , 40,000–80,000) was pooled, dialyzed against 20 mM Tris-HCl, pH 8.5/40 mM urea/1 mM benzamidine/1 mM EDTA, and subjected to anion exchange chromatography on a MonoQ column (Pharmacia) equilibrated with a 50 mM Tris-HCl, pH 7.5/6 M urea/1 mM EDTA/1 mM benzamidine. A major peak containing proteins (M , 40,000–80,000) was pooled, dialyzed against 20 mM Tris-HCl, pH 8.5/40 mM urea/1 mM benzamidine/1 mM EDTA, and subjected to anion exchange chromatography on a MonoQ column (Pharmacia) equilibrated with a 50 mM Tris-HCl, pH 7.5/6 M urea/1 mM EDTA/1 mM benzamidine. A major peak containing proteins (M , 40,000–80,000) was pooled, dialyzed against 20 mM Tris-HCl, pH 8.5/40 mM urea/1 mM benzamidine/1 mM EDTA, and subjected to anion exchange chromatography on a MonoQ column (Pharmacia) equilibrated with a 50 mM Tris-HCl, pH 7.5/6 M urea/1 mM EDTA/1 mM benzamidine. A major peak containing proteins (M , 40,000–80,000) was pooled, dialyzed against 20 mM Tris-HCl, pH 8.5/40 mM urea/1 mM benzamidine/1 mM EDTA, and subjected to anion exchange chromatography on a MonoQ column (Pharmacia) equilibrated with a 50 mM Tris-HCl, pH 7.5/6 M urea/1 mM EDTA/1 mM benzamidine. A major peak containing proteins (M , 40,000–80,000) was pooled, dialyzed against 20 mM Tris-HCl, pH 8.5/40 mM urea/1 mM benzamidine/1 mM EDTA, and subjected to anion exchange chromatography on a MonoQ column (Pharmacia) equilibrated with a 50 mM Tris-HCl, pH 7.5/6 M urea/1 mM EDTA/1 mM benzamidine. A major peak containing proteins (M , 40,000–80,000) was pooled, dialyzed against 20 mM Tris-HCl, pH 8.5/40 mM urea/1 mM benzamidine/1 mM EDTA, and subjected to anion exchange chromatography on a MonoQ column (Pharmacia) equilibrated with a 50 mM Tris-HCl, pH 7.5/6 M urea/1 mM EDTA/1 mM benzamidine. A major peak containing proteins (M , 40,000–80,000) was pooled, dialyzed against 20 mM Tris-HCl, pH 8.5/40 mM urea/1 mM benzamidine/1 mM EDTA, and subjected to anion exchange chromatography on a MonoQ column (Pharmacia) equilibrated with a 50 mM Tris-HCl, pH 7.5/6 M urea/1 mM EDTA/1 mM benzamidine. A major peak containing proteins (M , 40,000–80,000) was pooled, dialyzed against 20 mM Tris-HCl, pH 8.5/40 mM urea/1 mM benzamidine/1 mM EDTA, and subjected to anion exchange chromatography on a MonoQ column (Pharmacia) equilibrated with a 50 mM Tris-HCl, pH 7.5/6 M urea/1 mM EDTA/1 M

Staining of a cDNA Clone Coding for Peritrophin-44—The cDNA library in λ gt11 was screened with the 860-bp DNA fragment amplified by PCR (described above) from genomic DNA and labeled with digoxi

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CAAC-3' and the antisense primer was 5'-TAAAGTTTTGCTTGCTCGAAG-3'. The conditions used for PCR were identical to those described above. This combination of oligonucleotide primers specifically amplified a 1100-bp DNA product from tissues expressing mRNA coding for peritrophin-44. The total RNA prepared from each tissue was initially treated with DNase 1 to ensure the absence of any genomic DNA. This was achieved by using a PCR approach with primers specific for a L. cuprina gene (peritrophin-95) that contains an intron. The quantity of first strand cDNA used in PCR to examine the tissue-specific expression of peritrophin-44 directly reflected the total content of first strand cDNA derived from each tissue. For the examination of the developmental expression of peritrophin-44, the same quantity of first strand cDNA (0.9 μg) was used for all of the samples except eggs (3.2 ng). Direct comparison of the relative quantity of total cDNA in each of these developmental stage samples was also made using a PCR approach specific for cDNA coding for β-actin. In this instance the sense primer was 5'-CAGCATGTGGTACGCTCAAC-3' and the antisense primer was 5'-G(G/C)CCATCTC(C/T)TGCTCGAA(G/A)TC-3'. A 350-bp β-actin DNA fragment was amplified from each cDNA sample using the following conditions: one cycle of 2 min at 95°C, 1 min at 60°C, and 1 min at 72°C; 33 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C; and one cycle of 2 min at 95°C, 1 min at 60°C, and 1 min at 72°C. The β-actin expression level in eggs was markedly less than other tissues. The reasons for this are not clear. DNA fragments amplified from cardia and midgut using primers specific for peritrophin-44 were also cloned and sequenced to confirm that they coded for peritrophin-44.

Reacetylated Chitosan Affinity Chromatography—Crab shell chitosan was reacetylated (29) and homogenized in a Waring blender for 30 s, and 3 ml were poured into a column and equilibrated with TBS. Purified peritrophin-44 (10 μg) was dialyzed against the same buffer and applied to the reacetylated chitosan affinity column, which was washed (0.5 ml/min) with 10 ml of TBS and sequentially eluted with 10 ml of TBS/20 mM Glc, 10 ml TBS/20 mM methyl α-D-mannopyranoside, 10 ml TBS/20 mM GalNac, 10 ml TBS/20 mM GlcNac, and finally 10 ml of 50 mM sodium acetate, pH 3.0. The peritrophin-44 eluted in each wash was concentrated in a Centricon 10K cell (Amicon) to 200 g/ml (0.23 m) was measured in a Perkin-Elmer LS50B luminescence spectrometer at 25°C. All solutions were filtered (0.22-μm filter) before use. Excitation was at 280 nm, and both slit widths were 5 nm. Spectra were corrected for the small background signal from the appropriate solvent but remain uncorrected for the variation in detector efficiency with wavelength. The final concentration of peritrophin-44 was 10 μg/ml (0.23 μM). The effect of a range of tri-N-acetyl chitotriose concentrations (0–1 mM) on the intrinsic fluorescence intensity (emission at 347 nm; excitation at 280 nm) of peritrophin-44 (10 μg/ml) was measured and analyzed by the method of Scatchard (30). Briefly, the fractional change in intrinsic fluorescence (F) at a specific concentration of tri-N-acetyl chitotriose was defined as F = F - F, where F is the intensity in the absence of tri-N-acetyl-chitotriose, F is the intensity at specific tri-N-acetyl chitotriose concentration (s), and F is the total change in intensity at a saturating concentration of tri-N-acetyl chitotriose. The Scatchard analysis plotted r/s versus r. The slope of this line is a measure of the association binding constant for the interaction (i.e., Kd). The maximum correction for dilution effects in this experiment was obtained with 5% D-glucose (Glc), methyl α-D-mannopyranoside, and GalNac each at a concentration of 1 mM had no significant effect on the intrinsic fluorescence spectrum of peritrophin-44.

RESULTS AND DISCUSSION

Integral Peritrophic Membrane Proteins—The PM was progressively extracted with a series of buffers with increasing strengths of solubilization. The detergent (2% Zwittergent 3–14) and then 6 M urea extractions of the PM solubilized 9 and 22 mg protein/g dry weight of PM, respectively. A subsequent 6 M guanidine HCl extract of the PM solubilized a further 4 mg protein/g dry weight of PM. The total protein extracted was 35 mg protein/g dry weight of PM, which was substantially less than the yield (100 mg dry weight for the total protein content of PM reported for some other higher dipteran insects (1)). The reason for this difference is not clear but may reflect species-specific differences or indicate the presence of a substantial quantity of protein covalently attached to the PM that was not extracted by any of the buffers used in the present study. Another significant difference from previous studies was the use of PM obtained by larval culture rather than by direct dissection from larval or adult guts. The latter PM probably contains substantial quantities of contaminating proteins from the ingested proteins present in the gut of these insects. PM obtained by larval culture should be relatively free of these contaminating proteins because of the progress of this PM through the digestive environment of the gut before its egression from the larvae and subsequent collection.

The SDS-PAGE profile of the detergent extract showed the presence of two predominant proteins (55,000 and 40,000 Da) but in a background of a large number of lower abundant proteins (Fig. 1A). The sample also shows a strong general background staining that may indicate the presence of nonproteaceous material. The proteins in the detergent extract are probably representative of the proteins loosely bound to the PM or entrapped within the PM and are unlikely to be intimately involved with the maintenance of PM structure. Of primary interest in this study is the limited number of urea-extracted integral PM proteins (peritrophins) that are likely to be the major structural proteins of the PM as well as being intimately involved in the functions of the PM. Fig. 1b (lanes 2–4) shows a silver-stained SDS-PAGE profile of the proteins extracted by the 6 M urea buffer. This extract contained 6 major proteins of Mw = 95,000, 65,000, 48,000, 44,000, 35,000, and 33,000 (measured under reducing conditions). Two of the integral PM proteins of Mw = 44,000 and 48,000 (i.e. peritrophin-44 and peritrophin-48) were the most abundant in this extract, together representing >70% of the total urea-extractable protein (measured by densitometry). The size of the largest peritrophin (Mw = 95,000, peritrophin-95), unlike the other PM proteins, was variable ranging between 85,000 and 95,000 depending on the percentage of acrylamide used in the SDS-PAGE (result not shown). The PMs from larvae of Calliphora erythrocephala, Sarcophaga barbata, and Tricholopsia ni contain a similar repertoire of major integral PM proteins (6, 13), suggesting that these proteins may occur in many insect PMs. None of the major L. cuprina proteins was extracted by the nonurea-containing buffers initially used to wash the PM as determined by comparative SDS-PAGE and specific immunoblots. A number of lower abundance proteins in a regular ladder of higher molecular weights (i.e. Mw > 100,000) was also present in the 6 M urea extract from L. cuprina PM. This can be seen in the higher loadings of this sample in lanes 3 and 4 of Fig. 1b. The relative quantities of these minor high molecular weight bands were variable and depended on the age of the sample and the time taken for SDS-PAGE. One possibility is that these bands are due to nonspecific polypeptide associations mediated by reoxidation of cysteine residues during the actual running time of the electrophoresis, despite the sample being initially reduced.

Purification and Glycosylation of Peritrophin-44—The prevalence of peritrophin-44 and peritrophin-48 in the PM suggested that they play a major role in the structure and function of the PM. Antibodies to peritrophin-44 ingested by larvae
block the pores in the PM, indicating that this protein is probably involved in determining the porosity of PM (14). This possibility is consistent with the uniform distribution of peritrophin-44 in PM (see below) and the strong interaction between peritrophin-44 and the PM. Peritrophin-44, the most abundant integral PM protein, was purified from the 6M urea extract of detergent-washed PM by Superose 12 gel permeation chromatography and MonoQ anion exchange chromatography (Fig. 2a, lanes 2 and 5). The lack of focus of proteins such as peritrophin-44 on SDS-PAGE can indicate that the protein is glycosylated. To test this possibility, purified peritrophin-44 was incubated with N-glycosidase F or endoglycosidase H, which specifically removes N-linked oligosaccharides (31). Both enzymes significantly reduced the size of peritrophin-44 (Fig. 2a, lanes 2 and 5, respectively), thereby confirming the presence of oligosaccharides on this protein. N-Glycosidase F caused a shift in the size of peritrophin-44 to an $M_r = 37,500$, whereas endoglycosidase H caused a marginally smaller change to an $M_r = 40,000$. The former enzyme removes intact
dase H is specific for most high mannose oligosaccharides but is
unable to remove complex oligosaccharides.

To further characterize the glycosylation of peritrophin-44, it wa
subjected to SDS-PAGE, transferred to nitrocellulose by elec
tro-blotting, and then probed with a range of biotinylated lectins (Fig. 2b). Both biotinylated lentil lectin and biotinylated con
canavalin A reacted with peritrophin-44 (Fig. 2b, lanes 2 and 6, respectively). The binding of lentil lectin was completely inha
bited by preincubation of this lectin with 0.3 M methyl α-D-
mannopyranoside (lane 4), whereas the binding of concana
cavalin A was partially reduced by the same sugar (lane 8). Biotinylated wheat germ lectin, Phaseolus vulgaris E lectin, peanut agglutinin, Sophora japonica lectin, or Pisum sativum lectin did not bind to peritrophin-44 (result not shown). Incubation of peritrophin-44 with N-glycosidase F completely inhibited the binding of biotinylated lentil lectin (Fig. 2c). However, treatment of peritrophin-44 with endogly
cosidase H, while clearly removing some oligosaccharides as
evidenced by a mobility shift of the protein (Fig. 2a), had no effect
on the binding of biotinylated lentil lectin (Fig. 2c). One explana
tion for this result is that peritrophin-44 contains two classes of attached oligosaccharides. The first class consists of a high mannose oligosaccharide that is reactive with biotiny
lated lentil lectin and that is removed by either endoglycosi
dase H or N-glycosidase F. The second class is a complex oligosaccharide that also reacts with biotinylated lentil lectin but can only be removed by N-glycosidase F.

Resistance of Peritrophin-44 to Proteolysis—The PM is bathed in
relatively high concentrations of proteolytic enzymes particularly trypsins and chymotrypsins, which are present in the larval gut to digest ingested food (16, 23, 32–34). Proteins, such as peritrophin-44, which are an integral component of the PM should be exposed to these proteases and therefore subject
to proteolysis. However, immunoblots of 6 M urea extracts from
freshly dissected PM and PM obtained by larval culture using an
anti-serum to GST-peritrophin-44 identified a single 44-kDa immu
noreactive protein. Therefore, there was no evidence of signif
icant processing of peritrophin-44 as it slowly passed through
the gut while attached to the growing PM. The reason why peritrophin-44 was not subject to extensive proteolysis in this
harsh proteolytic environment was not immediately clear.
One possibility is that peritrophin-44 is inherently resistant to
digestive gut proteases. Indeed, peritrophin-44 was totally re
sistant to proteolysis by endoproteinase Lys-C over 2 h at 37 °C
(Fig. 3, lanes 2–6). However, after initial reduction with 5 mM
dithiothreitol, peritrophin-44 was readily digested by this pro
tease in less than 5 min (Fig. 3, lanes 7–11). This result demo
strates that disulfide bonds in peritrophin-44 play an impon
t role in protecting this protein from proteolysis. The
oligosaccharides attached to peritrophin-44 may also help pro
tect this protein from proteases. Another possible explana
ion for the lack of digestion of peritrophin-44 in vivo could involve
its lack of ready accessibility to digestive gut proteases either
because of a protective envelope of chitin within the PM or
because peritrophin-44 is present on the ecto- rather than the
endo-PM surface. The former surface may not be as readily
exposed to digestive proteases.

Immunolocalization of Peritrophin-44 on Peritrophic Mem
brane—Fig. 4 shows the immunolocalization of peritrophin-44 on
PM obtained from freshly dissected larvae. There is strong
and uniform immunogold labeling of the entire PM (Fig. 4b).
The corresponding control using prevaccination serum showed
very little gold labeling of the PM (Fig. 4a). The resistance of
peritrophin-44 to proteolysis in vivo, therefore, was not due to

Fig. 3. Resistance of peritrophin-44 to proteolysis. Time course
of digestion of peritrophin-44 with endoproteinase Lys-C. Peritro
phin-44 (5 μg) was incubated with endoproteinase Lys-C (0.25 μg) in
TBS for 0, 5, 20, 60, and 120 min at 37 °C (lanes 2–6, respectively).
Lanes 7–11 were identical to lanes 2–6 except that the peritrophin-44
was preincubated with 5 mM dithiothreitol for 20 min before the addi
tion of endoproteinase Lys-C. These samples were then analyzed by
SDS-PAGE. Lane 1 contained molecular mass standards. All samples
were also reduced immediately before SDS-PAGE.

a privileged location of this protein on the ecto-PM surface
where digestive proteases may not have ready access. The
electron-lucent and electron-dense layers in the PM, which
have been reported previously (1), can also be discerned in the
electron micrographs. At least for peritrophin-44, one of the
major integral PM proteins, there was no correspondence be
 tween its location and these layers. Fig. 4d shows the immu
nofluorescence localization of peritrophin-44 on freshly dis
sected PM. Again, peritrophin-44 was uniformly distributed
throughout the entire PM. There was little or no fluorescence
associated with the corresponding prevaccination control se
rum (Fig. 4c). Specific antibodies bound to peritrophin-44 on
intact, unfixed PM during the immunofluorescence localiza
tion experiments, thereby demonstrating that this protein was
exposed on the PM surface and not protected by a chitin
envelope. Therefore, peritrophin-44 should also be accessible to
digestive proteases. Consequently, the resistance of peritrophin-44
to proteolysis in vivo is primarily due to the inherent stabilit
y of the protein, which is mediated by intramolecular disulfide
bonds. The uniform distribution of peritrophin-44 in the PM
and its relative abundance are consistent with a major role of
this protein in maintaining the structure of the PM.

Amino-terminal Amino Acid Sequences of Peritrophin-44 and
Peritrophin-48—The amino-terminal amino acid sequences of
purified peritrophin-44 and peritrophin-48 were directly deter
mined (Fig. 5). There were 9 identical and a further 6 conserved
positions in the first 29 amino acids of these two sequences (i.e.
52% similarity). The probability that the sequence alignment is
a chance occurrence is 4.5 e−8 (SEQDP computer program;
ANGIS computer system, Sydney). Of particular note is the
conservation of 3 cysteine residues at positions 8, 23, and 29.
Cysteines are often conserved in related extracellular proteins
because of their involvement in intramolecular disulfide bonds
(35). The sequence information indicates that there is signifi
cant amino acid sequence similarity between peritrophin-44
and peritrophin-48, suggesting that they belong to a common
family of proteins. Searches of the National Biomedical Re
search Foundation and Swiss Protein sequence data bases did
not reveal any significant similarities of these sequences with other proteins.

Isolation of cDNA Clones Coding for Peritrophin-44—The cDNA coding for peritrophin-44 was isolated and sequenced to gain further information about the structure and function of this protein. Peritrophin-44 was reduced, alkylated, and digested with endoproteinase Lys-C or endoproteinase Glu-C, and the released peptides were purified and sequenced. These peptide amino acid sequences (and the amino-terminal sequence of peritrophin-44) were used to design suitable, degenerate oligonucleotide primers that were used in the PCR in conjunction with either cDNA or genomic DNA to amplify in

**Fig. 4. Localization of peritrophin-44 on PM.** Peritrophin-44 was localized to freshly dissected PM by immunogold labeling (a and b) and immunofluorescence labeling (c and d) using anti-serum to a recombinant GST-peritrophin-44 fusion protein. Controls using prevaccination serum are shown in a and c. b and d show the localization of peritrophin-44 using anti-serum raised to purified GST-peritrophin-44. B, bacterium; ECPS, ecto-PM space; ENPS, endo-PM space. The scale bars in a and b represent 500 nm. The PM used for the immunofluorescence localization of peritrophin-44 was freshly dissected from L. cuprina larvae.
both cases a 860-bp DNA fragment; a result that indicated that introns were not present in the genomic DNA fragment. The latter DNA fragment was sequenced and shown to contain a single open reading frame. The deduced amino acid sequence contained extensions of the peptide sequences used to design the oligonucleotide primers used in the PCR reaction as well as 10 additional peptide sequences that were obtained directly from purified peritrophin-44. Some of these peptide sequences were overlapping. The 860-bp genomic DNA fragment was then used to screen a L. cuprina larval cDNA library constructed in λ gt-11. Eight positive clones were isolated.

Complete Nucleotide Sequence of Peritrophin-44—The nucleotide sequence obtained from one cDNA clone containing the longest insert (1470 bp) had an open reading frame of 1068 nucleotides that coded for a protein of 356 amino acids (Fig. 6). A poly(A) signal sequence (AATAAA; Ref. 36) was located 69 nucleotides after the stop codon, although there was no poly(A) tail. Translation of the nucleotide sequence revealed 20 of the 23 peritrophin-44 peptide amino acid sequences (5 of the 20 identified peptide amino acid sequences are not shown in Fig. 6 because they were substantially or fully redundant with peptides already listed in the figure). Three low abundance peptide sequences from one of the four independent peritrophin-44 protein preparations (each of which were used to generate peptides) were not located within the deduced amino acid sequence. Recently, these three peptide sequences were located in the amino acid sequence of peritrophin-44. This result indicated that one of the four peritrophin-44 preparations also contained small amounts of peritrophin-48. There were 10 positional differences (out of a total of 177 unique positions) between the peritrophin-44 peptide amino acid sequences and the corresponding amino acid positions deduced from cDNA. Several of these differences (6 out of 10) were conservative substitutions and none involved cysteine residues (see below). These minor differences in sequence may reflect allelic variations because the cDNA, genomic DNA and purified peritrophin-44 were obtained from large numbers of individuals (16,000, 16,000, and 320,000 individuals, respectively). Alternatively, there may be a family of highly related peritrophin-44 genes. The existence of extensive intramolecular disulfide bonds (see Fig. 3) could maintain the tertiary structure of peritrophin-44 and therefore its principal function, while accommodating limited amino acid substitutions in flexible loops on the surface of the protein. Other gene sequences from L. cuprina, such as the excretory and secretory chymotrypsin, LCTb, also show sequence variation (23).

The amino acid sequence of peritrophin-44 deduced from cDNA contained a typical amino-terminal signal sequence of 23 amino acids (37). The amino terminus of the mature protein was verified by direct amino acid sequencing. The calculated molecular mass of the mature protein is 36,300 Da, which is significantly less than that measured by SDS-PAGE (Mₘ = 44,000) and consistent with the size of peritrophin-44 after deglycosylation with N-glycosidase F (Mₘ = 37,500; Fig. 2). The calculated pl of the mature protein is 5.2. Apart from the amino-terminal signal sequence, there are no other regions in the protein which are strongly hydrophobic. The sequence contains 2 potential N-linked glycosylation consensus sites (NX(S/T)X, where X is any amino acid except proline; Ref. 38), which is consistent with the knowledge that the native protein contains two classes of oligosaccharides (Fig. 2). The most striking feature of the deduced amino acid sequence is the abundance of cysteine residues (32 cysteines in the mature protein, or approximately 10 mole percent). The abundance of cysteine residues, their relatively close spacing, and the presence of appropriate potential β-turns in the secondary structure of the protein (39) strongly suggest that these cysteines are involved in extensive intramolecular disulfide bonding (35). Indeed, the results shown in Fig. 3 directly support this conclusion.

Domain Structure of Peritrophin-44 and Similarities with Other Proteins—Peritrophin-44 contains 5 nonidentical but related domains, each of approximately 70 amino acids (Fig. 7). The characteristic feature of each of these domains is a common register of 6 cysteine residues (except domain 2, which contains 8 cysteines). This register consists of the following consensus sequence C-X₃₋₅-C-X₉₋₁₀-C-X₁₀₋₁₄-C-X₁₀₋₁₄-C-X₅₋₁₂-C (where X is any amino acid except cysteine). There is a small amount of additional interdomain amino acid sequence similarity between each of these cysteine residues. In particular, there is strong conservation of an aromatic amino acid between cysteines 1 and 2, 2 and 3, and 4 and 5 (Fig. 7a). The conservation of the aromatic amino acids in each of these domains suggests that these residues are important for expression of the function of peritrophin-44. Fig. 7b shows a diagrammatic representation of the domain structure of peritrophin-44.

The National Biomedical Research Foundation amino acid sequence data base was searched using the FASTA program (40) for proteins that showed significant amino acid sequence similarity to peritrophin-44. A number of extracellular cysteine-rich proteins (e.g. epidermal growth factor precursor, laminin, and fibulin) showed similarity to peritrophin-44, but this was primarily due to the prevalence of cysteines in these proteins rather than overall sequence similarity. The characteristic 6-cysteine register in each of the 5 domains of peritrophin-44 was not present in any of these proteins. Moreover, there were no sequence similarities with any proteins derived from insect cuticle that lines the crop and hindgut of this insect. Cuticle proteins typically have no cysteine residues and are not glycosylated (41). The cysteine-rich domains are the major architectural feature of peritrophin-44 and related proteins would be expected to contain a similar arrangement of cysteine residues.

The computer program Scrutineer (42) was used to search the GenPep protein sequence data base (translated sequences from Genbank) for proteins containing the 6-cysteine domain consensus sequence described above (or minor variations thereof). There were only four matches, namely the cysteine-rich, carboxyl-terminal domains in chitinases from Brugia malayi (a parasitic nematode; Ref. 43), Mandra sexta (tobacco hornworm, an insect; Ref. 44), and Chelonus sp. (an endoparasitic wasp; Ref. 45) as well as a small hypothetical polypeptide from Autographa californica nuclear polyhedrosis virus (i.e. baculovirus; Genbank accession number L22858) (Fig. 7a). Each of the chitinases contains only one of these domains, which is located immediately adjacent to the carboxyl terminus. This region is not part of the catalytic domain of the chitinases and has no identified function.

Plant chitinases do not contain this particular 6-cysteine domain, but rather one group, the class 1 plant chitinases, contain an analogous domain containing 8 cysteines, which is strongly related to each of the cysteine-rich domains in wheat germ lectin. The single 8-cysteine wheat germ lectin-like domain is located at the mature amino termini of this class of
The baculovirus polypeptide sequence was deduced from an open reading frame and has no known function. The predicted polypeptide consists of an apparent amino-terminal signal sequence of 23 amino acids, followed by one copy of the 6-cysteine domain (76 amino acids). Both of these features suggest that the protein is secreted. There is also additional sequence similarity between this hypothetical polypeptide and peritrophin-44 based on the conservation of specific aromatic amino acids.

It is interesting to note that baculoviruses infect specific insect species via the insect gut and cross the PM during this process. It is possible that this viral polypeptide binds to chitin within the insect PM and facilitates the movement of the virus across the PM.

**Effect of Tri-N-Acetyl Chitotriose on the Intrinsic Fluorescence Spectrum of Peritrophin-44**

*Fig. 6. Nucleotide and deduced amino acid sequences of peritrophin-44 determined from cDNA.* Underlining denotes the identification of peptide amino acid sequences. Differences between peptide amino acid sequences and the deduced amino acid sequence are shown below the indicated peptides as lowercase letters. Cysteines are circled. A potential polyadenylation signal sequence is underlined twice, and the amino-terminal signal sequence of the protein is underlined with a broken line. Two potential N-linked glycosylation sites are boxed. The numbers at the end of each line refer to the nucleotide (upper) and amino acid (lower) sequences.

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Plant chitinases and has been shown to mediate binding of class 1 chitinases to chitin (46, 47). By analogy with the plant chitinases, it is likely that the single 6-cysteine domain at the carboxyl-terminal end of each of the three animal chitinases listed above and also the multiple 6-cysteine domains within peritrophin-44 mediate binding of these proteins to chitin. The major component of insect PM is chitin, a linear polymer of β-1,4-linked GlcNAc (1). Thus, it is likely that the domain structure of peritrophin-44 reflects its capacity to interact with chitin within the PM. Moreover, the multiple cysteine-rich domains in peritrophin-44 may allow multi-site binding to the GlcNAc polymer that makes chitin thereby producing a noncovalent binding interaction of considerable strength. This possibility may explain the necessity for strong denaturants such as 6 M urea to solubilize peritrophin-44 from PM.
Fig. 7. Related cysteine-rich domains in peritrophin-44. a, five contiguous domains from peritrophin-44 (PM44-I–PM44-V) each containing six cysteines (except domain 2, which contained an additional two cysteines) were aligned. The conserved cysteine residues are boxed and shaded boxes denote the five cysteine-rich domains in peritrophin-44. Potential N-linked glycosylation sites are represented by the y-shaped symbols.

b

![Diagram of cysteine-rich domain](image)

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| Sequence |
|----------|
| PM44-I  | QGQVDFPVQVYSCYQGYC |
| PM44-II | QGQVDFPVQVYSCYQGYC |
| PM44-III| QGQVDFPVQVYSCYQGYC |
| PM44-IV | QGQVDFPVQVYSCYQGYC |
| PM44-V  | QGQVDFPVQVYSCYQGYC |

The sugars Glc, methyl α-D-mannopyranoside, and GalNAc each at a concentration of 1 mM did not specifically bind to reacetylated chitosan (1). This lectin is composed of two subunits, each containing four very similar 8-cysteine domains (A–D). One region in domain A and an identical region in domain B of wheat germ lectin have the sequence CSQYGYC. Although this similarity, by itself, is

buffer (Fig. 9, lane 6). The sugars Glc, methyl α-D-mannopyranoside, and GalNAc each at 20 mM concentrations in TBS did not elute peritrophin-44 from the reacetylated chitosan affinity column (lanes 2–4). These results indicate that peritrophin-44 specifically binds to reacetylated chitosan and are also consistent with the results shown in Fig. 8. A small quantity of the applied peritrophin-44 did not bind and was found in the initial TBS wash of the reacetylated chitosan affinity column. Although this unbound peritrophin-44 is difficult to see in lane 1 of Fig. 9, higher loadings of this sample (not shown) indicate that it represents approximately 10% of the applied protein. This fraction may represent peritrophin-44, which was irreversibly denatured by the harsh conditions required to initially extract the protein from PM. The ability of GlcNAc to elute only a fraction (~70%) of the total peritrophin-44 bound to the reacetylated chitosan affinity column (the remaining 30% was eluted by the low pH buffer) may reflect the polydispersity of the latter with respect to GlcNAc polymer lengths and degrees of reacetylation. Indeed, the affinity of peritrophin-44 for GlcNAc polymer may depend on the length of the polymer (as for wheat germ lectin; Ref. 48). GlcNAc may efficiently elute peritrophin-44 from one population of small GlcNAc polymers, whereas a low pH buffer may be required to remove peritrophin-44, which had bound more strongly to longer polymers.

The specificity of the interaction between peritrophin-44 and reacetylated chitosan was also demonstrated with control experiments, which demonstrated that bovine serum albumin and soybean trypsin inhibitor did not bind to the reacetylated chitin affinity column (result not shown). Thus, peritrophin-44 binds specifically to reacetylated chitosan (Fig. 9) and tri-N-acetyl chitotriose (Fig. 8). GlcNAc (0.3 mM) did not elute peritrophin-44 directly from freshly isolated PM.

Wheat germ lectin has been used extensively as a probe for detection of chitin associated with PMs because of the ability of this lectin to strongly bind GlcNAc polymers including reacetylated chitosan (1). This lectin is composed of two subunits, each containing four very similar 8-cysteine domains (A–D). One region in domain A and an identical region in domain B of wheat germ lectin have the sequence CSQYGYC. A similar sequence is present in the first cysteine-rich domain of peritrophin-44, i.e., CSQYGYC. Although this similarity, by itself, is

Spectrum of purified native peritrophin-44 after excitation at 280 nm was measured in the presence and the absence of tri-N-acetyl chitotriose to determine whether this oligosaccharide bound to peritrophin-44 (Fig. 8a). In the absence of the oligosaccharide, the wavelength of maximal emission was 347 nm, which is characteristic of tryptophan emission in a polar environment (48) and suggests substantial exposure of the single tryptophan residue on the surface of peritrophin-44. The decrease in intrinsic fluorescence emission indicated that the oligosaccharide had bound to peritrophin-44 and perturbed the environment(s) of some of the aromatic amino acids within peritrophin-44 either directly through local contact or via alteration in the efficiency of energy transfer between tryosine residues and the single tryptophan residue in this protein. Titration of the change in intrinsic fluorescence of peritrophin-44 with a range of tri-N-acetyl chitotriose concentrations indicated that the binding was saturable. A Scatchard plot (30; Fig. 8b) was linear (r = −0.95), indicating a single class of specific binding sites with an association constant Ka = 5.5 ± 2.3 mM−1. A number of sugars including Glc, methyl α-D-mannopyranoside, and GalNAc, each at a concentration of 1 mM, had no significant effect on the intrinsic fluorescence spectrum of peritrophin-44. GlcNAc at a concentration of 1 mM caused approximately 6% quenching of the fluorescence spectrum. The binding constant for the interaction of GlcNAc with peritrophin-44 could not be accurately determined from this relatively small change in intrinsic fluorescence.

Binding of Peritrophin-44 to Recactylated Chitosan—The binding of peritrophin-44 to a reacetylated chitosan affinity column was directly measured to further test the proposal that peritrophin-44 binds chitin. Recactylated chitosan is an insoluble heterogeneous mixture of GlcNAc linear polymers. The peritrophin-44 present in various washes from the affinity column was analyzed by SDS-PAGE (Fig. 9). The majority of the peritrophin-44 (~80%) bound to the reacetylated chitosan affinity column and was specifically eluted by either 20 mM GlcNAc (Fig. 9, lane 5) or subsequently by a pH 3.0 acetate
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Fig. 9. Binding of peritrophin-44 to reacetylated chitosan. Purified peritrophin-44 (10 μg) was added to a reacetylated chitosan affinity column, which was then progressively washed with TBS, Glc, methyl α-D-mannopyranoside, GalNAc, and GlcNAc (each in TBS). Finally, the reacetylated chitosan affinity column was washed with 50 mM sodium acetate, pH 3.0. Each eluate was concentrated, and samples were subjected to SDSPAGE and then stained with silver. Lane 1, TBS wash; lane 2, 20 mM Glc eluate; lane 3, 20 mM methyl α-D-mannopyranoside eluate; lane 4, 20 mM GalNAc eluate; lane 5, 20 mM GlcNAc eluate; lane 6, 50 mM sodium acetate buffer, pH 3.0 eluate.

not particularly significant, it is noteworthy that in the wheat germ lectin sequence, the serine and both tyrosines are important ligands directly involved in binding GlcNAc (49). Moreover, there is absolute conservation of a tyrosine residue in the central position of this sequence similarity throughout all of the peritrophin-44 cysteine-rich domains (Fig. 7), suggesting that this position has an important involvement in the function of this protein. The intrinsic fluorescence spectra of both wheat germ lectin and peritrophin-44 including a small region of sequence similarity, the presence of multiple cysteine-rich domains, the ability to bind chitin and reacetylated chitosan, a similar relationship with the chitin-binding domains of chitinases, and the ability to bind to PM. However, peritrophin-44 does not have wheat germ lectin-like agglutination abilities (result not shown).

Expression of Peritrophin-44—Microscopic examination of the gut of many insects has indicated that the cardia is the primary site for synthesis of type 2 PMs (1). This small group of highly specialized cells (~1000) is usually situated in the anterior midgut region. However, it is not clear whether the proteins associated with this class of PM are synthesized in the cardia or produced by midgut cells and then added to the PM in a subsequent maturation step. To differentiate between these two possibilities, reverse transcriptase-PCR specific for peritrophin-44 was performed on first strand cDNA made from RNA derived from various gut tissues dissected from individual larva. Fig. 10a shows the amplification of a peritrophin-44-specific DNA fragment (1100 bp) from first strand cDNA derived from cardia (lane 2) and to a much smaller extent from midgut (lane 3). There was no expression of peritrophin-44 mRNA in hindgut (lane 4), crop (lane 5), salivary gland (lane 6), malphigian tubules (lane 7), or trachea (lane 8). The quantity of first strand cDNA used in PCR for each sample was directly related to the total mRNA content from that tissue. Thus, the dominant expression of peritrophin-44 in cardia is further underscored by the knowledge that this tissue contains relatively few cells (~1000) compared with each of the other tissues tested, particularly the midgut. It is therefore likely that the PM is produced predominantly from the larval cardia in a near mature form containing a full complement of integral PM proteins.

All three larval instars express peritrophin-44 (Fig. 10b, lanes 2–4), whereas virtually none is expressed in pupae or eggs (lanes 5 and 7). There is a very small level of expression of peritrophin-44 in adult flies (lane 6). These results indicate that peritrophin-44 expression is primarily restricted to larvae. The relative level of expression (per unit mass of total first strand cDNA) in the three larval instars is constant even though these larvae are enormously different in size. This suggests that peritrophin-44 is constitutively expressed and that the rate of growth of the larvae is directly linked to the rate of growth of the PM. Adult L. cuprina also produce a type 2 PM from cardia (1). Specific immunoblots of adult fly tissues and isolated PM failed to detect peritrophin-44 (result not shown). This result is consistent with the very low level of expression of peritrophin-44 in adult flies detected by reverse transcriptase-PCR and indicates that the structure of the larval and adult type 2 PMs from L. cuprina are very different. The reasons for this are not clear. One possibility is that the
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REFERENCES

1. Peters, W. (1992) Zoophysiology. Peritrophic Membranes (Bradshaw, S. D., Burggren, W., Heil, H. C., Ishii, S., Langer, H., Neuweiler, G., and Randall, D. J., eds) Vol. 130, Springer-Verlag, Berlin.
2. Stolz, D. B., and Summers, M. D. (1971) J. Biol. 8, 900–909
3. Paschke, J. D., and Summers, M. D. (1975) In Invertebrate Immunity: Mechanisms of Invertebrate Vector-Parasite Rivalries (Maramorosch, K., and Shope, R. E., eds.) pp. 75–112, Academic Press, New York.
4. Brandt, C. R., Adang, M. J., and Spence, K. D. (1978) J. Invertebr. Pathol. 32, 12–24.
5. Adang, M. J., and Spence, K. D. (1981) Cell. Tissue Res. 218, 141–147.
6. Derksen, A. C. G., and Granados, R. R. (1988) Virology 167, 242–250.
7. Billingstey, P. F., and Rudin, W. (1992) J. Parasitol. 78, 430–440.
8. Miller, N., and Lehane, M. J. (1993) Parasitol. Today 9, 45–50.
9. Lehane, M. J., and Msangi, A. R. (1991) Med. Vet. Entomol. 5, 495–501.
10. Sieber, K.-P., Huber, M., Kasalow, D., Banks, S. M., Torii, M., Akaiwa, K., and Miller, L. H. (1991) Exp. Parasitol. 72, 145–156.
11. Pearson, W. R., Billingstey, P. F., and Rudin, W. (1988) Parasitol. Today 4, 319–321.
12. East, I. J., Fitzgerald, C. J., Pearson, R. D., Donaldson, R. A., Vuocolo, T., Cadogan, L. C., Tellam, R. L., and Eisemann, C. H. (1993) J. Invert. Pathol. 23, 221–229.
13. Stamm, B. D’Haese, J., and Peters, W. (1978) J. Insect Physiol. 24, 1–8.
14. Tellam, R. L., Eisemann, C. H., and Tellam, R. L. (1993) Insect Mol. Biol. 1, 139–147.
15. Lieber, I. E., Sharon, N., and Goldstein, I. J. (1986) The Lipids: Properties, Functions, and Applications in Biology and Medicine, Academic Press, London.
16. Smith, D. B., and Johnson, K. S. (1988) Science 239, 487–491.
17. Liener, I. E., Sharon, N., and Goldstein, I. J. (1986) Science 239, 487–491.
18. Pearson, W. R., and Lipman, D. J. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 4477–4481.
19. Li, Y.-M., Jang, H.-K., Corbitt, K. J., and Muthukrishnan, S. (1993) Biochem. Mol. Biol. 20971–20976.
20. Tse, W. T., and Forget, B. G. (1990) Anal. Biochem. 190, 269–278.
21. Casu, R. E., Pearson, R. D., Jarmey, J. M., Cadogan, L. C., Riding, G. A., and Willadsen, P. (1990) Insect Mol. Biol. 3, 159–170.
22. Willadsen, P., Riding, G., McHenry, R., Kemp, D., and Tellam, R. L. (1993) Insect Mol. Biol. 1, 139–147.
23. Casu, R. E., Pearson, R. D., Jarmey, J. M., Cadogan, L. C., Riding, G. A., and Tellam, R. L. (1994) Insect Mol. Biol. 3, 201–211.
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
25. West, S., Schröder, J., and Kunz, W. (1990) Anal. Biochem. 190, 254–258.
26. Tse, T. W., and Forget, B. G. (1990) Gene 88, 293–296.
27. Hirano, S., Ohe, Y., and Ohno, H. (1976) Carbohydr. Res. 47, 315–320.
28. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660–672.
29. Chou, P. Y., and Fasman, G. D. (1978) Adv. Enzymol. 47, 25–40.
30. Pearson, W. R., and Lipman, D. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2444–2448.
31. Andersen, S. O., Hojrup, P., and Roepstorff, P. (1995) Carbohydr. Res. 283–294.
32. Elvin, C. M., Vuocolo, T., Smith, W. J. M., Eisemann, C. H., and Riddles, P. W. (1995) Insect Mol. Biol. 3, 159–170.
33. Casu, R. E., Jarmey, J. M., Elvin, C. M., and Eisemann, C. H. (1994) Insect Mol. Biol. 3, 159–170.
34. Elvin, C. M., Vuocolo, T., Smith, W. J. M., Eisemann, C. H., and Riddles, P. W. (1994) Insect Mol. Biol. 3, 105–115.
35. Thornton, J. M. (1981) J. Mol. Biol. 151, 261–287.
36. Proudfoot, N. J., and Brownlee, G. G. (1976) Nature 263, 105–115.
37. Briggs, M. S., and Giersch, L. M. (1986) Adv. Protein Chem. 38, 109–180.
38. Gavd, Y., and von Heijne, G. (1990) Protein Eng. 3, 433–442.
39. Chou, P. Y., and Fasman, G. D. (1978) Adv. Enzymol. 47, 45–148.
40. Pearson, W. R., and Lipman, D. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2444–2448.
41. Casu, R. E., Jarmey, J. M., Elvin, C. M., and Eisemann, C. H. (1994) Insect Mol. Biol. 3, 159–170.
42. Siibsald, P. R., and Argos, P. (1990) Comp. Appl. Biosci. 6, 279–288.
43. Fuhrman, J. A., Lane, W. S., Smith, R. F., Piessens, W. F., and Perler, F. B. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1548–1552.
44. Kramer, K. J., Corpuz, L., Choi, H. K., and Muthukrishnan, S. (1993) J. Insect Biochem. Mol. Biol. 23, 691–701.
45. Krishnan, A., Nair, P. N., and Jones, D. (1994) J. Biol. Chem. 269, 151–157.
46. Wright, H. T., Sandrasegaran, G., and Wright, C. S. (1991) J. Biol. Chem. 266, 283–294.

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2 C. H. Eisemann and R. L. Tellam, unpublished results.