Characterization of a Novel Gut-specific Chitinase Gene from the Human Malaria Vector Anopheles gambiae*

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Chitinases that function in the molting of the larval exoskeleton have been characterized previously. However, chitinase expression in an adult insect gut has not been described. Here we report on the initial characterization and cloning of a novel chitinase gene that is expressed specifically in the midgut of adult Anopheles gambiae females. Upon feeding, chitinase is secreted into the gut lumen as an inactive pro-enzyme that is later activated by trypsin. Thus, temporal regulation of chitinase activity is tightly coupled to the temporal pattern of trypsin secretion. The enzyme may play a role in structuring the chitin-containing extracellular peritrophic matrix, whose formation is also induced by feeding. A chitinase cDNA was cloned from a library enriched for gut-specific sequences. The open reading frame encodes a 523-amino acid protein comprised of a putative catalytic domain at the N terminus, a putative chitin-binding domain at the C terminus, and a threonine/serine/proline-rich amino acid stretch in between them. Northern analysis indicates that this chitinase is expressed exclusively in the guts of adult females and not in adult carcasses or in any larval or pupal tissues. The present findings suggest the possibility of using this chitinase as an antigen for a malaria transmission-blocking vaccine.

In addition to the exoskeleton, chitin is also an important component of the insect peritrophic matrix (PM). The PM is a sleeve-like extracellular layer that surrounds the food bolus in the gut of most arthropods (8–10). In adult mosquitoes, the PM contains proteins and proteoglycans (8–10). The function of the PM in insects is a matter of conjecture. It may provide a physical barrier to pathogens, facilitate digestion, and provide physical protection of gut epithelial cells from damage by food particles (8–10). It is unknown how the formation and structure of any PM are regulated.

Mosquitoes of the genus Anopheles are the sole vectors of human malaria. Malaria is caused by the protozoan Plasmodium sp. Transmission is initiated when the mosquito ingests an infected blood meal. In the gut, Plasmodium gametocytes mate and then develop into oocinates that cross both the PM and the gut epithelium. Increased PM thickness can impair Plasmodium development in the mosquito (11). Moreover, feeding mosquitoes with the chitinase inhibitor allosamidin results in a thicker PM that starts forming earlier and persists longer (12). The latter observation is consistent with the existence of a chitinase in the mosquito gut that modulates the physical properties of the PM. However, to date there has been no direct experimental evidence for this hypothesis. Here we demonstrate that upon feeding, adult A. gambiae females secrete a chitinasezymogen into the gut lumen that is subsequently activated by trypsin. Furthermore, we have isolated a cDNA clone that encodes a midgut-specific chitinase.

**EXPERIMENTAL PROCEDURES**

**Insects**—A colony of A. gambiae (G3 strain) was maintained as described previously (13). Experiments were normally conducted with 4–5-day-old mosquitoes.

**Feeding of Mosquitoes with Latex Beads and γ-Globulin**—Before feeding, mosquitoes were deprived of sugar for 18–24 h. Porcine γ-globulin (Sigma) was dissolved to 300 mg/ml in feeding buffer (120 mM NaCl and 20 mM NaHCO₃, pH 7.0) and dialyzed overnight against the same buffer. When indicated, soybean trypsin inhibitor (Sigma) was also added. The protein-free meal consisting of 10% (v/v) latex beads (Sigma) was prepared and administered as described previously (14), except that the jacketed glass feeder was covered with Parafilm.

**Detection of Chitinase Activity after Polyaacylamide Gel Electrophoresis (PAGE)**—A chitinase assay modified from that of Trudel and Asselin (15) was used. Native substrate PAGE was performed using a Tris-glycine buffer system (pH 8.8). Glycol chitin (0.02%) was incorporated into 8% (w/v) polyacrylamide mini-gels (50 × 140 × 0.75 mm). Samples contained 10% glycerol and 0.001% bromphenol blue. Electrophoresis was performed at room temperature for 2 h at 15 mA. After electrophoresis, the gel was immersed in 100 mM sodium acetate (pH 5.0) and incubated at 37 °C for 2 h. The gel was then stained for 15 min in 0.01% Calcofluor White M2R (Sigma; a fluorescent dye that binds chitin) and destained in water for 1 h. The activity band was visualized under an UV transilluminator and photographically documented with a Gel Doc 1000 imaging system (Bio-Rad).

**Time Course of Gut Chitinase Activity after γ-Globulin Feeding**—Two

*The abbreviations used are: PM, peritrophic matrix; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s).
groups of about 100 female mosquitoes were fed with γ-globulin or γ-globulin plus 5 mg/ml soybean trypsin inhibitor. Fifteen mosquitoes were collected from each group at different times after feeding. The guts were immediately dissected and homogenized in 30 μl of 50 mM Tris-HCl (pH 7.5). Five μl from each sample were loaded onto a native polyacrylamide gel for the activity assay.

**Activation of Chitinase by Trypsin**—Mosquitoes were fed with γ-globulin plus 5 mg/ml soybean trypsin inhibitor and immediately dissected on a dry glass slide, and the fluid from the gut lumen was collected with a fine glass pipette. Different amounts (0, 1, and 3 μl) of trypsin (1 mg/ml) were then added to a fixed amount (5 μl) of fluid. The volume of each sample was adjusted to 10 μl with H2O and then incubated at 37°C for 30 min. After incubation, the activity was assayed by substrate native PAGE as described above. Whole-gut extracts from mosquitoes dissected 5 h after feeding with γ-globulin containing 5 mg/ml soybean trypsin inhibitor were also tested for activation by the same procedure.

**Subtraction cDNA Library**—The PCR-Select Subtraction kit (CLONTech, Palo Alto, CA) was used to generate a mini-library enriched for gut-specific sequences according to the manufacturer’s instructions. Briefly, the cDNA synthesized from 0.1 μg of carcass polyadenylated RNA was used as the driver, and the cDNA from 0.1 μg of gut polyadenylated RNA was used as the tester. The subtracted cDNAs were cloned into pGEM-T Easy vector (Promega). Forty random clones were partially sequenced, and a BLAST search (18) was performed to identify similarity to the sequences in the database.

**Characterization of a Chitinase cDNA Clone**—A cDNA insert from the subtraction library that had similarity to chitinases was labeled with [α-32P]dCTP and used to screen the A. gambiae midgut ZAP II cDNA library that was described by Lemos et al. (13). Inserts from two positive phages were sequenced. To obtain the sequence of the 5’ end of the cDNA, a polymerase chain reaction reaction was performed using whole library DNA as a template and a chitinase-specific oligonucleotide (5’-CCGGAAAATCTCCAGGGC-3’) plus a T3 universal primer as primers. The polymerase chain reaction was performed using the Taglong kit (Stratagene) for 30 cycles at 94°C for 20 s, 58°C for 30 s, and 72°C for 1 min. The resulting fragment was subcloned and sequenced. The amino acid sequence alignment was carried out using the ClustalV program (17).

**Northern Analysis**—Total RNA (5 μg each) from larval guts, larval carcasses, whole pupae, adult female guts, and adult carcasses was fractionated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with an α-32P-labeled probe derived from the 1.2-kilobase cDNA insert of a chitinase cDNA clone. The final wash of the filter was done at 65°C and 0.1× SSC. The same blot was then stripped and rehybridized with an A. gambiae mitochondrial rRNA probe to serve as a loading control (13).

**The Effect of Chitinase Inhibitor on PM**—The mosquitoes were fed with rabbit serum with or without adding allosamidin to 0.5 mM (18). The PMs were dissected at different times after feeding and examined by light microscopy.

**RESULTS**

**Chitinase Activity in Midguts Is Low Early after Feeding and Increases Significantly after the PM Has Formed**—Chitinase activity was measured by electrophoresis of gut extracts in native polyacrylamide gels containing the synthetic substrate glycol chitin. After electrophoresis, the gels were incubated at 37°C to allow substrate hydrolysis by chitinases and then stained with a fluorescent dye that binds the substrate (Fig. 1). brightly fluorescent areas represent regions with no active enzyme, whereas dark areas represent regions where enzyme activity degraded the substrate. The wells of the gel contain no substrate and are always dark (Fig. 1, top). Enzyme activity was distributed somewhat heterogeneously near the top of the gel (Fig. 1). This may be because the enzyme interacts with the substrate (glycol chitin) during electrophoresis and/or because of the low pH associated with the activity assay.

**FIG. 1.** Changes of gut chitinase activity after feeding. Mosquitoes were fed with γ-globulin only (upper panel) or with γ-globulin plus soybean trypsin inhibitor (STI, lower panel). Groups of 15 guts dissected at 5, 10, 20, and 32 h after feeding were homogenized in 30 μl of 50 mM Tris-HCl (pH 7.5), and 5 μl of each sample were analyzed by electrophoresis on substrate activity gels (see the text). The dark areas at the top of each lane are the loading wells. Dark areas below the wells indicate chitinase activity. Five μl of the γ-globulin solution (300 mg/ml) used to feed the mosquitoes were loaded in the control lane.

**FIG. 2.** Chitinase activation by trypsin. Chitinase activity was measured by the substrate gel activity assay (see the text). The dark areas at the top of each lane are the loading wells. Dark areas below the wells indicate chitinase activity. A, samples were the luminal contents of mosquito guts dissected within 30 min of feeding on a meal containing γ-globulin plus 5 mg/ml soybean trypsin inhibitor. B, samples were whole-gut extracts obtained from mosquitoes dissected 5 h after feeding with γ-globulin plus 5 mg/ml trypsin inhibitor. For both A and B,amples (3 μl) were incubated for 30 min at 37°C after the addition of H2O (lane 1) or trypsin to either 0.1 mg/ml (lane 2) or 0.3 mg/ml (lane 3).

5 h after feeding, but it was easily detectable at 10 h. However, when soybean trypsin inhibitor was included in the meal, chitinase activity was much lower at early times (5 and 10 h after feeding) and increased only at later times (20 and 32 h). This delay in the increase of chitinase activity suggested that trypsin may be required for chitinase activation.

**Feeding Induces the Secretion into the Gut Lumen of an Inactive Form of Chitinase That Can Be Activated by Trypsin**—In the experiments described in the previous paragraph, chitinase activity was assayed in whole-gut homogenates. To determine whether chitinase is secreted into the gut lumen in response to food ingestion, the luminal contents of guts dissected within 30 min after feeding were collected and assayed.
for chitinase activity (Fig. 2A). No activity was detected when the sample was analyzed without treatment with trypsin (Fig. 2A, lane 1). However, when the sample was treated with trypsin, significant activity was detected (Fig. 2A, lanes 2 and 3). This result indicates that upon feeding, chitinase is released into the gut lumen in an inactive form and that trypsin can activate it.

The activation of the chitinase by trypsin was also observed with whole-gut extracts collected 5 h after feeding a g-globulin meal containing trypsin inhibitor (Fig. 2B). There was no detectable chitinase activity in the sample (Fig. 2B, also shown in Fig. 1), however, treatment of the sample with trypsin resulted in a significant increase of chitinase activity (Fig. 2B, lane 3).

Together, these results indicated that chitinase is secreted into gut lumen in an inactive form and that trypsin is likely to be involved in the activation of the enzyme.

Isolation of AgChi-1, an Adult Gut-specific cDNA—A gut-enriched cDNA mini-library was constructed by subtractive cDNA cloning. About 40 individual clones from this library were partially sequenced. One of them had an insert of 120 bp that shares sequence similarity with chitinases from various sources. This cDNA fragment was then used as a probe to screen a gut cDNA library. Two clones were isolated (clone 181 and clone 182). These two cDNAs were incomplete and were missing a 5' sequence. To determine the missing 5' sequence, a cDNA fragment was obtained by polymerase chain reaction using total library DNA as a template. The overlapping sequence between this cDNA and clone 181 is identical, indicating that it is part of the same gene, named AgChi-1. Fig. 3 presents the full AgChi-1 cDNA sequence and the deduced amino acid sequence. The cDNA is 1725 bp long and contains an open reading frame of 525 amino acids. It includes 62 bp of 5'-untranslated sequences and 86 bp of 3'-untranslated sequences. Also, there is a predicted polyadenylation signal (AATAA) 29 bp upstream of a stretch of A residues. The size of this cDNA is close to the mRNA size estimated from Northern blots.

The AgChi-1 cDNA Encodes a Chitinase—A BLAST search with the deduced amino acid sequence revealed similarity with chitinases from a number of organisms, including arthropods, plants, fungi, and bacteria. Sequence alignment analysis identified a putative catalytic domain at the N terminus, a putative chitin-binding domain at the C terminus, and a serine/threonine/proline-rich stretch in between them. In the catalytic domain, all chitinases have a stretch of conserved amino acids (underlined in Fig. 4A). These include three aspartic acid residues and one glutamic acid residue, some or all of which have been suggested to be involved in the enzymatic hydrolysis of glycosidic bonds (19–21). This conserved stretch of amino acids also appears in AgChi-1, suggesting that this gene encodes a chitinase. The putative chitin-binding domain of insect chitinases was reported to be similar to each of the five chitin-binding domains of the PM protein peritrophin-44 (22). Six cysteine and three aromatic residues are conserved among

Fig. 3. Nucleic acid and deduced amino acid sequences of the AgChi-1 cDNA. The arrowhead after Ala-19 indicates the predicted signal peptide cleavage site; the arrowheads after Lys-31 and Lys-32 indicate possible trypsin activation sites. The putative polyadenylation signal (AATAA) is in bold. The serine/threonine/proline-rich domain is underlined.
them. AgChi-1 has all of these conserved residues (Fig. 4B).

Whereas the putative chitin-binding domain of most insect chitinases is at the C terminus, that of *Penaeus japonicus* chitinase is at the N terminus (23). The serine/threonine/proline-rich stretch (residues 401–466; underlined in Fig. 3) has a highly biased amino acid composition. 45% of the amino acids in this region are serines or threonines, 20% are prolines, and 12% are glycines. The high proline and glycine content probably suppresses the formation of a secondary structure in this region of the protein, thus providing a flexible swivel between the chitin-binding domain and the catalytic domain. This swivel may allow the enzyme to catalyze chitin hydrolysis at multiple nearby sites after binding to chitin. The serine/threonine residues may be sites of O-linked glycosylation (6).

The deduced AgChi-1 N-terminal sequence contains a highly hydrophobic amino acid stretch that is likely to function as a signal peptide (Fig. 3). This indicates that the protein is secreted and is consistent with recovery of chitinase activity in the gut lumen after ingestion of a meal (Fig. 2). Using von Heijne's rules (24), we predict that the signal peptide is cleaved after Ala-19. Activation of the gut chitinase by trypsin in our activity assay suggests the presence of a pro-peptide after the signal peptide. Two consecutive lysine residues were found 11 amino acids after the predicted signal peptide cleavage site (Fig. 3). These two lysine residues may be the trypsin cleavage sites for pro-enzyme activation. Pro-chitinase activation by trypsin was also observed in other species, such as the fungus *Mucor mucedo* (25), the molting chitinase of *Bombyx mori* (26), and the chitinase from *P. gallinaceum* (27).

The AgChi-1 Gene Is Specifically Expressed in the Adult Midgut—The tissue and developmental specificity of chitinase expression was determined by Northern analysis (Fig. 5). AgChi-1 mRNA is only detectable in adult guts. No AgChi-1 mRNA was detected at any larval or pupal stage of development, suggesting that this chitinase does not play a role in molting. Moreover, the mRNA is not detected in the guts of

![Characterization of an Insect Gut-specific Chitinase](https://example.com/characterization)

**Fig. 4.** Amino acid sequence alignment of the catalytic and chitin-binding domains of arthropod chitinases. The sequences were retrieved from GenBank. The chitinase sequences listed are from *A. gambiae* (this work), *M. sexta* (Ref. 6; S64757), *B. mori* (M. K. Kim, H. Y. Park, and S. W. Shin, unpublished observations; U86876), *Chelonus* sp. (Ref. 7; U10422), and *P. japonicus* (Ref. 23; D84250). PM-44 is a peritrophic matrix protein from *Lucilia cuprina* (Ref. 22; LUCPER144P). A, alignment of the putative catalytic domains; the best conserved sequence, which may be involved in catalysis, is underlined. B, alignment of putative chitin-binding domains. An asterisk indicates that the residues are identical; a period indicates that the residues are conserved; a dash indicates a gap introduced to maximize sequence identity.

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**Fig. 5.** Tissue and developmental specificity of AgChi-1 mRNA accumulation. About 5 μg of total RNA were fractionated by agarose gel electrophoresis, blotted to a nylon membrane, and hybridized with a radioactive AgChi-1 probe (upper panel). An autoradiogram is shown. The source of RNA in each lane is as follows: lane 1, fourth instar larval carcasses (whole bodies minus gut); lane 2, fourth instar larval guts; lane 3, whole pupae; lane 4, adult carcasses; lane 5, adult female guts. The origin of the upper band in lane 5 is unknown; its relative intensity is variable in different experiments. The probe was stripped after autoradiography, and the blot was rehybridized with a mitochondrial rRNA probe used as a loading control (lower panel).
larvae, indicating that AgChi-1 plays a specific role in the adult gut.

Feeding a Chitinase Inhibitor Delays Degradation of the PM—After ingestion of a meal, the PM forms rapidly (within minutes) and gradually thickens and matures. By 7 or 24 h after ingestion of the meal, the PM can be easily dissected. However, as time progresses, the PM becomes more fragile. By 72 h, the PM has completely disappeared (in 55% of the mosquitoes) or become very small and brittle (Fig. 6A). When allosamidin (a chitinase inhibitor) was included in the meal, degradation of the PM was substantially delayed such that at the 72-h time point, all guts had large and intact PMs (Fig. 6B). These results suggest that the Anopheles gut chitinase is a regulator of PM formation and degradation.

DISCUSSION

Detection of Chitinase Activity—The substrate gel electrophoresis activity assay is sensitive because it uses fluorescence for detection of the substrate. High sensitivity is an advantage for these experiments, because it reduces the number of guts that need to be dissected. Moreover, electrophoretic fractionation of the enzyme before the activity assay separates the enzyme from other gut proteinases. Proteinases can influence chitinase activity either by activating thezymogen or by degrading the enzyme. For example, a chitinase zymogen from the fungus M. mucedo is activated when a crude extract is stored at −20 °C over a period of several days (25). The detection system used for the present experiments differs from that of Trudel and Asselin (15) in that the substrate (glycol chitin) was incorporated directly into the separating gel instead of using an overlay substrate gel for enzyme detection. A disadvantage of incorporating the substrate into the gel is that it may cause retardation of enzyme migration and some smearing. However, it has the advantage of being faster and more sensitive, because it does not require the enzyme to migrate out of the separating gel and into the overlay substrate gel. We investigated the effect of pH during incubation of the gel after electrophoresis and found no significant difference in activity from pH 5 to pH 9 (data not shown). In the present experiments, the pH at the beginning of the incubation was pH 8.8 (the pH of the electrophoresis running buffer) and then gradually decreased to pH 5.0 during incubation. In the acetate buffer (pH 5.0), attempts to detect enzyme activity after boiling the sample in the presence of SDS and performing SDS-PAGE followed by renaturation in the presence of Triton X-100 (15) were not successful. In conclusion, the present substrate native PAGE activity assay is relatively simple and sensitive and represents the actual activity in the gut at the time of dissection.

Does the Cloned cDNA Encode the Chitinase Detected by the Activity Assays?—The evidence that the enzyme whose activity was detected in the gut is encoded by the AgChi-1 gene is circumstantial. The possibility that AgChi-1 encodes a chitinase is supported by the similarity of the predicted amino acid sequence to a number of known chitinases and, more importantly, by the fact that key amino acids required for function are conserved in AgChi-1. Furthermore, enzyme and mRNA are both found in the same tissue. Final proof of identity can be obtained by purification of the enzyme from Anopheles guts followed by microsequencing of the protein or by immunological methods. Experiments that address this issue are in progress.

Significance of Chitinase Secretion as an Inactive Zymogen—Upon feeding, mosquitoes secrete a PM composed of chitin, proteins, and proteoglycans. Chitin is of critical importance, because it provides structural support for the PM. Therefore, temporal modulation of chitinase activity in the guts (relative to food ingestion) is important to maintain a functional PM, because inappropriate levels of chitinase activity could interfere with PM formation. Temporal modulation could occur by at least two different mechanisms: 1) secretion of an active enzyme only late in the digestion cycle or 2) secretion of the enzyme as an inactive zymogen that is later activated. The present data support the latter hypothesis. In An. gambiae, chitinase is secreted as a zymogen that can be activated by trypsin (Fig. 2). In this mosquito, trypsin activity rises only slowly during the first 10 h after the blood meal and rises rapidly thereafter (13). Similarly, chitinase activity is low during the first 10 h and rises rapidly thereafter (Fig. 1). These observations are consistent with a causal relationship between the increase of trypsin activity and chitinase activation. Moreover, it is likely that the temporal pattern of chitinase activation is physiologically significant. An inactive chitinase would allow the initial organization and formation of the chitin-containing PM without the interference of hydrolytic activity. Later, after the PM has formed, maturation of the PM would occur as a balance of PM secretion and degradation of the PM scaffold. Finally, toward the end of the digestion cycle, PM secretion would cease, and the chitinase would destroy the remaining PM structure (Fig. 6).

Chitinase May Be a Regulator of PM Structure and Function—Because chitin is an important structural component of the PM, it was at first surprising that food ingestion should trigger chitinase secretion. It seems likely that chitinase acts to modulate PM thickness and permeability. In support of this conjecture, we found that PMs were stronger and persisted longer in the guts when the mosquitoes were fed with chitinase inhibitor. This agrees with the observation made by Shahabuddin et al. (12). Conversely, Regev et al. (28) have shown that administration of chitinase to Spodoptera littoralis larvae causes perforation of the PM and in this way greatly facilitates the action of a Bacillus thuringiensis δ-endotoxin. During digestion, enzyme molecules secreted by the gut epithelium must traverse the PM to reach the food bolus, and digestion products must cross the PM in the opposite direction to be absorbed by the epithelial cells. A thicker PM may have adverse effects on digestion and food absorption by hampering the traffic of these essential molecules across the PM.

Chitinase as a Candidate Antigen for a Transmission-blocking Malaria Vaccine—Previous work has demonstrated that to cross the PM, the Plasmodium ookinetes secrete a pro-chitinase that is activated by the mosquito trypsin (29). However, the PM still represents a significant barrier for penetration by Plasmodium. For instance, experimental induction of a thicker PM by providing multiple blood meals also had a detrimental effect on parasite transmission (11). It is possible that the gut chitinase characterized here may facilitate Plasmodium pene-
tation of the PM by generating a thinner, more porous PM. If antibodies directed against the AgChi-1 protein inhibit gut chitinase activity in vivo, then the recombinant protein may serve as an antigen for a transmission-blocking vaccine. Experiments to test the effectiveness of such antibodies in hindering transmission are in progress.

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