The Journal of Experimental Biology 210, 3636-3643
Published by The Company of Biologists 2007
doi:10.1242/jeb.008334

A chymotrypsin-like serine protease interacts with the chitin synthase from the midgut of the tobacco hornworm

Gunnar Broehan, Lars Zimoch, Anton Wessels, Beyhan Ertas and Hans Merzendorfer*
Department of Biology/Chemistry, University of Osnabrück, D-49069 Osnabrück, Germany
*Author for correspondence (e-mail: merzendorfer@biologie.uni-osnabrueck.de)

Accepted 6 August 2007

Summary

The chitin portion of the peritrophic matrix in the midgut of the tobacco hornworm, *Manduca sexta*, is produced by chitin synthase 2 (CHS2), a transmembrane family II glycosyltransferase, located at the apical tips of brush border microvilli. To look for proteins that potentially interact with CHS2, we performed yeast two-hybrid screening, identifying a novel chymotrypsin-like protease (CTLP1) that binds to the extracellular carboxyterminal domain of CHS2. The occurrence of this interaction *in vivo* is supported by co-localization and co-immunoprecipitation data. Based on our findings we propose that chitin synthesis is controlled by an intestinal proteolytic signalling cascade linking chitin synthase activity to the nutritional state of the larvae.

Key words: chitin, chitin synthase, *Manduca sexta*, chymotrypsin-like protease, midgut, peritrophic matrix.

Introduction

Next to cellulose, chitin is the most important biopolymer in nature. It is produced by numerous species belonging to different taxonomic groups, including fungi, molluscs and arthropods. The ability to form chitin strictly depends on the expression of chitin synthase (EC 2.4.1.16), a transmembrane family II glycosyltransferase, which is the key enzyme in chitin metabolism as it catalyses the polymerization of *N*-acetylglucosamine using uridine-5′-diphosphate *N*-acetylglucosamine as the activated sugar donor (Merz et al., 1999; Merzendorfer, 2006). Due to the lack of sufficient purification procedures and heterologous expression systems, current knowledge on the structure, function and regulation of chitin synthases is still very limited, particularly in insects. Most of what we know about chitin synthases originates from studies performed in yeast and other fungi. Site-directed mutagenesis has revealed several amino acids that are essential for chitin synthesis and phenotypic screenings have yielded a few proteins that appear to modulate enzyme activity (Merzendorfer, 2006; Roncero, 2002). Additionally, fungi have been most valuable in elucidating the trafficking routes of chitin synthase, involving special intracellular storage vesicles called chitosomes (Bartnicki-Garcia, 2006; Henar Valdivieso et al., 1999). In fungi, chitin synthase isoenzymes are encoded by different numbers of genes ranging from one in *Schizosaccharomyces pombe* to up to seven in some filamentous fungi, such as *Aspergillus fumigatus* (Roncero, 2002). In contrast, only two genes, *CHS1* and *CHS2*, encode chitin synthases in insects (Merzendorfer, 2006). As reported for *Manduca sexta* and *Tribolium castaneum*, *CHS1* is exclusively expressed in the epidermis and the tracheal system while *CHS2* is expressed only by midgut columnar cells, where chitin is produced as a component of the peritrophic matrix protecting the epithelium from mechanical damage, radical oxygen species and infectious microorganisms (Arakane et al., 2005; Barbehenn and Stannard, 2004; Hogenkamp et al., 2005; Lehane, 1997; Zimoch et al., 2005). The expression of insect chitin synthase genes is differentially regulated during development. In *Manduca*, *CHS1* is expressed only during moult and pupation, whereas *CHS2* is expressed during the intermoult stages of feeding larvae and down-regulated during moult (Zimoch et al., 2005). While most studies that have analysed chitin synthesis in insects focused on epidermal and tracheal cuticles, we have focused on the midgut chitin synthase.

In the *Manduca* midgut, chitin is synthesized by columnar cells along with other components of the peritrophic matrix (Hopkins and Harper, 2001; Lehane, 1997). Immunocytochemistry has revealed that the chitin synthase from feeding larvae is localized at the very apical tips of the brush border microvilli formed by midgut columnar cells (Zimoch and Merzendorfer, 2002). As well as transcriptional regulation of chitin synthase expression, post-translational mechanisms also appear to be involved in regulating the enzyme. Proteolytic activation of zymogenic chitin synthases is one post-translational mechanism that is discussed controversially in the literature (Roncero, 2002). The zymogenic nature of chitin synthases was mainly deduced from the observation that the addition of trypsin stimulates chitin synthesis in many systems (Cabib and Farkas, 1971; Choi et al., 1994; Cohen and Casida, 1980; Duran et al., 1975; Mayer et al., 1980; Ruiz-Herrera and Bartnicki-Garcia, 1976; Schekman and Brawley, 1979; Ward et al., 1991; Zimoch et

al., 2005). In addition, proteolytic fragments associated with chitin synthase activity have been identified in some fungal systems (Kang et al., 1984; Machida and Saito, 1993; Uchida et al., 1996). However, so far no protease has been identified that would bind and process chitin synthase *in vivo*. In the case of *Manduca*, we have previously shown that trypsin stimulates chitin synthesis in crude midgut extracts but not in membrane fractions (Zimoch et al., 2005). When we added the soluble fraction to the membrane fractions, we could recover trypsin-mediated activation, suggesting that chitin synthase is not directly affected by trypsin but by an unknown soluble factor, which is cleaved by trypsin and interacts with chitin synthase.

Based on our previous results, we decided to screen for proteins that potentially interact with chitin synthase and thus might be involved in trypsin-mediated activation of chitin synthesis. Yeast two-hybrid screening revealed a novel chymotrypsin-like protease, CTLP1, which binds to the extracellular carboxyterminal domain of CHS2. As CTLP1 is a secretory protease that co-localizes with CHS2 at the brush border microvilli of columnar cells and is activated by tryptic cleavage, it might be part of an intestinal proteolytic signalling cascade that controls chitin synthase activity in the lepidopteran midgut.

Materials and methods

Insects

Larvae of *Manduca sexta* Linné 1763 (Lepidoptera, Sphingidae) were reared under long-day conditions (16 h of light) at 27°C using a synthetic diet modified as described previously (Bell and Joachim, 1974).

Yeast two-hybrid screening

Screening for potential interaction partners of CHS2 was done with the Matchmaker two-hybrid system (Clontech, St-Germain-en-Laye, France) according to the manufacturer's protocol and the Yeast Protocol Handbook (Clontech). The cDNAs encoding the cytoplasmic catalytic domain (B, amino acid positions 478–904) or the two carboxyterminal extracellular domains (C5 and C7, amino acid positions 1045–1244 and 1325–1524, respectively) were inserted into the yeast expression vector pGBKT7 (see Fig. 1). Yeast cells of the strain Y187 were transformed with the resulting plasmids. For construction of a cDNA library, mRNA was purified from anterior midguts of fifth instar larvae using the QuickPrep Micro mRNA purification kit (GE Amersham, Munich, Germany). First and second strand cDNA synthesis was performed as recommended by the manufacturer (Clontech).

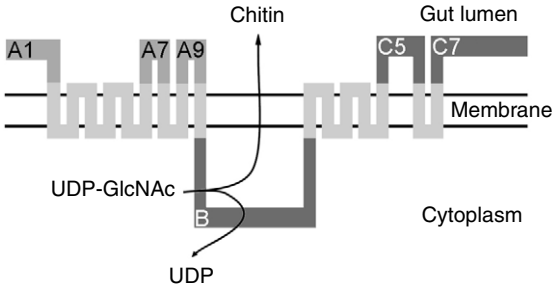

Fig. 1. Domain architecture of the midgut chitin synthase CHS2. The two black horizontal lines represent the apical brush border membrane of midgut columnar cells. Horizontal bars represent intra- or extracellular domains and vertical bars transmembrane helices. (A) Aminoterminal transmembrane region; (B) cytoplasmic catalytic domain; (C) carboxyterminal transmembrane region; the numbers refer to single domains within each region. Those domains that were tested as baits in the yeast two-hybrid system are coloured dark grey. UDP, uridine 5′-diphosphate; GlcNAc, *N*-acetyl-D-glucosamine.

Yeast AH109 cells were transformed with double-stranded cDNAs and linear pGADT7rec to obtain recombinant plasmids by homologous recombination that represent the transcriptome of the anterior midgut. Selection and testing of positively transformed AH109 cells was carried out according to the Clontech manual. To screen the midgut cDNA library for proteins that interact with the chitin synthase domains, the transformed Y187 and AH109 cells were mated and plated on SD (–His, –Leu, –Trp) agar plates and finally selected on SD (–Ade, –His, –Leu, –Trp, +X-α-Gal) plates. The mating efficiency was 30% and the calculated number of independent clones that was screened was 1.8×10^7. To test the indicated interactions between CTLP1 and CHS2, the CTLP1 cDNA was cloned into pGADT7rec and transformed into AH109 cells, which in turn were mated with Y187 cells containing the pGBKT7-CHS_{C7} plasmid. The opposite experiment was also carried out. For this purpose the CTLP1 cDNA was cloned into pGBKT7 and Y187 cells were transformed, while the cDNA encoding the CHS_{C7} domain was ligated into pGADT7 and AH109 cells were transformed.

Immunological methods

Polyclonal antibodies to the recombinant catalytic domain of the *Manduca* chitin synthase were generated previously (Zimoch and Merzendorfer, 2002). To generate anti-CTLP1 antibodies, the peptide IVGGTQAPSGSHPH (amino acid positions 41–54) was synthesized, coupled to Keyhole limpet haemocyanin and used for the immunization of rabbits (Charles River, Sulzfeld, Germany). Semi-dry electroblotting of the polyacrylamide gels onto nitrocellulose membranes (Millipore, Schwalbach, Germany) was carried out with the buffer system according to previous protocols (Kyhse-Andreson, 1984), modified by the addition of 20% methanol. Blot membranes were stained with 0.02% (v/v) Ponceau S (Sigma, Taufkirchen, Germany). Immunoblots were performed as described previously (Zimoch and Merzendorfer, 2002). The immunoreactions were carried out with polyclonal anti-CHS antibodies (1:1000 dilution), anti-CTLP1 antibodies (1:1000 dilution) or monoclonal antibodies to the V-ATPase subunit A [1:10 dilution; also named 221-9 (Klein et al., 1991)]. The secondary antibodies were anti-rabbit or anti-mouse antibodies (1:10 000 and 1:30 000 dilution, respectively; whole molecules conjugated with alkaline phosphatase, Sigma). Cryosectioning of tissues and immunocytochemistry were carried out as described previously (Zimoch and Merzendorfer, 2002). Immunoprecipitation was performed with the protein G Immunoprecipitation kit (Sigma) following the instructions of the manufacturer.

Other methods

Protein concentrations were determined by the Amido Black method (Wieczorek et al., 1990), and SDS polyacrylamide gel electrophoresis was performed according to Laemmli (Laemmli, 1970). Determination of chitin synthase activity was performed as described by Zimoch et al. (Zimoch et al., 2005). To express CTLP1 in the form of the mature protease (amino acid positions 41–281), the corresponding cDNA was ligated into pET29b (Novagen, Madison, WI, USA). Protein expression was performed in *E. coli* Rosetta (DE3) pLysS cells as described previously (Merzendorfer et al., 2000). Synthesis of RNA probes and *in situ* hybridization were performed according to previous protocols (Zimoch and Merzendorfer, 2002), with the exception that the detection of RNA probes was carried out with anti-Dig-gold antibodies (Roche) and silver enhancement (IntenSE^{TM} silver enhancement kit, GE Amersham). RNA probes were complementary to the cDNA nucleotide positions 1–178 of CTPL1 (GenBank accession no. AM419170) and 2094–2303 of CHS2 (GenBank accession no. AY821560). The missing 5′ region of CTLP1 was completed by 5′RACE using the FirstChoice RLM-RACE Kit (Ambion) and the sequence-specific primers 5′-GTAGGCACAATGATCTCCAG-3′ and 5′-GAAGGTGCACAGCTCGATGT-3′ designed on the basis of the cDNA sequences of the pGADT7rec inserts.

Results

Yeast two-hybrid screening reveals a novel chymotrypsin-like serine protease binding to the carboxyterminal domain of chitin synthase

To identify candidate proteins interacting with chitin synthase, we performed a yeast two-hybrid screen. For this purpose, we constructed a cDNA library starting with mRNA from the midguts of fifth instar larvae, and transformed the library into AH109 cells. The cDNAs encoding the soluble B, C5 and C7 domains of CHS2 (see also Fig. 1) were fused in-frame to the GAL4 DNA binding domains. Screening 1.7×10^7 clones with the C7 bait on nutritionally deficient plates yielded 29 colonies, which were still positive after quintuple selection on leucine-, histidine-, tryptophan- and adenine-deficient media containing X-α-Gal (Table 1). In contrast, screening for interaction partners of the catalytic B domain and the extracellular C5 domain did not reveal positive colonies. The plasmids from positive yeast colonies were isolated and the sequences of the cDNA inserts were analysed. Twenty-seven of the isolated plasmids contained identical cDNA inserts, encoding the carboxyterminal half of a previously unidentified serine protease. As BLAST and ClustalW (NCBI-EBI) analysis revealed highest similarities to insect chymotrypsins (see also Fig. 3), we termed the putative protein chymotrypsin-like protease 1 (CTLP1). To verify the protein interaction in yeast, we transfected Y187 cells with pGBKT7-CTLP1 and AH109 cells with pGADT7-CHS2$_{C7}$, and performed two-hybrid tests by mating and selection. As expected, the transformants grew on triple and quintuple selective media (Table 1). The converse experiment, mating Y187 cells transfected with pGBKT7-CHS2$_{C7}$ with AH109 cells transfected with pGADT7-CTLP1 also yielded positive colonies on triple and quintuple selective media (Table 1). Finally, a second independent screening for proteins interacting with the C7 domain of CHS2 yielded in total 22 colonies growing on triple and quintuple selective media. Sequencing the inserts after isolating the pGADT7 plasmids revealed that again CTLP1 cDNAs had been isolated. These results suggest that the carboxyterminal, extracellular C7 domain of CHS2 interacts with the carboxyterminal half of CTLP1 in yeast.

Isolation and sequencing of the cDNA encoding CTLP1

The isolated cDNAs were 409 base pairs (bps) in length and encoded 109 amino acids of the carboxyterminal half of CTLP1 and the complete 3′ untranslated region but lacked the aminoterminal end and the 5′ untranslated region. To complete the cDNA sequence we performed 5′ RACE. Nested PCR yielded a single product of about 600 bps, which was cloned and sequenced. The RACE product included the 5′ untranslated region and the open reading frame encoding 192 amino acids of the aminoterminal half of CTLP1 as deduced from 59 nucleotides overlapping with the 5′ end of the truncated CTLP1 cDNA. To confirm the nucleotide sequence, we amplified the complete cDNA by RT-PCR using midgut mRNA as a template. The resulting cDNA sequence was identical to the sequence that we obtained by joining the 5′ and 3′ cDNA fragments. The CTLP1 cDNA comprises 939 nucleotides encoding an open

Table 1. *Yeast two-hybrid screen to identify interaction partners of soluble CHS2 domains*

			–T	–T,H	–T,A	–L,T,H	–L,T,H,A
	Strain Y187 (Bait)	Strain AH109 (Prey)	+Gal	+Gal	+Gal	–Gal	+Gal
Screen 1	pGBKT7-B	pGADT7-cDNA library				–	–
	pGBKT7-C5	pGADT7-cDNA library				–	–
	pGBKT7-C7	pGADT7-cDNA library				+	+, blue 29 colonies
Screen 2	pGBKT7-C7	pGADT7-cDNA library				+	+, blue 22 colonies
Controls	pGBKT7-C7		+, white	–	–		
		pGBKT7-C7	+, white	–	–		
	pBKT7-CTLP1		+, white	–	–		
		pBKT7-CTLP1	+, white	–	–		
	pGBKT7-CTLP1	pGADT7-C7				+	+, blue
	pGBKT7-C7	pGADT7-CTLP1				+	+, blue

```
1   5'TACTTCGGGCTACACAATGTACGTGAAAGTAGCACTTCTGTTGGTAGCCCTCAT   54
                    M  Y  V  K  V  A  L  L  L  V  A  L  I   13
55    TGCTGGGAGCTGGGCCTTCCCAAAGCTCGAAGATGAGCAGGACATGTCCATCTT  108
      A  G  S  W  A↓F  P  K  L  E  D  E  Q  D  M  S  I  F   31
109   CTTCACGCAGCTCGATTCGAGCGCGCGTATCGTGGGTGGTACCCAGGCCCCCAG  162
      F  T  Q  L  D  S  S  A  R↓I  V  G  G  T  Q  A  P  S   49
163   CGGAAGTCACCCTCACATGGTGGCGATGACCACCGGTACCTTCATCAGGAGCTT  216
      G  S  H  P  H  M  V  A  M  T  T  G  T  F  I  R  S  F   67
217   CAGCTGTGGAGGCTCAGTTGTCGGTAGACGTTCCGTTCTGACTGCGGCTCATTG  270
      S  C  G  G  S  V  V  G  R  R  S  V  L  T  A  A  H  C   85
271   CATCGCTGCTGTTTTCAGTTTCGGTTCCCTCGCCAGTACCCTCCGCTTGACGGT  324
      I  A  A  V  F  S  F  G  S  L  A  S  T  L  R  L  T  V  103
325   CGGCACCAACTTCTGGAACCAGGGAGGCACCATGTACACCGTCGCTCGCAACAT  378
      G  T  N  F  W  N  Q  G  G  T  M  Y  T  V  A  R  N  I  121
379   AACCCACCCCCACTACGTCTCTGCGACCATCAAGAACGACATCGGTCTGTTCAT  432
      T  H  P  H  Y  V  S  A  T  I  K  N  D  I  G  L  F  I  139
433   CACTCACAACAACATCATCGACACGACTGTCGTCCGCAGCATCCCTCTTAACTT  486
      T  H  N  N  I  I  D  T  T  V  V  R  S  I  P  L  N  F  157
487   TGACTATGTGCCCGGTGGTGTTCTCACTAGAGTCGCCGGATGGGGCAGGATCAG  540
      D  Y  V  P  G  G  V  L  T  R  V  A  G  W  G  R  I  R  175
541   GACCGGCGGTGCCATCTCTCCCTCTCTGCTGGAGATCATTGTGCCTACTATCAG  594
      T  G  G  A  I  S  P  S  L  L  E  I  I  V  P  T  I  S  193
595   TGGAAGCGCATGCGTAGCCAGTGCAATCCAAGCTGGCATCGATCTGAACATGAG  648
      G  S  A  C  V  A  S  A  I  Q  A  G  I  D  L  N  M  R  211
649   ACCACCTCCCGTCGAGCCTCACATCGAGCTGTGCACCTTCCACGGTCCTAACGT  702
      P  P  P  V  E  P  H  I  E  L  C  T  F  H  G  P  N  V  229
703   AGGCACTTGTAATGGTGACTCCGGCAGCGCTCTTGCCCGCCTAGACAACGGCCA  756
      G  T  C  N  G  D  S  G  S  A  L  A  R  L  D  N  G  Q  247
757   GCAGATCGGTGTGGTATCGTGGGGCTTCCCGTGCGCACGCGGCGGTCCCGACAT  810
      Q  I  G  V  V  S  W  G  F  P  C  A  R  G  G  P  D  M  265
811   GTTCGTCAGGGTCAGCGCCTACCAATCCTGGCTGCAGCAGAGCATCGTATAATC  864
      F  V  R  V  S  A  Y  Q  S  W  L  Q  Q  S  I  V  *      282
865   TCCGAACCGAACCTGATCTTAACTGTAAATAAAATAAGCGATACCCAAAAAAAA  918

919   AAAAAAAAAAAAAAAAAAAA 3'                                 939
```

Fig. 2. Primary structure of CTPL1. cDNA sequence and deduced amino acid sequence (standard: zymogen; bold: mature protease) of the *M. sexta* CTLP1. Italic characters indicate the 5′ and 3′ untranslated regions of the cDNA and the putative signal peptide for the secretory pathway of the protein. Arrows mark the predicted cleavage sites of the signal peptidase and trypsin.

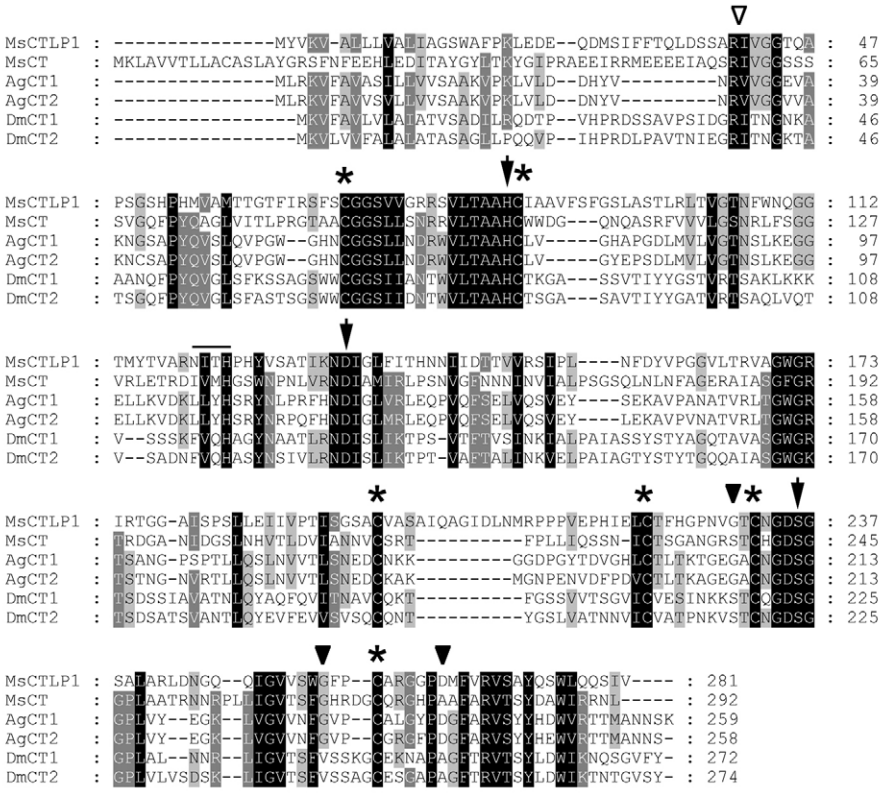

Fig. 3. ClustalW alignment of insect chymotrypsin-like proteases. Amino acids that are conserved, highly conserved or identical in all sequences are highlighted in light grey, grey or black, respectively. Open triangle, conserved trypsin cleavage site; filled triangles, conserved residues of the S1 specificity pocket; arrows, residues of the catalytic triad of serine proteases; asterisks, conserved cysteines; bar, putative *N*-glycosylation site for CTLP1. Ms, *Manduca sexta*; Ag, *Anopheles gambiae*; Dm, *Drosophila melanogaster*; CT, chymotrypsin.

reading frame of 281 amino acids, yielding a soluble protein with a deduced molecular mass of 29.8 kDa (Fig. 2, EMBL accession no. AM419170). BLAST and ClustalW analysis revealed highest similarities to unassigned insect S1A peptidases (serine proteases of the chymotrypsin family) of the MEROPS peptidase database (Fig. 3). The deduced amino acid sequence of CTLP1 showed the highest similarity to two *Anopheles gambiae* chymotrypsins (AgCT1 and AgCT2; GenBank accession nos Z18887 and Z18888), exhibiting 33% identical amino acids. The similarity to a previously reported *Manduca* chymotrypsin (MsCT, GenBank accession no. L34168) (Peterson et al., 1995) was somewhat lower, with 27% identical amino acids, and was thus comparable to that of two *Drosophila* chymotrypsin-like proteases (GenBank accession nos CG10477 and CG6483).

CTLP1 meets all essential structural requirements of digestive serine proteases, as it possesses conserved histidine, aspartate and serine residues forming the catalytic triad (Kraut, 1977; Law et al., 1977). Moreover, it contains six cysteine residues typically present in invertebrate serine proteases (Fig. 2) (Yan et al., 2001). The presence of the three amino acids glycine, glycine and aspartate in the primary specificity pocket suggests that CTLP1 may exhibit a chymotrypsin-like substrate specificity with a glycine at the bottom of the pocket as the primary determinant (Perona and Craik, 1997). However, the occurrence of an aspartate residue at the side of the pocket may alter the specificity of CTLP1 significantly. CTLP1 is a secretory protein, since the premature form contains a signal peptide, which is predicted to be cleaved at the carboxyterminal end of the amino acid at position 18 (Bendtsen et al., 2004; von Heijne, 1990). Furthermore, like all

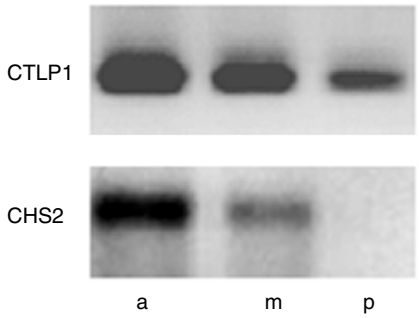

Fig. 4. Expression of CTLP1 and CHS2 in the midgut of *M. sexta*. Poly(A) RNA from the anterior (a), median (m) and posterior (p) midgut was reverse transcribed and used as a template for PCR (25 cycles) to amplify the cDNAs encoding CTLP1 and CHS2. Control reactions showed the absence of contaminating RNA and DNA (not shown).

members of this protease family, CTLP1 appears to be a zymogen proteolytically activated by tryptic cleavage between amino acid positions 40 and 41 (Fig. 3) (see also Kraut, 1977; Law et al., 1977; Lehane et al., 1996; Peterson et al., 1995).

Immunocytochemistry and co-immunoprecipitation reveal that CTLP1 co-localizes and interacts with CHS2

To determine the localization of CTLP1 and CHS2 expression, we performed RT-PCR, *in situ* hybridization and immunocytochemistry. RT-PCR indicated that both genes are mainly expressed in the anterior and median midgut of fifth instar larvae (Fig. 4). *In situ* hybridization showed, moreover, that CTLP1 and CHS2 transcripts are localized at the apical half of midgut columnar cells (Fig. 5). As the C7 domain of CHS2 faces the extracellular space, CTLP1 has to be secreted by the columnar cells in order to reach and bind to the C7 domain. To analyse the localization of CTLP1 at the protein level, we generated monospecific antibodies using a CTLP1-specific peptide as an antigen. In immunoblots, the anti-CTLP1 antibodies specifically stained the recombinant CTLP1, which was expressed as the mature protease (Fig. 6, lane 1). Its apparent molecular mass was about 30 kDa, which is in good agreement with the expected mass of the mature CTLP1 (25.3 kDa) plus that of two tags (4.3 kDa) at the amino- and carboxyterminal ends provided by the pET29b expression vector. In the gut contents, a single protein of similar size was detected indicating that CTLP1 is secreted and proteolytically processed (Fig. 6, lane 2), as is well established for zymogenic chymotrypsins (Kraut, 1977). However, the apparent molecular mass was in this case higher than expected. The decreased motility in SDS-PAGE might be explained by *N*-glycosylation, as CTLP1 exhibits the putative *N*-glycosylation site NITH at amino acid positions 120–123 (Fig. 3). Additionally, the antibodies detected a single protein band in tissue extracts of the anterior midgut (Fig. 6, lane 3), which represents the zymogenic form of CTLP1, as it exhibits a significantly higher molecular mass than the mature protease. In this case, the apparent molecular mass was also higher than the theoretical molecular mass. This finding may be explained by the activation peptide's high content of charged amino acids (32%) in contrast to that of the mature protein (13.2%), which might cause secondary structures accounting for the unusual migration properties observed by SDS-PAGE (Lepier et al., 1996).

When we analysed the distribution of CTLP1 and CHS2 in cryosections of the anterior midgut from fifth instar larvae using the anti-CTLP1 and anti-CHS antibodies (Zimoch and Merzendorfer, 2002), we detected both proteins at the brush border microvilli formed by columnar cells (Fig. 7, arrows). In

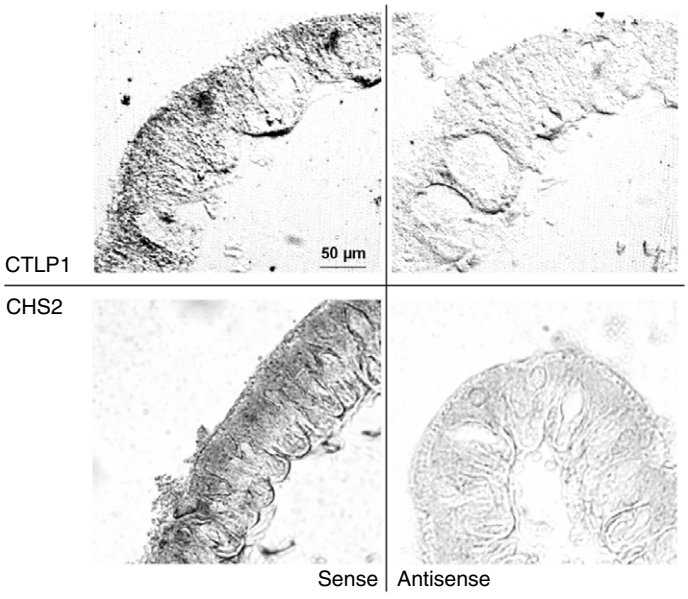

Fig. 5. Localization of CTLP1 and CHS2 mRNAs in the anterior midgut of *M. sexta*. *In situ* hybridization was performed under high stringency conditions using 20 μm cryosections of the anterior midgut and antisense RNA probes to detect the sense RNAs for CTLP1 and CHS2 (left). As a negative control, sense RNA probes were use to detect the corresponding antisense RNAs (right).

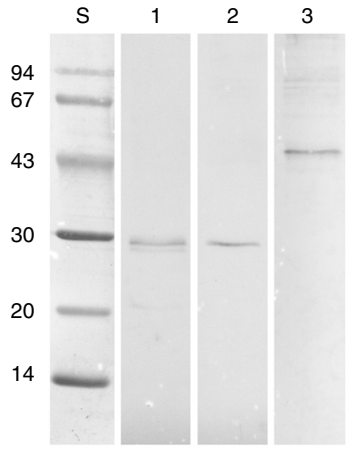

Fig. 6. Immunoblots demonstrating that CTLP1 is secreted and proteolytically processed. Proteins from CTLP1-expressing bacterial cells (1), the gut contents (2) and the anterior midgut (3) were separated by SDS-PAGE, blotted onto nitrocellulose and stained with monospecific antibodies to CTLP1. Standard proteins with molecular masses indicated in kDa are also shown (S).

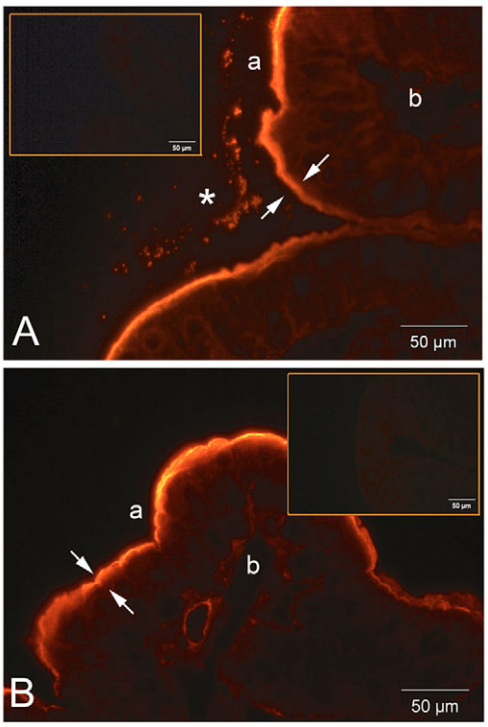

Fig. 7. Immunolocalization of CTLP1 and CHS2 in the midgut of *M. sexta*. Midgut cryosections of 10 µm thickness from fifth instar larvae were stained with either anti-CTLP1 antibodies (A) or anti-CHS antibodies (B) directed to the recombinant catalytic domain of chitin synthase (Zimoch and Merzendorfer, 2002). Arrows mark the apical brush border of the midgut epithelium, the asterisk marks immunoreactive material in the gut lumen. The insets show corresponding negative controls performed in the absence of primary antibodies. a, apical; b, basal.

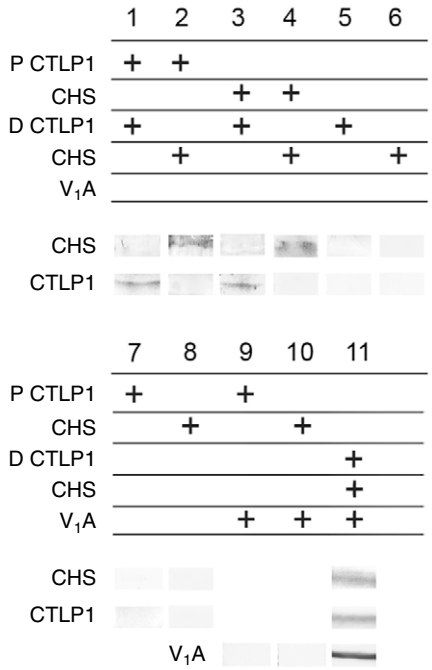

Fig. 8. Co-immunoprecipitation of CTLP1 and CHS2. For immunoprecipitation, cell lysates of the anterior midgut were incubated with the indicated precipitating antibodies (P) and then bound to protein-G–agarose. Unbound proteins were washed away, then bound proteins were eluted, separated by SDS-PAGE and analysed by immunoblotting using detecting antibodies (D) to CTLP1, CHS2 and V-ATPase subunit A (V_1A). Lanes 1–4, co-immunoprecipitation of CHS2 using anti-CTLP1 antibodies (lanes 1,2) and of CTLP1 using anti-CHS antibodies (lanes 3,4); lanes 5–8, control reactions in the absence of precipitating (lanes 5,6) or detecting antibodies (lanes 7,8); lanes 9,10, control reactions for non-specific precipitation of V-ATPase. As a positive control, midgut cell lysates were used (lane 11).

contrast to CHS2, which is a membrane-integral protein, CTLP1 is also detected within the gut contents, supporting the idea that CTLP1 is secreted into the midgut lumen (Fig. 7A, asterisk). To test whether CTPL1 and CHS2 also interact *in vitro*, we performed co-immunoprecipitation assays, taking advantage of the availability of monospecific antibodies to both proteins (Fig. 8). In contrast to negative control reactions in the absence of precipitating antibodies (Fig. 8, lanes 5 and 6), we observed co-immunoprecipitation of CHS2 when we added anti-CTLP1 antibodies to midgut cell lysates (Fig. 8, lanes 1 and 2). When we used the anti-CHS antibodies for immunoprecipitation, we observed co-immunoprecipitation of CTPL1 (Fig. 8, lanes 3 and 4). As V-ATPases are expressed in the midgut of the tobacco hornworm in exceptionally high densities (Beyenbach and Wieczorek, 2006), we used antibodies to the A subunit of the V-ATPase (V_1A) as a control for non-specific precipitation by the anti-CHS and anti-CTLP1 antibodies. As shown in Fig. 8 (lanes 9 and 10), V-ATPase A subunits were not co-immunoprecipitated by either anti-CHS or anti-CTLP1 antibodies.

Discussion

In insects, trypsin-mediated stimulation of chitin synthesis has been reported, in support of the enzyme's zymogenic nature (Cohen and Casida, 1980; Mayer et al., 1980; Ward et al., 1991). Chitin synthesis is also stimulated in the midgut of the tobacco hornworm upon trypsin treatment. However, trypsin does not directly act on chitin synthase but on a soluble factor, which in turn affects chitin synthase activity (Zimoch et al., 2005). From this finding we concluded that trypsin-mediated activation of chitin synthesis involves either an inhibitor protein of chitin synthase that is inactivated, or an activator protein that is activated by tryptic cleavage. By performing a yeast two-hybrid screen on a *Manduca* midgut cDNA library, we have now identified a novel chymotrypsin-like protease (CTLP1) that interacts with the extracellular carboxyterminal domain of CHS2 *in vitro*. Several lines of argument suggest that CTLP1 also interacts with CHS2 *in vivo*. Firstly, CTLP1 is expressed in the same tissues as CHS2, predominantly in the anterior midgut. Secondly, CTLP1 is evidently secreted into the gut lumen; thus, it can interact with the extracellular C7 domain of CHS2 (see also Fig. 1). Thirdly, CTLP1 and CHS2 co-localize at the brush border microvilli of the columnar cells. Fourthly and lastly, co-immunoprecipitation experiments demonstrated that the two proteins interact specifically in midgut cell lysates. Most important, however, is the fact that chymotrypsins are produced as zymogens, which are activated by tryptic cleavage

(Kraut, 1977; Law et al., 1977; Lehane et al., 1996). The highly conserved trypsin cleavage site is also present in the CTLP1 amino acid sequence, suggesting that the precursor of CTLP1 is activated by trypsin (Fig. 3). Tryptic cleavage of CTLP1 is additionally supported by the immunological detection of the proteolytically processed CTLP1 in the gut contents, which exhibits a significantly smaller molecular mass than CTLP1 detected in extracts from midgut epithelial cells (Fig. 6). However, as yet we have no direct evidence that CTLP1 exhibits a proteolytic activity that is induced by tryptic cleavage. Nevertheless, its binding to CHS2 may be the first clue for the participation of CTLP1 in the proteolytic stimulation of chitin synthesis (Zimoch et al., 2005). For control of the activity of midgut chitin synthase, we therefore propose a simplified and hypothetical model in which trypsin activates the zymogenic form of CTLP1, being aware that the activation of CTLP1 might require further proteases in addition to trypsin. However CTLP1 is secreted and proteolytically activated, our experimental data suggest that it binds to the extracellular C7 domain of CHS2. This interaction either could result in a direct conformational change inducing chitin synthase activity, or might be a crucial step in the processing of CHS2 involving the proteolytic activity of CTLP1. In order to test whether CTLP1 directly cleaves CHS2, we tried to express CTLP1 as an active, recombinant enzyme. Although we could purify CTLP1 from *E. coli* inclusion bodies (data not shown), we have not so far been able to refold the recombinant protease sufficiently to obtain an enzyme exhibiting significant proteolytic activity. Therefore, we tested bovine chymotrypsin for its ability to stimulate chitin synthesis in midgut extracts. As shown in Fig. 9, chitin synthesis was stimulated in the presence chymotrypsin, suggesting that a chymotrypsin-like activity is involved in the activation of chitin synthesis. Chymotrypsin stimulated chitin synthesis comparably as well as trypsin, which was shown previously to stimulate chitin synthesis in *Manduca* midgut extracts (Zimoch et al., 2005). Since Pefabloc SC (Biomol, Hamburg, Germany), a known inhibitor of serine proteases, impairs chitin synthesis, proteolytic activity appears to be necessary for chitin synthesis (Fig. 9).

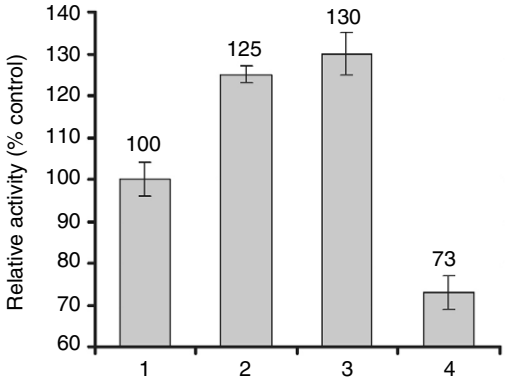

Fig. 9. Stimulation of chitin synthesis in midgut extracts by serine proteases. Relative chitin synthase activity was measured by the incorporation of *N*-acetyl-D-[U-^{14}C]glucosamine into alkali-insoluble material obtained from crude extracts of the anterior midgut of fifth instar larvae. (1) Activities of untreated extracts (0.4 nmol mg^{-1} h^{-1}) were set to 100%; (2–4) relative activities in the presence of trypsin at a concentration of 10 μg μl^{-1} (2), chymotrypsin at a concentration of 10 μg μl^{-1} (3), and 5 mmol l^{-1} Pefabloc SC serine protease inhibitor (4). Mean values (±s.e.m.) from three independent experiments are given as a percentage of the control.

The hypothetical model for the proteolytic activation of chitin synthesis is attractive, for it couples the control of chitin synthase activity in the midgut to the nutritional state of the larvae. This is because trypsins are known to be secreted in response to dietary protein entering the midgut (Law et al., 1977; Lehane et al., 1996). Even though the precise mechanisms that lead to the activation of gut proteases in response to nutrient uptake are not fully understood, particularly not in insects, it seems clear that trypsins initiate the activation of other gut proteases such as chymotrypsins (Lehane et al., 1996). Insect midgut proteases may therefore act not only to digest nutrients but also to specifically modulate non-digestive gut enzymes such as chitin synthase, which is necessary for peritrophic matrix production. This notion may also be important for a better understanding of the structural and functional diversity of insect midgut proteases, particularly observed in lepidopteran systems (Srinivasan et al., 2006). Thus, protease inhibitors produced by plants as a defence against herbivorous insects could interfere not only with digestive gut proteases but also with intestinal proteolytic signalling cascades controlling chitin synthesis and thus peritrophic matrix formation, which is necessary to protect the insect digestive tract from mechanical damage and infection by pathogens.

The authors are grateful to Dr Helmut Wieczorek for critical comments on the manuscript and to Judith Krysiak for her support in constructing and testing yeast two-hybrid vectors. The technical help of Margret Düvel and Ulla Mädler is also gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 431 and GRK 612).

References

Arakane, Y., Muthukrishnan, S., Kramer, K. J., Specht, C. A., Tomoyasu, Y., Lorenzen, M. D., Kanost, M. and Beeman, R. W. (2005). The *Tribolium* chitin synthase genes *TcCHS1* and *TcCHS2* are specialized for synthesis of epidermal cuticle and midgut peritrophic matrix. *Insect Mol. Biol.* **14**, 453-463.

Barbehenn, R. V. and Stannard, J. (2004). Antioxidant defense of the midgut epithelium by the peritrophic envelope in caterpillars. *J. Insect Physiol.* **50**, 783-790.

Bartnicki-Garcia, S. (2006). Chitosomes: past, present and future. *FEMS Yeast Res.* **6**, 957-965.

Bell, R. A. and Joachim, F. G. (1974). Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. Entomol. Soc. Am.* **69**, 365-373.

Bendtsen, J. D., Nielsen, H., von Heijne, G. and Brunak, S. (2004). Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.* **340**, 783-795.

Beyenbach, K. W. and Wieczorek, H. (2006). The V-type H$^+$ ATPase: molecular structure and function, physiological roles and regulation. *J. Exp. Biol.* **209**, 577-589.

Cabib, E. and Farkas, V. (1971). The control of morphogenesis: an enzymatic mechanism for the initiation of septum formation in yeast. *Proc. Natl. Acad. Sci. USA* **68**, 2052-2056.

Choi, W. J., Sburlati, A. and Cabib, E. (1994). Chitin synthase 3 from yeast has zymogenic properties that depend on both the *CAL1* and the *CAL3* genes. *Proc. Natl. Acad. Sci. USA* **91**, 4727-4730.

Cohen, E. and Casida, J. E. (1980). Properties of *Tribolium* gut chitin synthetase. *Pestic. Biochem. Physiol.* **13**, 121-128.

Duran, A., Bowers, B. and Cabib, E. (1975). Chitin synthetase zymogen is attached to the yeast plasma membrane. *Proc. Natl. Acad. Sci. USA* **72**, 3952-3955.

Henar Valdivieso, M., Duran, A. and Roncero, C. (1999). Chitin synthases in yeast and fungi. *EXS* **87**, 55-69.

Hogenkamp, D. G., Arakane, Y., Zimoch, L., Merzendorfer, H., Kramer, K. J., Beeman, R. W., Kanost, M. R., Specht, C. A. and Muthukrishnan, S. (2005). Chitin synthase genes in Manduca sexta: characterization of a gut-specific transcript and differential tissue expression of alternately spliced mRNAs during development. *Insect Biochem. Mol. Biol.* **35**, 529-540.

Hopkins, T. L. and Harper, M. S. (2001). Lepidopteran peritrophic membranes and effects of dietary wheat germ agglutinin on their formation and structure. *Arch. Insect Biochem. Physiol.* **47**, 100-109.

Kang, M. S., Elango, N., Mattia, E., Au-Young, J., Robbins, P. W. and Cabib, E. (1984). Isolation of chitin synthetase from *Saccharomyces cerevisiae*. Purification of an enzyme by entrapment in the reaction product. *J. Biol. Chem.* **259**, 14966-14972.

Klein, U., Löffelmann, G. and Wieczorek, H. (1991). The midgut as a model system for insect K^+-transporting epithelia: immunocytochemical localization of a vacuolar-type H^+pump. *J. Exp. Biol.* **161**, 61-75.

Kraut, J. (1977). Serine proteases: structure and mechanism of catalysis. *Annu. Rev. Biochem.* **46**, 331-358.

Kyhse-Andreson, J. (1984). Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer from proteins to nitrocellulose. *J. Biochem. Biophys. Methods* **10**, 203-209.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Law, J. H., Dunn, P. E. and Kramer, K. J. (1977). Insect proteases and peptidases. *Adv. Enzymol. Relat. Areas Mol. Biol.* **45**, 389-425.

Lehane, M. J. (1997). Peritrophic matrix structure and function. *Annu. Rev. Entomol.* **42**, 525-550.

Lehane, M. J., Müller, H. M. and Crisanti, A. (1996). Mechanisms controlling the synthesis and secretion of digestive enzymes in insects. In *Biology of the Insect Midgut* (ed. M. J. Lehane and P. F. Billingsley), pp. 195-205. Cambridge: Chapman & Hall.

Lepier, A., Gräf, R., Azuma, M., Merzendorfer, H., Harvey, W. R. and Wieczorek, H. (1996). The peripheral complex of the tobacco hornworm V-ATPase contains a novel 13-kDa subunit G. *J. Biol. Chem.* **271**, 8502-8508.

Machida, S. and Saito, M. (1993). Purification and characterization of membrane-bound chitin synthase. *J. Biol. Chem.* **268**, 1702-1707.

Mayer, R. T., Chen, A. C. and DeLoach, J. R. (1980). Characterization of a chitin synthase from the stable fly, *Stomoxys calcitrans* (L.). *Insect Biochem.* **10**, 549-556.

Merz, R. A., Horsch, M., Nyhlen, L. E. and Rast, D. M. (1999). Biochemistry of chitin synthase. *EXS* **87**, 9-37.

Merzendorfer, H. (2006). Insect chitin synthases: a review. *J. Comp. Physiol. B* **176**, 1-15.

Merzendorfer, H., Reineke, S., Zhao, X. F., Jacobmeier, B., Harvey, W. R. and Wieczorek, H. (2000). The multigene family of the tobacco hornworm V-ATPase: novel subunits a, C, D, H, and putative isoforms. *Biochim. Biophys. Acta* **1467**, 369-379.

Perona, J. J. and Craik, C. S. (1997). Evolutionary divergence of substrate specificity within the chymotrypsin-like serine protease fold. *J. Biol. Chem.* **272**, 29987-29990.

Peterson, A. M., Fernando, G. J. and Wells, M. A. (1995). Purification, characterization and cDNA sequence of an alkaline chymotrypsin from the midgut of *Manduca sexta*. *Insect Biochem. Mol. Biol.* **25**, 765-774.

Roncero, C. (2002). The genetic complexity of chitin synthesis in fungi. *Curr. Genet.* **41**, 367-378.

Ruiz-Herrera, J. and Bartnicki-Garcia, S. (1976). Proteolytic activation and inactivation of chitin synthetase from *Mucor rouxii*. *J. Gen. Microbiol.* **97**, 241-249.

Schekman, R. and Brawley, V. (1979). Localized deposition of chitin on the yeast cell surface in response to mating pheromone. *Proc. Natl. Acad. Sci. USA* **76**, 645-649.

Srinivasan, A., Giri, A. P. and Gupta, V. S. (2006). Structural and functional diversities in lepidopteran serine proteases. *Cell. Mol. Biol. Lett.* **11**, 132-154.

Uchida, Y., Shimmi, O., Sudoh, M., Arisawa, M. and Yamada-Okabe, H. (1996). Characterization of chitin synthase 2 of *Saccharomyces cerevisiae*. II: Both full size and processed enzymes are active for chitin synthesis. *J. Biochem.* **119**, 659-666.

von Heijne, G. (1990). The signal peptide. *J. Membr. Biol.* **115**, 195-201.

Ward, G. B., Mayer, R. T., Feldlaufer, M. F. and Svoboda, J. A. (1991). Gut chitin synthase and sterols from larvae of *Diaprepes abbreviatus* (Coleoptera, Curculionidae). *Arch. Insect Biochem. Physiol.* **18**, 105-117.

Wieczorek, H., Cioffi, M., Klein, U., Harvey, W. R., Schweikl, H. and Wolfersberger, M. G. (1990). Isolation of goblet cell apical membrane from tobacco hornworm midgut and purification of its vacuolar-type ATPase. *Meth. Enzymol.* **192**, 608-616.

Yan, J., Cheng, Q., Li, C. B. and Aksoy, S. (2001). Molecular characterization of two serine proteases expressed in gut tissue of the African trypanosome vector, *Glossina morsitans morsitans*. *Insect Mol. Biol.* **10**, 47-56.

Zimoch, L. and Merzendorfer, H. (2002). Immunolocalization of chitin synthase in the tobacco hornworm. *Cell Tissue Res.* **308**, 287-297.

Zimoch, L., Hogenkamp, D. G., Kramer, K. J., Muthukrishnan, S. and Merzendorfer, H. (2005). Regulation of chitin synthesis in the larval midgut of *Manduca sexta*. *Insect Biochem. Mol. Biol.* **35**, 515-527.