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THE IMMUNOPROTEIN SCOLEXIN AND ITS SYNTHESIZING SITES – THE MIDGUT EPITHELIUM AND THE EPIDERMIS*

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Scolexin is one of the bacterial induced hemolymph proteins of tobacco hornworm *(Manduca sexta)* larvae, that has hemocyte coagulation-provoking activity. The 72 kDa scolexin complex is composed of two 36 kDa subunits. To examine the protein secretory pathways in insect epithelia a polyclonal antibody was raised against the 36 kDa hemolymph protein. This MsH36 antibody recognised a 36 and a 72 kDa protein in tissue homogenates. On the basis of the characteristic labelling pattern observed on immunoblots and immunocytochemical sections we concluded that the 36 kDa protein in the hemolymph, in the midgut and in the epidermis was identical with the scolexin subunit. In present paper we report a labelling shift in the midgut epithelium between goblet and columnar cells that may be controlled by the hormonal system. A 72 kDa protein showed similar epitops and molecular weight to the scolexin complex and was detected in epidermis and in cuticle under both reducing and non-reducing conditions. Tissue localization of 36 kDa and 72 kDa MsH36 antibody labelling proteins indicated the possibility that the epidermal cells produce two kinds of scolexin-like proteins. The complex composed of 36 kDa subunits are transported basolaterally into the circulation and display hemocyte coagulation inducing activity while the 72 kDa form contains two subunits linked covalently secreted apically into the cuticle.

Keywords: Scolexin – secretory pathway – midgut – epidermis – cuticle

INTRODUCTION

Scolexin is one of the several inducible immunoprotein of the tobacco hornworm *(Manduca sexta)* larvae. It was originally described as a glycosylated hemolymph protein by Hughes et al. [9] in 1983. Its molecular mass was determined as 36 kDa on SDS polyacrylamide gel [9]. This band was found to contain at least three proteins. Two of them were inducible by bacteria [10], by yeast and lipopolysaccharide injections or baculovirus infection in larvae [5, 6]. Existence of two scolexin isoforms was detected during native PAGE analysis. Both of them consist of two 36 kDa subunits and have molecular mass of 72 kDa [11]. Unlike the inducible lysozyme and other antibacterial peptides, scolexin does not possess a bacteriolytic activity, but

*Dedicated to Professor János Kovács on the occasion of his 70th birthday.

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shows a potent hemocyte coagulation-inducing activity [15]. Combination of infection-inducibility and coagulation activity suggests that scolexin may represent the first identified member of an insect blood clotting system [7].

By their DNA sequences scolexin isoforms represent a new subfamily of chymotrypsin-like serine proteinases. According to the sequence analysis their activation mechanism seems to be different from the typical serine proteinases: activation occurs not through proteolysis of the zymogen form but via substrate or cofactor binding [7].

Epidermis is considered as a primary site of scolexin biosynthesis. Its expression is inducible and is under hormonal control, too [11]. Scolexin proved to be inducible by bacteria only in larvae but not in pupae [10]. In non-immunized larvae its expression level progressively decreases in epidermis during the feeding period of the last larval stage and becomes non detectable from the first day of the wandering period [11].

The only other tissue where the scolexin has been detected is the midgut [10, 16]. The hemocytes and the fat body cells do not synthesize its mRNA [16].

In our laboratory several antisera were developed against hemolymph proteins of *Manduca sexta* larvae to examine the secretory pathways in the insect epithelia. One of these antibodies, called as MsH36 [antibody against **36** kDa **M**anduca **s**exta **h**emolymph protein) recognised a 36 kDa scolexin subunit. In the present work we demonstrate the immunocytochemical localization of the MsH36 antibody labelled proteins in midgut cell types and the possibility of the existence of a scolexin related 72 kDa protein in the integumental epidermis and in the neighbouring cuticle.

MATERIALS AND METHODS

Experimental animals

Tobacco hornworm *(Manduca sexta)* eggs were kindly provided by Prof. S. E. Reynolds (University of Bath). Larvae were reared as described previously [2]. Different stages of development were recognised by a staging scheme adopted from Samuels and Reynolds [19].

Preparation of tissue samples for immunoblots

Hemolymph

Animals in the fifth larval stage were bled from wounded proleg into 400 µl of chilled anticoagulant buffer [22]. Hemocytes were pelleted by centrifugation (180 g for 10 minutes, Spinchron R Centrifuge, Beckman) and the supernatants were stored at –20 °C. Hemolymph samples were boiled for 2 minutes with equal volume of SDS-sample buffer [12] containing β-mercaptoethanol or without β-mercaptoethanol.

Internal organs and tissues

Chilled larvae were dissected in PBS under a stereomicroscope. Fat body and midgut were removed as described by Borhegyi et al. [2]. Isolated midguts were cut to anterior, middle and posterior parts and were handled separately. Epidermis and cuticle were prepared as described by Csikós et al. [4]. Organs were rinsed in a large volume of ice-cold homogenization buffer (100 mM sucrose, 1% dextran, 40 mM Tris maleate (pH = 7.2), 0.5 mM $MgCl_2$, 100 mM KCl, 10 mM NaCl and 10 mM phenylmethylsulfonyl fluoride [PMSF]). Tissues were homogenized in a glass-teflon homogenizer and the debris was removed by a short centrifugation. Samples were frozen and stored at –20 °C.

Isolation of MsH36 protein from hemolymph

Hemolymph of one- or two-day-old fifth instar larvae was collected as described above. The protein was separated on a 8, 10 and 12% preparative acrylamide column (Bio-Rad Prep Cell, Model 491). Collected 1.5 ml fractions were loaded on SDS polyacrylamide gel to analyze their protein content. Fractions (678–686 of 8% gel, 596–618 of 10% gel and 199–220 of 12% gel) containing exclusively the 36 kDa protein were pooled, dyalized against distilled water, concentrated in a SpeedVac system (RC10–10, Jouan) and tested for purity again on SDS analytical polyacrylamide gel.

MsH36 antibody development and its immunospecificity

100 μg of MsH36 protein was dissolved in 100 μl phosphate buffered saline (PBS) mixed with 100 μl Freund's complete adjuvant (Difco Laboratories, Detroit, MI) was injected subcutaneously in C57Black mice. Animals were boosted again three weeks later with the same amount of protein in 100 μl PBS emulsified with 100 μl Freund's incomplete adjuvant. One week later the titers of the antibodies were tested in blood samples collected from the tip of the tail. After completion of immunization, mice were sacrificed and the immunoglobulin fraction was separated from the sera with ammonium sulphate precipitation.

Immunoblotting of SDS-gels

Total homogenates were treated with SDS-sample buffer and β-mercaptoethanol. Samples prepared for examinations under non-reducing conditions were not mixed with β-mercaptoethanol. Tissue homogenates were run on Minigel (Bio-Rad Mini Protean II. System) according to the method of Laemmli [12] and electrophoretically transferred onto nitrocellulose membrane (Hybond-C Super, 0.2 μm pore size,

Amersham) in Towbin-buffer [21] (Bio-Rad Mini Trans-Blot Cell, 75 V, 1.5 h). Non-specific binding sites were blocked with 5% Carnation non-fat dry milk powder diluted in Tris-buffered saline (TBS: 0.15 M Tris-HCl, 0.5 M NaCl, pH = 7.0) for 1 hour at room temperature (RT). Membranes were incubated with the primary antibody overnight at 4 °C (dilution was 1 : 1500 in 3% milk powder/TBS). After washing three times with TTBS (TBS containing 0.5% Tween-20) the nitrocellulose sheets were incubated in the presence of the second antibody (AP-conjugated anti-mouse IgG developed in rabbit, Sigma Immuno-Chemicals) in 1 : 3000 dilution in 3% milk powder/TTBS for 1 hour at RT. Following TTBS and TBS rinsing the immunoreaction was visualised using freshly prepared NBT-BCIP (5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium, Bio-Rad) as substrate.

Immunohistochemistry for light microscopy

Animals were fixed in Bouin fixative for 6 hours at RT. Fixative was injected into the body cavity and the animals were immersed into large volume of it. After 2 hours the partly fixed body was cut into three pieces and fixed further. Fixed materials were embedded in Paraplast (Dulbecco). Five μm serial sections were cut and placed on poly-L-lysine-coated slices and dried overnight at 39 °C. After rehydration and blocking (5% Carnation non-fat dry milk powder in PBS) sections were incubated overnight at 4 °C with primary antibody diluted 1 : 100 in 3% Carnation non fat dry milk powder in TBS. After washing three times in TTBS the sections were incubated for 1 hour at RT in the presence of second antibody (same as described at immunoblotting) diluted 1 : 100 in 3% milk powder/TTBS. Slices were washed twices in TBS than TTBS and developed using NAMP-Fast-Red tablet sets (Sigma Fast, Sigma Chemical Co.). Control sections were incubated with non-immune mouse serum or only with the second antibody. These sections remained negative. The slices were embedded in Mowiol (Polysciences) and the microphotographs were taken with a Zeiss Axioskop (Germany, MC80. Microscope Camera, OPTON).

RESULTS

Isolation of the MsH36 protein and developing of the MsH36 antibody

A 36 kDa protein was isolated from the hemolymph of tobacco hornworm larvae on the first two days of the last larval stage. The isolated protein proved to be homogenous and pure on SDS polyacrylamide gel (Fig. 1A). This protein fraction was used to develop polyclonal antibody in mice. The antisera were tested on SDS treated total hemolymph samples by immunoblots: the MsH36 antibody recognised a single 36 kDa band (Fig. 1B).

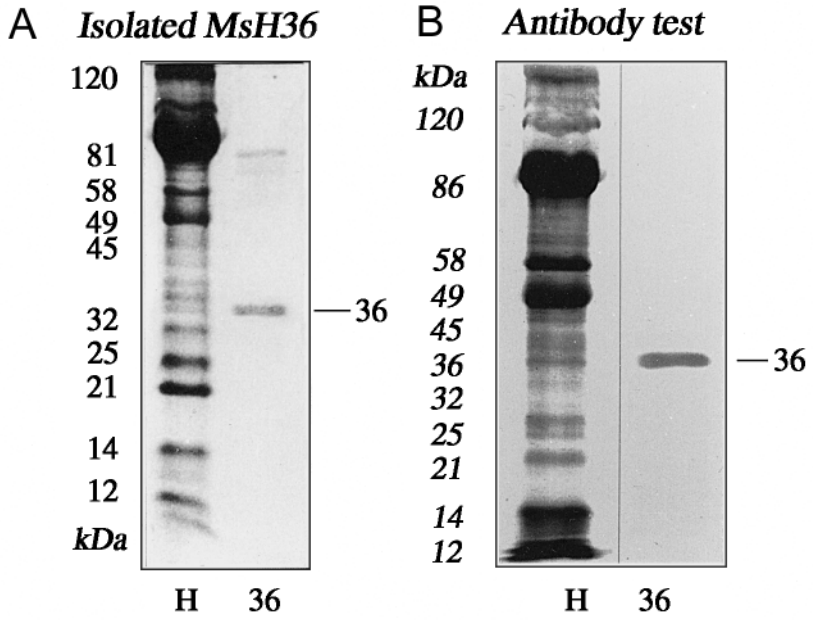

Fig. 1. A: Coomassie stained analytical SDS polyacrylamide gel of the total hemolymph sample and the isolated MsH36 protein. The band above 81 kDa in the isolated protein fraction was originated from the applied SDS. Numbers indicate the molecular mass of the hemolymph proteins. H: total hemolymph sample, 36: isolated MsH36 protein. B: Immunospecificity of the MsH36 antibody. H: total hemolymph sample (Coomassie-stained nitrocellulose), 36: MsH36 antibody labelling on total hemolymph sample. Bars indicate the position of the MsH36 protein

Tissue specificity of the MsH36 antibody on immunoblots

MsH36 protein was identified in SDS treated homogenates of the midgut, the epidermis and the cuticle. Two immunopositive bands which appeared characteristically in these samples contained β-mercaptoethanol. The original 36 kDa band was detected in the hemolymph samples during the whole last larval stage, in the epidermis of the feeding larvae and the midgut prepared from insects during the feeding and the wandering period. However, the immunolabelling was remarkably stronger in the anterior part of the midgut. An additional immunopositive signal at 72 kDa was observed in the epidermis and in the cuticle homogenates. The cuticle sample prepared from insects on the 5th day of the last larval stage contained a larger amount of 72 kDa protein than the homogenate prepared from the feeding period (Fig. 2A). Without β-mercaptoethanol only the 72 kDa bands appeared on immunoblots (Fig. 2B). The MsH36 antibody did not bind to fat body proteins.

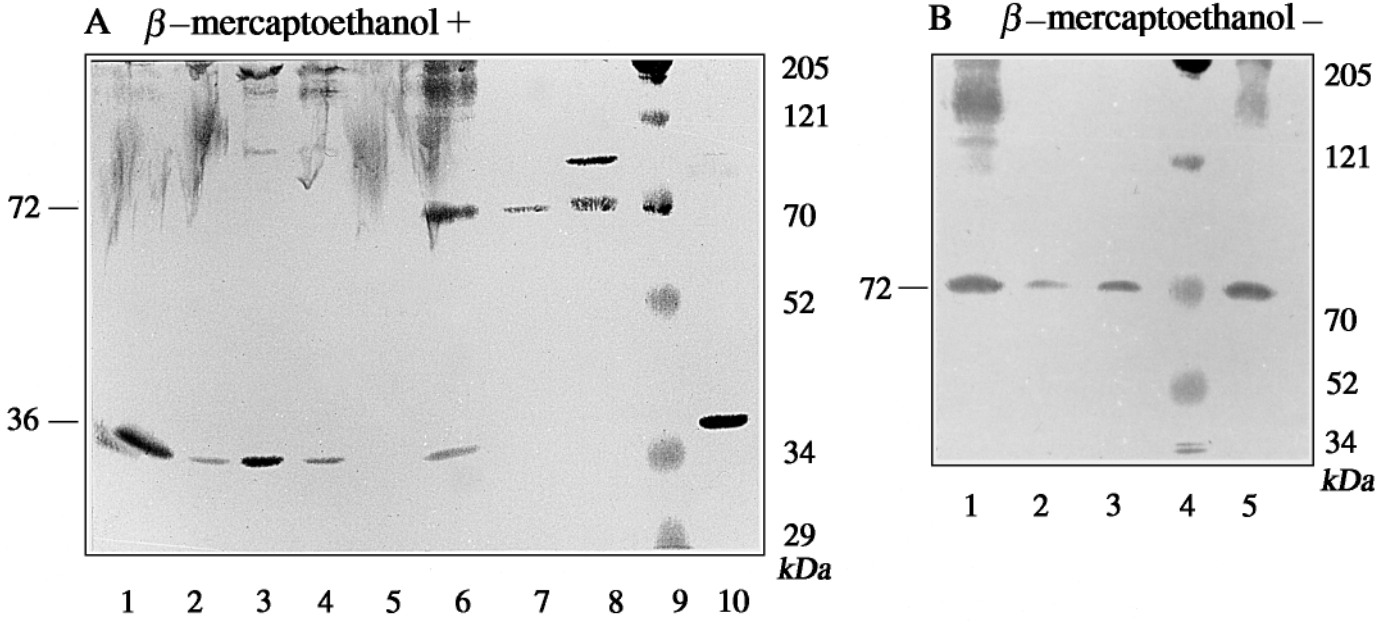

Fig. 2. Immunoblots of midgut, fat body, epidermis and cuticle homogenates. A: SDS and β-mercaptoethanol treated samples. 1: anterior part of the midgut originated from the feeding period (2nd day of the last larval stage), 2: posterior part of the same midgut, 3: anterior part of the midgut prepared from wandering larva (6th day of the last larval stage), 4: posterior part of the same midgut, 5: fat body homogenate, 6: epidermis sample originated from the feeding period (2nd day of the last larval stage), 7: integumental cuticle homogenate from the same day, 8: integumental cuticle homogenate prepared from wandering larva (6th day of the last larval stage), 9: Bio-Rad Broad Range Prestained Standard (205, 121, 70, 52, 34, 29 kDa), 10: hemolymph sample. B: SDS-treated tissue samples under non-denaturing conditions. 1: epidermis sample originated from the feeding period (2nd day of the last larval stage), 2: integumental cuticle homogenate from the same day, 3: integumental cuticle homogenate prepared from wandering larva (6th day of the last larval stage), 4: Bio-Rad Broad Range Prestained Standard (205, 121, 70, 52, 34, 29 kDa), 5: hemolymph sample originated from the 1st day of the last larval stage. Bars indicate the immunolabelled 36 and 72 kDa bands

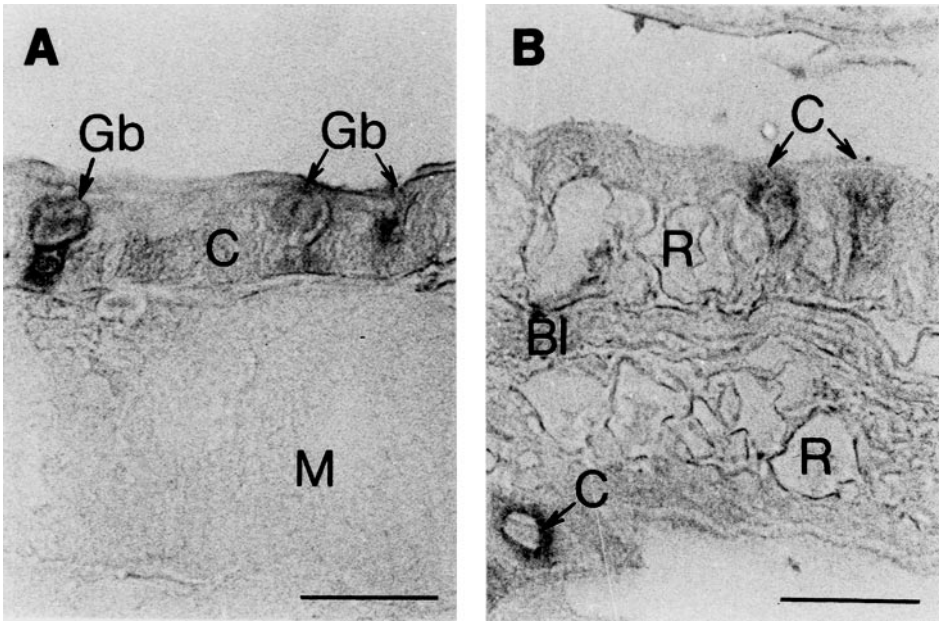

Fig. 3. MsH36 antibody labelled midgut cells. A: Anterior part of the midgut during the feeding period. Goblet cells were strongly labelled (arrows). B: Folded midgut epithelium on the 4th day of the last larval stage. Columnar cells became immunopositive (arrows). Gb: goblet cells, C: columnar cells and R: regenerative cells in the midgut epithelium, Bl: basal lamina, M: muscle. Scale bars: 50 μm

Immunocytochemistry for light microscopy

MsH36 protein was localized in the hemolymph, the hemocytes, the epithelial cells of the midgut, the Malpighian tubes (not shown), the integumental and tracheal epidermis. The labelling pattern showed typical and clear differences between cell types of the midgut epithelium. The immunopositive cells proved to be goblet cells with homogeneous cytoplasmic staining in feeding larvae, and columnar cells with cytoplasmic and granular staining in wandering insects (Fig. 3). MsH36 immunopositivity was observed in the integumental and the tracheal cuticle during the whole last larval stage (Fig. 4). After the larval-pupal moult, only the new tracheal cuticle contained the labelled protein while the integumental cuticle became negative (Fig. 4E). Immunolabelled hemocytes were observed during the wandering period. Hemolymph, epidermis, tracheal cuticle and hemocytes remained immunopositive after the larval/pupal moult (Fig. 4E). MsH36 antibody did not label the fat body lobes (Fig. 4D, E).

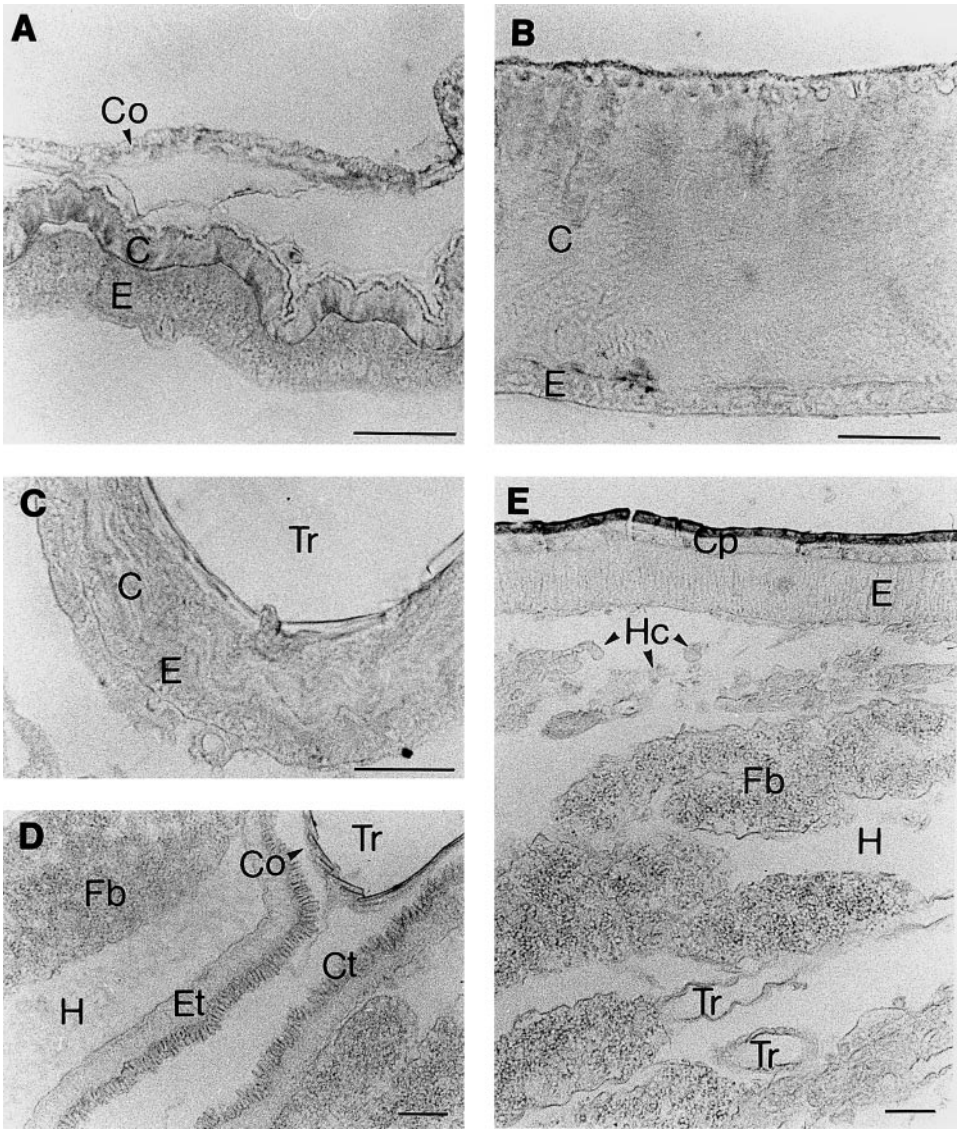

Fig. 4. MsH36 antibody labelling integumentum and tracheae. A: Integumentum at the time of the larval-larval moult between the 4th and the 5th stage. B: Integumental epidermis and cuticle and C: trachea at the beginning of the wandering period. D: Moulting trachea: new cuticular layer, epidermis and hemolymph were immunopositive. E: Integumental tissues and fat body lobes of green pupa. Epidermis, hemolymph, hemocytes and tracheae were weakly immunopositive, fat body and cuticle remained negative. Dark colour of the epicuticle was the result of its structure, not the immunoreaction. C: larval cuticle, Cp: cuticule of green pupa, Co: old cuticle (arrowheads in Figs A, D), E: epidermis, Tr: trachea, H: hemolymph, Fb: fat body, Hc: hemocytes (arrowheads in Fig. E). Scale bars: 50 μm

DISCUSSION

Hemolymph proteins synthesized by insect larval epithelial cells, midgut [17] and/or epidermis [4, 13, 20] may possess a sorting signal that directs them to a basolateral secretory pathway. To examine these signals in epithelial tissues a set of hemolymph proteins had been isolated from *Manduca sexta* larvae. Synthesis, secretion and transport of these polypeptides were followed during the last larval stage by polyclonal antibodies raised against them.

One of the hemolymph proteins, the 36 kDa MsH36 was detected in the hemolymph, in the hemocytes, in the anterior and posterior part of the midgut epithelium, in the Malphigian tubes, in the epidermis and in the integumental and tracheal cuticle. MsH36 antibody labelled bands were localized at 36 kDa in SDS and β-mercaptoethanol treated hemolymph, midgut and epidermis homogenates. A 72kDa immunopositive protein was observed in the hemolymph, in the epidermis and the in cuticle samples under non-reducing conditions. This result indicated that two 36 kDa subunits associated to form the native 72 kDa complex.

Scolexin complexes composed of two 36 kDa subunits are characterised as 72 kDa proteins [11]. The presence of the subunits' mRNA were detected in epidermal and midgut epithelial cells [10, 11, 16]. On the basis of characteristic tissue localization and subunit composition we concluded that the MsH36 protein was identical with the 36 kDa scolexin subunit and the 72 kDa protein with the scolexin complex.

The midgut of feeding larvae may be one of the target site of bacterial invasion [1, 8]. Western blot analysis confirmed the existence of the 36 kDa protein in the midgut homogenates. Detailed immunocytochemical examinations revealed a remarkable change in the labelling pattern of this epithelium: during the feeding period the goblet cells, while from the beginning of the wandering period the columnar cells were strongly immunopositive. This change ran parallel with the exchange of the larval midgut epithelium to the imaginal epithelium: the regenerative cells begin to proliferate and form a monolayer, while the larval epithelium is sloughed off into the lumen of the gut [18]. On the other hand, this alteration occurs when the hormonally regulated scolexin mRNA biosynthesis is stopped in the epidermis [11]. Taken together these data support the hypothesis that the observed switch in the midgut epithelium may be induced by the commitment peak of the 20-hydroxyecdysone.

MsH36 immunopositivity was detected in the hemocytes of wandering larvae and green pupae on the light microscopical sections. The scolexin mRNA was not detected in these cells [16], so this accumulation may be the result of its uptake from the hemolymph that might be induced by the 20-hydroxyecdysone.

Epidermal cells were labelled by MsH36 antibody in the course of the whole last larval stage. Similarly to the hemolymph, both the 36 kDa subunit and the 72 kDa complex were detected on immunoblots. However, one 72 kDa immunopositive band was observed in SDS and β-mercaptoethanol treated epidermis homogenates. This protein seemed to be 72 kDa in native form (under non-reducing conditions) although it should not be identical with known scolexin complexes due to its different molecular structure: while the scolexin complexes described earlier dissociate

into two subunits after β-mercaptoethanol treatment, the above-mentioned 72 kDa protein does not. Both the integumental and the tracheal cuticle proved to be immunopositive with the MsH36 antibody by the immunocytochemical results. The integumental cuticle homogenates contained exclusively the 72 kDa protein while the 36 kDa subunits were not detectable.

The cuticle proteins synthetized by the epidermal cells [4, 20] are secreted apically into the exoskeleton, so they are detectable only in the epithelium and in the cuticle. Other proteins accumulated in the cuticle are synthesized not exclusively by the epidermis or are produced in other tissues and organs (hemocytes, fat body). These polypeptides are secreted into the hemolymph than transported through the epidermal layer via transcytosis [4, 20], so they become detectable in the hemolymph, too. On the basis of the absence of the 72 kDa immunolabelled cuticular protein in the circulation we concluded that this protein originated from the epidermis and was identical with a one type of a 72 kDa protein detected in the epidermis homogenates.

The immunolabelling of a 36 and a 72 kDa protein in the epidermis is the result of crossreaction caused by resembling epitops or conformational motifs. Our polyclonal MsH36 antibody recognised both proteins with equal intensity. This raises the possibility of the relationship between the scolexin isoforms and this 72 kDa protein. Considering these data we suggest two explanations.

According to the first interpretation, the epidermis synthesizes two sets of 72 kDa proteins. One set is identical with the earlier described scolexin complexes that are formed by association of two 36 kDa subunits and transported basolaterally into the hemolymph. The other 72 kDa protein is composed of the same 36 kDa scolexin isoforms, but however, these subunits are linked covalently so this bond is not scissible by β-mercaptoethanol. The latter form differs only in its structure from the known scolexin complexes of the hemolymph and is secreted apically into the cuticle.

Recently, several data have been published about cuticle proteins that are transported unusually by the epidermal cells. Marcu and Locke [14] reported a 22 kDa cuticular protein secreted first basolaterally by epidermal cells in *Calpodes ethlius* larvae. In the late intermoult, during the wandering period this protein is taken up from the hemolymph by the integumental epithelium and transported through the epidermis into the cuticle. Another example was provided by Csikós et al. [4]. The 12.3 kDa cuticular protein of *Manduca sexta* is originally secreted into the cuticle layers. After the feeding period this protein is withdrawn by the epidermal cells from the cuticle and secreted into the hemolymph. These data prove that the same protein may be transported into different directions in the epidermis [20]. In addition, the three dimensional structure of the described scolexin complex and this 72 kDa scolexin related protein may be not identical. This conformational difference may be enough to distinguish these two molecules and to change the secretion pathway of them.

Finnerty et al. [7] sequenced two cDNA clones originated from epidermis that code for scolexin isoforms in 1999. The scolexin A clone corresponded to the scolexin previously isolated from hemolymph after induction by bacteria [11] or baculovirus [5]. The scolexin B proved to be new isoform with unknown function [7]

and has not yet been detected in the circulation. This data raises a possibility of another hypothesis: the epidermis may synthesize two different 72 kDa scolexin for two different functions. Scolexin A subunits associate to form a complex characterised with hemocyte coagulation-inducing activity and secreted into the hemolymph. Scolexin B molecules are linked covalently and form another 72 kDa protein complex that transported apically into the cuticle.

On the basis of their sequences, the scolexin A and B isoforms belong to the subfamily of the chymotrypsin like serine proteinases. Although the proteolytic activity of plasma-derived or recombinant scolexins have not yet been detected [7], considering the presence of both the scolexin related 72 kDa protein and the prophenoloxidase system in the cuticle is indicate some interesting functions. Scolexin sequences are unusual in lacking consensus sequence for activation peptide cleavage site, so activation may occur via substrate or cofactor binding [7]. A protein activated not by another enzyme and has putative proteinase activity is good candidate for being the first member of the enzymatic cascade in the cuticular prophenoloxidase system.

In the future, sequence analysis is required to verify the hypothesis that the MsH36 protein is identical with the scolexin and the 72 kDa protein observed in epidermis and cuticle is composed of two scolexin subunits. Identification of factors that control its biosynthesis in the epidermis is essential for the determination the exact function of this 72 kDa protein. The synthesizing rate of this scolexin-related cuticular protein may be regulated by not exclusively bacterial or viral infections. Increased immunoprotein synthesis and secretion after mechanical injury of cuticle may play an essential role and serve as an initial step for effective defense mechanism.

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