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IN VIVO BIOSYNTHESIS OF A STAGE-SPECIFIC CUTICLE GLYCOPROTEIN DURING EARLY METAMORPHOSIS OF THE MEDFLY *Ceratitis capitata*

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SUMMARY: Cuticle proteins of an insect pest, the Medfly *Ceratitis capitata*, were resolved in polyacrylamide gels and partially characterized. The pupal cuticle was found to be different from cuticles of other insects since more than 80 % w/w of the protein is a single mannose-containing polypeptide (PCG-100). The temporally-regulated *in vivo* biosynthesis and deposition of cuticle proteins was studied by microinjection of [^{35}S]methionine followed by hand dissection of pupal cuticles. The major pupal glycoprotein, PCG-100, is cuticle- and stage-specific and was the earliest to be labeled and deposited. Its synthesis was maximal at around 46 hours after pupariation and then it decreased. The deposited PCG-100 and other minor pupal cuticle proteins become non-extractable at the end of the instar (7 days after pupariation) probably by sclerotization phenomena. These results provide insight into the temporal control of gene expression programs involved in cuticle deposition during medfly metamorphosis.

The insect cuticle is an extracellular multilamellate structure covering the animal. It provides both physiochemical protection and body support. The underlying epidermis sequentially deposits the cuticle. The thick cuticle inner layer, called procuticle, is composed mainly of proteins and chitin (poly N-Acetylglucosamine) [1].

As in other dipterans, five different cuticles are formed during the development of the Medfly *Ceratitis capitata*. The first three correspond to larval instars. At the end of the third larval instar the cuticle changes by sclerotization and tannification processes [2] to become the puparium i.e. the water-proof capsule providing protection during metamorphosis. After morphogenesis has started, pupal cuticle is deposited by both larval epithelia and imaginal discs cells [3]. About 7 days after pupariation at 23 °C, the pupal cuticle separates from the underlying epithelium and a new cuticle is then deposited . The

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ABBREVIATIONS: LCP and PCP, Larval and Pupal Cuticle Protein(s), respectively; LCG and PCG, Larval and Pupal Cuticle Glycoprotein(s); SDS, Sodium Dodecyl Sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PAS, Peryodic Acid-Schiff's reagent; ConA, Concanavaline A; CBB, Coomassie Brilliant Blue.

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pharate adult is thus enclosed within three cuticular structures: the innermost adult cuticle, the shed pupal cuticle, and the transformed larval cuticle, the puparium.

Studies in *Drosophila* indicate that the molting hormone, 20-hydroxyecdisone, interacts with chromatin triggering the transcription of stage-specific genes [4,5]. In particular, *Drosophila* hormone-regulated cuticle protein genes have been described and temporally regulated cuticle deposition was shown to occur. These genes specify several different urea-soluble cuticle polypeptides [5,6,7]. The cuticle protein fraction of other insects is also heterogeneous; as in *Drosophila*, each developmental stage contains its own set of cuticle proteins [8]. The exact timing of cuticle components synthesis during metamorphosis is not well known, since most of the studies are usually carried out using tissue-culture or cell-free systems.

In the present study we report the developmentally regulated *in vivo* biosynthesis and deposition of cuticle components during *Ceratitis* metamorphosis. To our knowledge, this is the first time that microinjection of puparia with radiolabeled precursors has been accomplished to examine cuticle protein synthesis and deposition under physiological conditions. The data presented herein indicate that pupal cuticle of *Ceratitis* is distinctive since most of the proteic material is constituted by a single polypeptide instead of several ones as in other cuticles. We found that this cuticle- and stage-specific glycoprotein is synthesized early and then exported and deposited to form the new cuticle. Our results provide the basis for further studies on the control of cuticle protein gene expression during *Ceratitis* metamorphosis.

MATERIALS AND METHODS

Insects: Different wild type strains of the Medfly *Ceratitis capitata* (Wied.) were kindly provided by the CICA/INTA (Castelar). The "INTA Arg. 17" was used as wild type reference strain. Small and mass-rearing colonies were grown by standard methods on a carrot-based food [9].

Solubilization of cuticular proteins: Mass preparation of larval or pupal cuticles were performed as described [6], with minor modifications. Individual cuticles were dissected in a drop of cold Ringer solution containing Butyl-hydroxytoluene, Phenyl-thiourea and Phenyl methyl sulphonylfluoride. As discussed below, denaturing agents were required to extract proteins from cuticles. One percent SDS was used in the experiments shown here. When necessary, SDS was precipitated out from the extracts with KCl [10]. Apparently, no loss of protein occurred. However, in absence of detergent, solubilized PCPs slowly tend to aggregate. This also was observed in dialyzed samples, thus ruling out a salt-dependent effect. After detergent elimination, no precipitation was observed at 25 ^{o}C for 2 hs, whereas overnight incubation at the same temperature coagulates proteins. Routinely, proteins were stored in SDS 1%.

Protein determination was performed either by the Lowry or Bradford assay. To avoid interference, SDS was eliminated with KCl [10]. Single bands in CBB-stained gels were quantified by measuring the absorbance at 595 nm of the eluted dye [11].

Electrophoresis and glycoprotein detection: Denaturing SDS-PAGE was carried out as described [12]. Gels were stained for protein with CBB or specifically for glycoprotein by the peryodic acid-Schiff's reagent technique [13]. Mannose containing proteins were detected by Con A affinoblotting: proteins were electrotransferred to nitrocellulose sheets and located according to the method of Faye and Chrispeels [14], using 4-Chloro-1-naphtol as chromogen.

Microinjection and *in vivo* labeling: Synchronized pupae were microinjected with 0,5 μl of a solution containing 0,2 μCi of [^{35}S]methionine (800 Ci/mmol, NEN). Error in microinjection was less than 20 %. After a 4-hours pulse at 25 ^{o}C, the cuticles were carefully dissected and proteins were extracted and subjected to 10 % SDS-PAGE. Gels were stained, treated with fluorography enhancer and dried. Radioactivity was detected using X-OMAT-R film. Bands of interest were excised from the gel and the incorporated radioactivity was counted in a liquid scintillator using a toluene-0.4% Omnifluor mixture.

RESULTS AND DISCUSSION

Extractability and solubility of cuticular proteins.

Extractability of dipteran and other insect cuticle proteins has been examined previously [15,16]. Most of *Ceratitis* pupal cuticle proteins (PCP) are water-insoluble. Figure 1 shows that few PCP were extracted with low (lane 1) or high (lane 2) ionic strength solutions. Sodium dodecyl sulfate or 7M urea were required to solubilize the extractable proteins (Fig. 1, lanes 3,4 and 5). Further extraction did not increase significantly the amount of released protein (not shown). Detergent is required for both extraction from the cuticle and maintenance of proteins in solution (see methods).

Developmental profile.

The SDS-PAGE pattern of *Ceratitis* mass-isolated cuticular proteins from different instars is shown in Fig. 2 A. The bulk of LCP appears to be of low molecular weight (lane L). This is consistent with the findings in *Drosophila* [16] and other insects [8,17]. The early pattern during puparial stage (P_2-P_6) was found to correspond to pupal cuticle

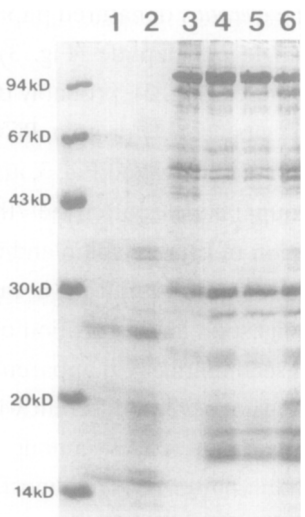

Fig. 1: Extraction of pupal cuticle proteins. SDS-PAGE (12.5 %) of proteins extracted from individual cuticles of five days old pupae. Extraction was overnight at 25 ^{o}C, with insect Ringer solution (1), NaCl 0.5M (2), SDS 1% (3), Urea 7M (4) and SDS 1% plus urea 7M (5), or 10 min. at 100^{o}C, with sample buffer (6).

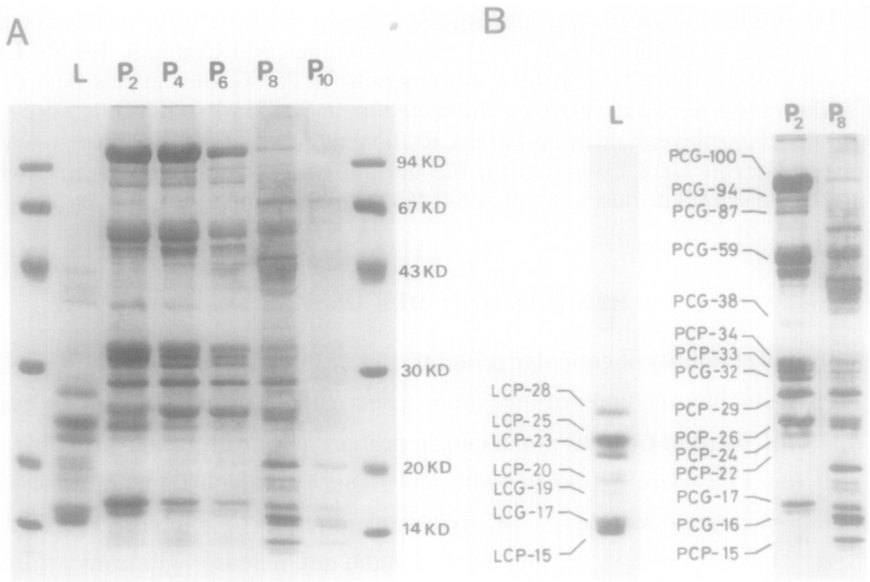

Fig. 2: Developmental profile. **A**: Electrophoresis of denatured proteins extracted from mass isolated cuticles of third instar larvae (L), pupae (P_2-P_6) and pharate adult (P_8-P_{10}).The numbers indicate days after pupariation.(SDS-PAGE 12.5%). **B**: Same as in **A** but indicating the apparent size of the denatured representative proteins or glycoproteins from 3rd larval and pupal instar (LCPs, LCGs, PCPs and PCGs respectively). Early pharate adult cuticle protein pattern (P_8) was shown for comparison.

material, whereas the late pattern (P_8-P_{10}) corresponds to pharate adult cuticle. This was clearly demonstrated by hand dissection of cuticles through the life cycle (data not shown). As above mentioned, cuticles of insects studied up to now contain several proteins [7,8,17]. Surprisingly, more than 80 % of the *Ceratitis* denatured pupal cuticle protein behaves in one-dimension electrophoresis as a single polypeptide (Fig. 3). Massive preparations of PCP become enriched in minor polypeptides (Fig. 2), probably due to the flotation method used for cuticle isolation [6]. This major pupal cuticle protein, PCG-100, was the first one detected at the beginning of the pupal instar (see below and Fig. 3, arrow, 46 hs., lane A). No proteins whatsoever were extracted from pupal cuticles after the 7th day of the puparial stage. This should be attributed to the formation of protein-chitin and/or protein-protein cross-linkages due to sclerotization phenomena [18] since significant breakdown by putative ecdisial fluid enzimes was not detected. Thus, digestion and resorption of cuticle that has been proposed to be a generalized event during insect molting [19] apparently do not occurs.

Cuticle mass preparations of several different wild-type lines of *Ceratitis capitata* were analyzed to detect putative differences among populations in (glyco)protein composition. Pupal and pharate adult patterns were almost identical in all the tested strains (not shown). Furthermore, hand dissected cuticles from more than 50 pupae from a wild type population were individually analyzed by SDS-PAGE, and no substantial variations in the major PCP were observed within the population (not shown). When the same studies were performed in two strains of cuticle color mutants, **niger** (nig) and **Black pupa** (B)[20],

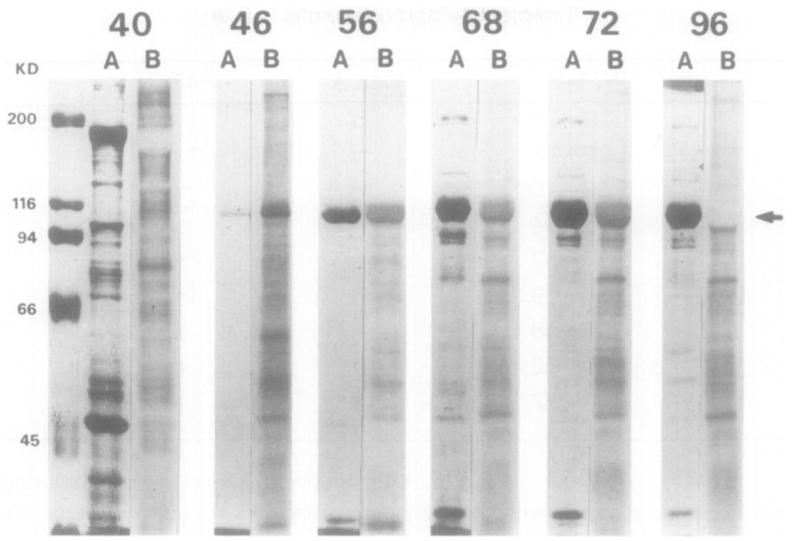

Fig. 3: Synthesis of ^{35}S-labeled cuticle proteins. Synchronized pupae were microinjected and pulse-labeled for 4 hs. at 40 to 96 hours after pupariation. Proteins from dissected cuticles (4 per lane) were extracted and separated in SDS-PAGE (Methods). **A**: lanes stained with CBB. **B**: corresponding fluorography.

only slight differences in mobility of some minor bands were observed (not shown). Thus, the bands seen in SDS-PAGE when mass-prepared cuticle proteins were analyzed are apparently not due to the overlapping of polymorphisms. Therefore, the one-dimensional electrophoresis pattern of the main extractable larval, pupal and pharate adult denatured (glyco)proteins was established (Fig. 2B). (Glycosylation was assessed as described below). PCG-100 was found to be present only in pupal cuticles and in all the above mentioned strains.

Mannosylated pupal cuticle proteins.

Table I summarizes the results of tests aimed at detection of glycosyl moieties. Mannose-containing bands were located by affinoblotting with Concanavaline A and visualized using the peroxidase/4-Chloro-1-naphtol reaction. The presence of α-bound mannoses was confirmed by digestion with mannosidase (not shown). Larval proteins did not bind Con A but two of them, LCG-17 and LCG-19 seem to contain saccharides as judged by the PAS stain. Among pupal proteins, PCG-59, PCG-87, PCG-94 and PCG-100 were found to be mannoproteins. PCG-100 was found to be highly Con A positive (table I). It seems to be an Asn-bound glycoprotein since preliminary results indicate that it is sensitive to β-(1,4)endo-Acetylglucosaminidase H digestion.

Spatial expression of PCP.

It is accepted that dipteran pupal cuticle is synthesized by two different cell lineages: a) the imaginal cells which synthesize the cephalothoracic cuticle (imaginal discs) as well as discrete parts of the abdominal cuticle (histoblasts), and b) the larval cells which form most of the abdominal epithelium [21]. The banding pattern of PCP extracted from different body regions was analyzed (not shown). A subset of minor low molecular weight proteins

Table I: Glycoprotein identification

Protein	Con A	PAS
LCG–19	–	++
LCG–17	–	++
PCG–100*	++	–
PCG–94	+	–
PCG–87	+	–
PCG–59	+	+
PCG–38	–	+
PCG–32	–	+
PCG–17	–	+

Con A binding was assessed by affinobloting. PAS indicates the staining of gels with the periodic acid-Schiff's reagent technique. Symbols indicate no (-), low (+) or high (++) densitometric signal. (*) sensitive to digestion with endo-β-N-acetylglucosaminidase H.

identified as PCP-22, PCG-17, PCG-16 and PCP-15, was detected only in abdominal cuticle, thus suggesting that they might be synthesized by cells of larval lineage. A similar situation was described for *Drosophila*, where LCP are of low molecular weight and inmunologically related to (low molecular weight) abdominal PCP [6]. However, PCG-100 was found to be present in all three body regions (not shown). Abdominal expression of this glycoprotein is probably due to the imaginal cell lineage, but the possibility that the corresponding gene might be activated in larval cell lineage cannot be ruled out yet.

***In vivo* synthesis of PCG-100.**

Several authors previously reported that insect cuticle proteins lack sulfur [22] and do not incorporate [^{35}S]Methionine [23]. However, we were able to incorporate [^{35}S]-Methionine *in vivo* into several *Ceratitis* pupal cuticular proteins. As major cuticle component, PCG-100 was found to be the earliest and most strongly labelled PCP (Fig. 3). Figure 4 shows both the time dependent rate of synthesis of PCG-100 and its accumulation through the pupal instar. Synthesis occurs mostly from 40 to 56 hours after pupariation and later decays (Fig. 4). The deposited PCG-100 could be extracted up to the end of the pupal instar (7 days) when it becomes non extractable, probably by sclerotization phenomena. The beginning of synthesis of PCG-100 is the earliest detectable biochemical event marking the initiation of pupal procuticle deposition in *Ceratitis*. Expression of this glycoprotein was found only in pupal cuticle; therefore, PCG-100 could be considered a developmentally regulated specific instar marker. We are purifying PCG-100 in order to prepare antibodies and to investigate if it shares common epitopes with other cuticle proteins.

In summary, the results presented here extend the knowledge on insect cuticle protein to an important pest, the Medfly, *Ceratitis capitata*. The fact that *Ceratitis* pupal

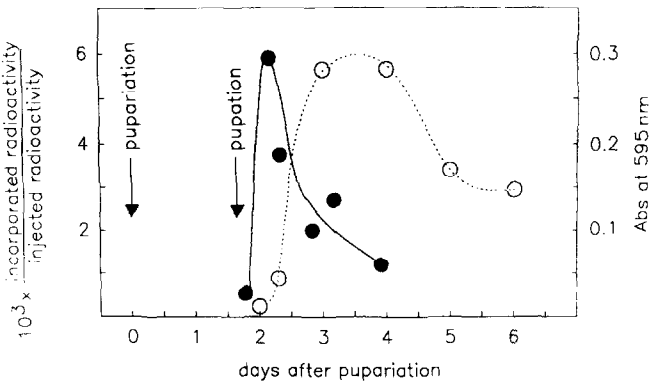

Fig. 4: Synthesis and accumulation of PCG-100. Radioactivity incorporated into PCG-100 bands excised from gels as in Fig. 3 was measured and related to total injected precursor. Each point (●) represents the average of at least 4 injected individuals of the same age. Accumulation of PCG-100 in cuticles was measured by excision of the CBB-stained band and quantification of the eluted dye (o) by spectroscopy (methods).

cuticle is constituted mostly by a single protein was unexpected since it is well known that the number of major cuticle proteins range from less than ten in *Drosophila* [16] or *Dacus* [8] to many more in other insects [24]. Thus, the synthesis and extracellular deposition of the main pupal cuticle polypeptide, PCG-100 was examined as a model for cuticle formation. It is important to point-out that puparia microinjection allowed us to pulse-label cuticle components under physiological levels of hormones that control metamorphosis, as opposed to the usual protocols using in vitro techniques [5-9,17,23,24]. As far as we know, this is the first time that microinjection has been used to radiolabel a fly pupal cuticle. From data as in Fig. 3 and microscope observations we can postulate that epicuticle material is first deposited to form a macroscopic membrane-like structure (40 hours after pupariation). Then, procuticle material i.e. chitin (not shown) and PCG-100 (Fig. 3, 46 hs.) stars to be synthesized and exported. Figures 3 and 4 strongly suggest that the whole process of synthesis, traffic inside the epithelial cell, export and deposition takes no more than three hours under strict physiological conditions at 25 °C.

These results provide a useful system for further studies on the molecular events involving the synthesis and deposition of cuticle components. Our data support the notion that *Ceratitis* genomic programs involved in cuticle protein genes expression are similar to those of evolutionary distant flies like *Drosophila* and *Lucilia* [7,16,17]. This, in turn, suggests that regulation of "metamorphosis genes" was probably conserved through the evolution of Dipterans.

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