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Original article

In vitro secretion of ecdysteroid-dependent proteins and of a 70 kDa subunit reactive to anti-prophenoloxidase serum by *Apis mellifera* integument

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Abstract – A tissue culture approach was used to monitor changes in protein secretion by integuments from *Apis mellifera* along the pupal stage. A dramatic change in epidermal protein secretion was correlated with the variation in endogenous ecdysteroid from a low level at the beginning of the pupal stage to a maximum level in the middle of this stage. Only a fraction of these proteins, however, turned out to be directly regulated by ecdysteroids when we exposed cultured integument to 20-hydroxyecdysone. An antiserum raised against a honey bee activated prophenoloxidase (proPO) found in hemolymph recognized a 70 kDa subunit in pupal integument incubations, strongly suggesting that honey bee epidermis synthesizes a genuine proPO. The 70 kDa subunit is not developmentally regulated by the ecdysteroid titer. Its constitutive expression throughout the pupal stage makes it difficult to reconcile the function of this protein with progressive cuticle pigmentation in late honey bee pupae.

***Apis mellifera* / integument / epidermis / cuticle / cuticular protein / prophenoloxidase**

1. INTRODUCTION

At the larval-pupal transition, the epidermal cells of holometabolous insects switch their pattern of protein expression, demonstrating that specific genes are inactivated whereas others initiate transcription. Thus, as development proceeds, epidermal cells synthesize and secrete different proteins in order to make stage-specific cuticles that differ considerably in structure (Cox and Willis, 1985; Kiely and Riddiford, 1985). Larval- or pupal-specific proteins have been identified for different insects (Nakato et al., 1990, 1994; Ochieng et al., 1993; Binger and Willis, 1994; Lampe and Willis, 1994; Sridhara, 1994; Rebers et al., 1997; Charles et al., 1998; Dotson et al., 1998). In *Manduca sexta* (Hiruma et al., 1991), *Drosophila melanogaster* (Apple and Fristrom, 1991) and *Bombyx mori* (Nakato et al., 1992), cuticle protein gene expression has been shown to be under the control of ecdysteroids and juvenile hormone. It is known that a pulse of ecdysteroids in the

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presence of juvenile hormone coordinates the synthesis of cuticle proteins during the larval molts.

Less attention, however, has been paid to the change in epidermal protein expression pattern within the pupal stage, in which adult exoskeleton differentiation takes place driven by ecdysteroids, in absence of juvenile hormone. The characterization of the sequential epidermal protein patterns during the pupal stage permits the identification of developmentally regulated protein expression. If the timed modulation of ecdysteroid titer within this stage is known, as is the case for *Apis mellifera* (Feldlaufer et al., 1985; Zufelato et al., 2000), a correlation between the variation in the pattern of protein expression and different hormone levels can be established. In this respect, stage-specific proteins can in turn serve as markers for the actual hormonal titer.

In previous studies (Zufelato et al., 2000; Santos et al., 2001), we showed that injection of 20-hydroxyecdysone (20E) in honey bee pupae promoted a prolongation in the time of expression of specific low molecular weight epidermal proteins, which normally cease to be expressed at the end of the pupal stage. 20E injection in honey bee pupae also arrested pigmentation and hardening of the cuticle. These effects were attributed to the maintenance of a high ecdysteroid level favored by the injection at a time when this hormone normally decreases in hemolymph. There are examples in other insects showing that a decline in ecdysteroid titer is required for the late events of cuticle differentiation and adult eclosion. Treatment with 20E delayed adult eclosion in *Manduca sexta* (Truman, 1981; Truman et al., 1983) and *Tenebrio molitor* (Sláma, 1980 in Truman et al., 1983). By reducing ecdysteroid levels in *M. sexta* through abdomen ligation, Schwartz and Truman (1983) observed acceleration in abdominal tissue differentiation, an effect reversed by injection of 20E.

The present study was conducted to compare the patterns of protein secretion by the honey bee epidermis at different stages of pupal development, characterized by low, high or declining endogenous ecdysteroid levels. The goal was to identify specific epidermal proteins regulated by this hormone as a first step in the study of developmentally regulated pupal-specific genes. For this purpose, an in vitro incubation system for honey bee integument was used to screen epidermal proteins synthesized de novo and secreted in the presence or absence of 20E. Also, since cuticle differentiation during the pupal stage requires melanin synthesis by epidermal cells, the in vitro synthesis and secretion of prophenoloxidase (proPO) by the epidermis was investigated using an antibody against a hemolymph proPO. ProPO is the inactive precursor of phenoloxidase (PO), a key enzyme for melanin synthesis in arthropods (Ashida and Brey, 1995).

2. MATERIALS AND METHODS

2.1. Staged pupae

Apis mellifera L. pupae were collected from colonies of Africanized stocks kept in the apiary of the Department of Genetics, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Brazil. The pupal phases used were distinguished by eye and cuticle color according to the criteria established by Michelette and Soares (1993) for Africanized honey bees. In the early pupal phases eye color develops from white (Pw) to pink (Pp) and finally brown (Pb), while the pupae still show an unpigmented cuticle. The older brown-eyed pupae (Pbl, Pbm and Pbd) progressively show a darker body due to melanin deposition in the cuticle.

2.2. In vitro incubation of the integument

Staged pupae were surface-sterilized by rapid immersion in 70% alcohol and their abdominal dorsal integuments were dissected in Ringer saline. The fat body adherent to the integuments was carefully removed and discarded. The resulting cuticles with their subjacent epidermis were incubated for 15–30 min in complete culture medium (2 pieces/mL) developed for bee tissues (Rachinsky and Hartfelder, 1998), and then transferred to 1 mL of incubation medium (complete medium without leucine), containing 7.5–8 μCi L-[4, 5-^{3}H] leucine (5 mCi/mmol, Amersham/Pharmacia).

For each set of experiments, 5 μg of 20E in 2.5 μL ethanol (10^{-5} M in incubation media) was added to half of the incubations and the other half received 2.5 μL ethanol as control. After culturing for 25 h at 34 °C under shaking, the incubation media and integuments were separately collected. Incubation media were centrifuged at 10 000 *g* for 10 min at 4–7 °C. The integuments were homogenized

(2 integuments/40 μL H_2O) before centrifuging at these same conditions.

2.3. TCA precipitation

Aliquots of incubation media or integument extracts were mixed (3:1, v/v) with 0.1% bovine serum albumin in 0.9% NaCl before adding 15% TCA. After 45 min on ice, and centrifugation at 10 000 *g* at 4–7 °C for 2 min, the pellets were washed twice with 10% TCA.

2.4. Quantification of proteins synthesized de novo and secreted by the epidermis in vitro

The TCA-pellets were left in 100 μL tissue solubilizer (Serva) overnight at room temperature. Radioactivity was then quantified using a Scintillation Counter (LS 6500, Beckman) after adding 5 μL glacial acetic acid and 1 mL 0.5% 2,5-diphenyl-oxazole in toluol.

2.5. Immunoprecipitation

Pre-immune rabbit serum (20 μL) was added to 100 μL aliquots of the culture media where the integuments had been incubated, or to integument extracts. After 60 min at 4 °C, 20 μL protein A (*S. aureus* cell suspension) was added to remove complexed proteins. The mixture was centrifuged at 12 000 *g* for 5 min, and 20 μL anti-proPO serum were added to the supernatant. After 180 min at 4 °C, protein A (30 μL) was again added to the mixture, which was maintained at 4 °C for 60 min more. After centrifuging under the same conditions as mentioned above, pellets were washed three times in 50 mM Tris, pH 8.0, 0.5 M NaCl and 1% NP40.

2.6. Electrophoresis and fluorography of TCA- and immunoprecipitated samples

SDS-PAGE was performed according to Laemmli (1970), with some modifications. TCA-precipitated proteins (12 000 cpm) were neutralized in 3 μL 1N NaOH, and mixed with 17 μL Laemmli's SDS-sample buffer. Immunoprecipitated proteins were dissolved in 20 μL of the same sample buffer. After boiling for 2 min, proteins were separated on 7–15% polyacrylamide gels (100 × 120 × 0.9 mm), prepared without SDS, which was added only to the running (0.1%) and sample buffers (2%). Electrophoresis was carried out at a constant current of 15 mA at 7–10 °C for 2:30 h. After electrophoresis, the gels were fixed in glacial acetic acid for 10 min, incubated in 20% 2,5-diphenyl-oxazole in glacial acetic acid for 90 min, washed in water, and dried (Skinner and Griswold, 1983). Radioactive proteins were detected after exposing the gels to X-OMAT AR film (Kodak) at –80 °C for 4 to 15 days. The molecular weight of the de novo synthesized proteins was determined by comparison with known ^{14}C-labeled standards.

2.7. Electrophoresis of hemolymph

Hemolymph was collected from an incision in the dorsal abdominal cuticle. Hemolymph from several Pbd pupae was pooled and centrifuged at 15 000 *g* for 5 min at 10 °C. The supernatants were mixed with sample buffer (Laemmli, 1970), and used for SDS-PAGE as described above. Since the hemolymph samples were not heated, the procedure is referred to here as non-denaturing electrophoresis. The gels were stained with 1 mM L-Dopa in 0.2 M sodium acetate buffer, pH 6.0. For convenience, this L-Dopa activity is referred to here as activated proPO.

The activated proPO bands detected on the gels were cut out, frozen in liquid nitrogen, pulverized, and extracted by homogenizing the gel pieces in water. After centrifugation at 15 000 *g* for 10 min at 4–10 °C, the supernatants were mixed with sample buffer and boiled for 2 min. Following a new electrophoresis under the same conditions as described above, the gels were stained with silver (Caetano-Anollés and Gresshoff, 1994). The molecular weight of the proPO subunits was determined by comparison with known standards.

2.8. Preparation of antibody against prophenoloxidase (anti-proPO)

Hemolymph from Pbd pupae was used as the proPO source for rabbit immunization. Activated proPO bands were cut out of the polyacrylamide gels stained with L-Dopa, frozen in liquid nitrogen and pulverized. The enzyme was extracted by homogenizing the gel pieces in water and by centrifugation at 15 000 *g* for 10 min at 4–10 °C. The supernatant (250 μL) was added to 450 μL of 0.9% NaCl and 700 μL of complete Freund adjuvant. This mixture was emulsified and a volume containing approximately 30 μg protein, measured using bicinchoninic acid (Smith et al., 1985), was injected subcutaneously into female rabbits. The injections were repeated twice at one week intervals. Subsequently, a booster dose of the enzyme preparation was injected, and one week later the rabbits were bled. The serum (anti-proPO) was separated by centrifugation at 400 *g* for 5 min at 4–10 °C.

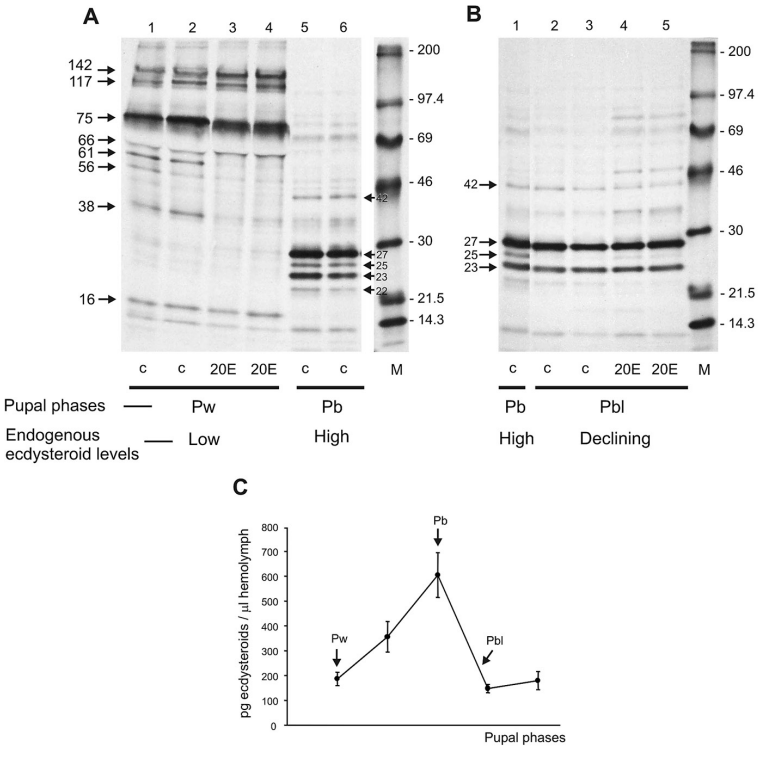

Figure 1. (A, B) Pattern of protein secretion by integuments from Pw, Pb and Pbl pupae. SDS-PAGE-fluorography of TCA-precipitated proteins synthesized de novo and secreted in vitro. Integuments were incubated during 25 h in the absence (c) or presence of 5 µg 20-hydroxyecdysone (20E). All samples were loaded at 12 000 cpm. Arrows indicate the proteins specifically mentioned in the text (see Results). Pw and Pb: pupae at the beginning and in the middle of the pupal stage showing still unpigmented cuticle; Pbl: pupae initiating cuticle pigmentation. Below the fluorographies, the endogenous ecdysteroid titer (low, high and declining) of each studied pupal stage is indicated, and the measurements obtained by Zufelato et al. (2000) are showed in (C) in order to set the times that Pw, Pb and Pbl integuments were explanted (indicated by arrows), and the specific hormone titer of each pupal phase studied. Each point of the graph corresponds to the mean and standard error of 6 samples analyzed.

2.9. Western blot

Integuments and hemolymph from staged pupae were separately pooled. Integuments were homogenized (6 integuments/50 µL water), and these extracts as wel as the hemolymph were centrifuged at 10 000 *g* for 2 min at 10 °C. The supernatants were mixed with sample buffer, heated for 2 min at 100 °C and used for SDS-PAGE. The specificity of the antibody (anti-proPO) was tested by Western blotting after transferring hemolymph or integument proteins from polyacrylamide gels onto PVDF membranes (Millipore) using the method of Towbin et al. (1979). A secondary pig anti-rabbit immunoglobulin (Dako, Denmark) antibody labeled with peroxidase and developed with diaminobenzidine was used for the detection of the primary antibody bound to proPO.

3. RESULTS

3.1. Synthesis and secretion of epidermal proteins: dependence on pupal developmental timing and 20E

The proteins secreted by the epidermis from Pw and Pb pupae are markedly different. Heavier proteins (or polypeptides) were mainly synthesized and secreted by Pw epidermis (Fig. 1A, lanes 1 and 2), whereas epidermis from the older Pb pupae secreted a set of low molecular weight proteins (Fig. 1A, lanes 5 and 6; Fig. 1B, lane 1). This change in protein pattern as development proceeds is

correlated with the variation in endogenous ecdysteroid level, indicated below Figures 1A and 1B, and shown in detail in Figure 1C. Taken together, Figures 1A and 1B suggest that the hormone peak in Pb pupae specifies the change in the pattern of secreted proteins.

Only the pupal phase-specific proteins (indicated by arrows in Figs. 1A and 1B), were consistently observed and considered in this study. Figure 1A shows that the secretion of Pw-specific proteins was inhibited (142, 117, 75, 66, 61, and 16 kDa proteins) or partially inhibited (56 and 38 kDa proteins) in Pb epidermis exposed to the high level of endogenous ecdysteroid (compare lanes 1, 2 with lanes 5, 6). However, exposure of Pw epidermis to 20E in vitro (premature exposure to 20E) failed to inhibit the synthesis and secretion of the majority of its specific proteins (compare lanes 1 and 2 with lanes 3 and 4 in Fig. 1A). But some proteins responded to 20E added to the in vitro incubations. In Figure 1A, lanes 1 and 2 show the 61 kDa protein secreted by Pw epidermis, which had not yet been exposed to the endogenous ecdysteroid peak. The secretion of this protein was inhibited when 20E was added to the incubations (Fig. 1A, lanes 3 and 4). Consistent with this result, the 61 kDa protein also was not secreted by Pb epidermis (Fig. 1A, lanes 5 and 6; Fig. 1B, lane 1), thus providing evidence for its negative control by ecdysteroids. The secretion of the 56 and 38 kDa proteins was also inhibited by 20E (compare lanes 1 and 2 with lanes 3 and 4 in Fig. 1A), although partially. Differently from the 61 kDa protein that completely disappeared from Pb epidermal secretion, the 56 and 38 kDa proteins were still evident, but as weaker bands (Fig. 1A, lanes 5 and 6).

Figure 1A also shows that the premature exposure to 20E did not induce the secretion of the low molecular weight proteins typical of older (Pb and Pbl) pupal stages (compare lanes 5 and 6 with lanes 3 and 4). However, the secretion of one of these low molecular weight proteins shown to be dependent on high levels of endogenous ecdysteroids. The 25 kDa protein was secreted only by Pb epidermis, exposed to high endogenous ecdysteroid titer (Fig. 1A, lanes 5 and 6; Fig. 1B, lane 1). This protein stopped to being secreted by Pbl epidermis, when the ecdysteroid titer decreased (Fig. 1B, lanes 2 and 3), and appeared to be again induced, although not fully, in the presence of exogenous 20E (Fig. 1B, lanes 4, 5).

In contrast to the transiently secreted 25 kDa protein, the other low molecular weight proteins (42, 27, 23, and 22 kDa) continued to be secreted even when the ecdysteroid level declined (Fig. 1B, lanes 2 and 3), indicating that their secretion was permanently activated.

Taken together, the above results showed (1) a clear change in the epidermal protein secretion from Pw to Pb pupal phases, characterized by low and high endogenous ecdysteroid levels, respectively; (2) that some Pw-specific proteins (61, 56 and 38 kDa) are downregulated by 20E, and (3) that the secretion of the 25 kDa protein occurs and is maintained only under high endogenous ecdysteroid titer.

It is important to note that Pb epidermis (exposed to high ecdysteroid level in vivo, prior dissection) incubated in absence of 20E (Fig. 1A, lanes 5 and 6; Fig. 1B, lane 1) showed identical secretion pattern when incubated in presence of 20E. For simplification of Figure 1, the lanes corresponding to Pb incubated with 20E were not shown.

3.2. Quantification of epidermal proteins secreted in vitro: correlation with the pupal endogenous ecdysteroid level and 20E added in vitro

Figure 2 shows that integuments from Pb pupae secreted into the incubation medium significantly higher levels of total protein than integuments from Pw or Pbl pupae. Since the endogenous titer of ecdysteroids is higher in Pb than in Pw or Pbl pupae, a correlation between high endogenous hormone level and high protein secretion could be established. However, when 20E was added to Pw and Pbl integuments incubated in vitro, their protein secretion activity did not change significantly (Fig. 2). Also, Pb integuments exposed to exogenous 20E did not significantly change its secretory activity (data not shown).

Even without a significant effect on total protein secretion, 20E changed the secretion of specific epidermal proteins, as described above. When comparing Figure 2 to Figure 1, it is important to note that the difference in the quantity of proteins secreted in vitro by pupal

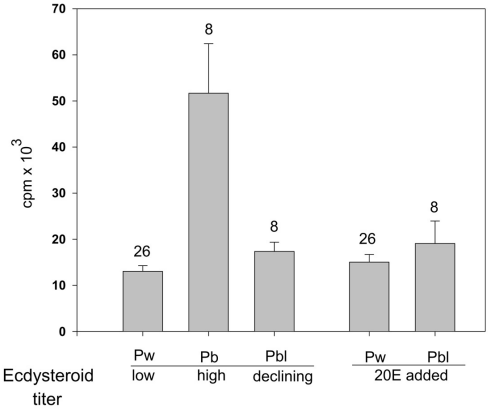

Figure 2. Quantity of proteins secreted by pupal integuments incubated in vitro during 25 h, with or without 20E. Pw, Pb and Pbl: successive phases of the pupal stage. Integuments from Pb pupae, characterized by high endogenous ecdysteroid levels, secreted significantly more proteins than integuments from Pw and Pbl pupae, presenting low and declining, respectively, ecdysteroid levels (Two way ANOVA, $P \leq 0.001$). When added to the incubation media, 20E did not significantly modify the secretory activity of Pw and Pbl integuments (Two-way ANOVA, $P = 0.27$). Cpm measured in samples of incubation medium. The number of samples analyzed is indicated above the bars.

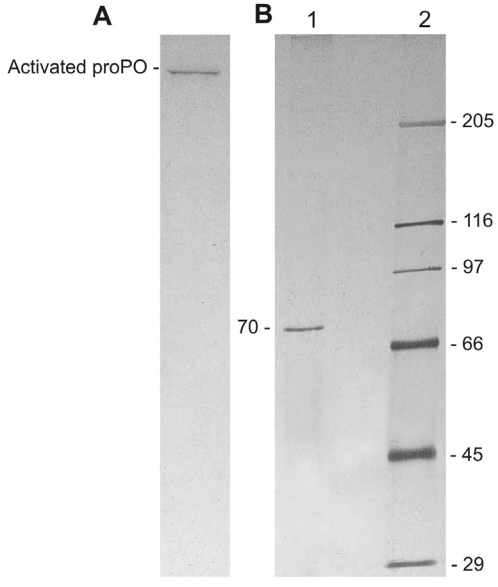

Figure 3. (A) Activated proPO in pupal hemolymph, and the (B) 70 kDa subunit. The activated proPO band (A) detected in unheated hemolymph (separated by SDS-PAGE, and stained with L-Dopa), was cut out of the gel, heat-denatured, and submitted to silver stained SDS-PAGE, yielding the 70 kDa subunit shown in (B). Molecular weight markers are showed at the right in Figure 3B.

integuments (Fig. 2) was not reflected in the gels (Figs. 1A and 1B) because all samples in the gels were normalized to 12 000 cpm.

3.3. The integument synthesizes and secretes in vitro a 70 kDa protein that reacts specifically with anti-proPO serum

Figure 3A shows the activated proPO band from which the proPO anti-serum was prepared (see Materials and Methods). This activity was detected in unheated hemolymph samples separated on SDS-polyacrylamide gels, subsequently stained with L-Dopa. We assumed that the L-Dopa activity resulted from conformational change of proPO occurring during electrophoresis. As stated by Ashida and Brey (1995), the insect PO is synthesized as inactive zymogen (proPO), naturally activated via a serine protease cascade. In biochemical assays, proPO has been usually activated with trypsin. The activation implies cleavage of the zymogen with consequent formation of the PO enzyme (Ashida and Brey, 1995). The hemolymph samples separated on the gels shown in Figure 3A were not treated with trypsin or any other activating agent. This is the reason for the assumption that the observed bands result from conformational changes in proPO, permitting reaction with the substrate, probably by exposing the active site. As a consequence, the observed L-Dopa activity (Fig. 3A) was termed "activated proPO".

The L-Dopa activity band (Fig. 3A) yielded subunits of 70 kDa (Fig. 3B) after it was cut out from the polyacrylamide gels, heated in sample buffer, and again submitted to SDS-PAGE stained with silver.

The activated proPO, as detected in pupal hemolymph (Fig. 3A), was never observed in integument samples submitted to the same electrophoretic conditions and L-Dopa gel

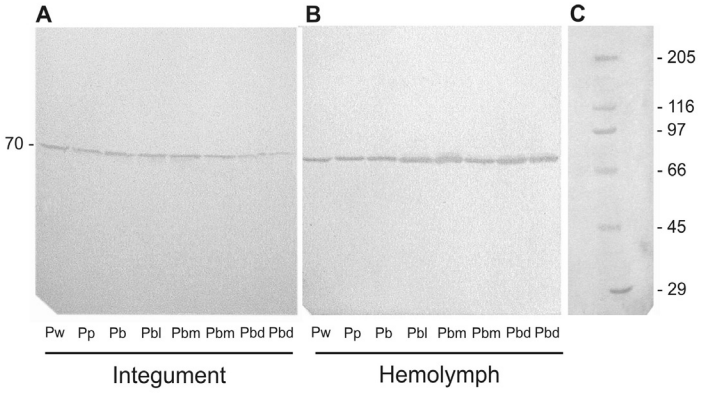

Figure 4. Western blotting using anti-proPO as antibody. The 70 kDa subunit band in (A) integuments, and (B) hemolymph from all pupal phases, was recognized by the antibody raised against activated proPO from hemolymph. Immunoreactivity against the hemolymph 70 kDa-subunit was used to test the specificity of the anti-proPO antibody. Pw, Pp and Pb: successively older unpigmented pupal phases; Pbl, Pbm, Pbd: progressively pigmented pupal phases. (C) Molecular weight markers.

staining used for hemolymph. At first glance, this proPO could thus be considered to be specific for hemolymph. However, a band having identical subunit molecular weight (70 kDa) and reactivity against anti-proPO serum was consistently detected in Western blots using integument extracts. This band was expressed by integuments (as well as by hemolymph samples) of every pupal phase analyzed (Fig. 4). Thus, although undetectable with the substrate L-Dopa, an integumental isoform was recognized by the antibody raised against the hemolymph proPO. This result shows that the 70 kDa subunits of different origins (hemolymph or integument) share immunological identity.

To determine whether this 70 kDa band is synthesized by the epidermis, integuments were incubated in vitro in the presence of radioactive leucine. The radiolabeled proteins synthesized de novo were precipitated with anti-proPO and submitted to electrophoresis and fluorography. The fluorographies revealed a band of 70 kDa, which was much more intense in samples of incubation media (Fig. 5) than in samples prepared with incubated integuments (data not shown). The reactivity with anti-proPO indicates that the integument (or epidermis) synthesizes and secretes a protein of

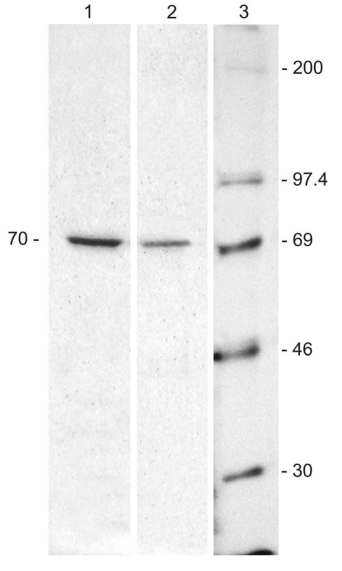

Figure 5. The 70 kDa subunit, reactive to anti-proPO, was synthesized de novo and secreted by pupal integuments incubated in vitro for 25 h. Lanes 1, 2: SDS-PAGE-fluorography of samples from incubation medium precipitated with antiserum against honey bee hemolymph proPO. Lane 3: molecular weight markers.

molecular weight and immunological properties identical to those of the activated proPO detected in hemolymph.

4. DISCUSSION

4.1. Patterns of protein secretion by pupal integuments: dependence on ecdysteroids

As shown in the present study, as honey bee pupae develops, the epidermal cells change their pattern of protein secretion, an event correlated with a shift in endogenous ecdysteroid level from a low titer at the beginning of the pupal stage (Pw pupae) to a high level in the middle of this stage (Pb pupae). A set of low molecular weight epidermal proteins (42, 27, 25, 23, and 22 kDa) started to be secreted when pupal development reached the Pb pupal phase, and the endogenous ecdysteroids peak. But only the 25 kDa protein stopped being secreted when the hormone titer declined thus showing the dependence of the 25 kDa protein on high levels of ecdysteroid. By considering the Ashburner model (see O'Connor, 1985) of hierarchical regulation of gene expression by ecdysteroids, the gene encoding the 25 kDa protein could be a late gene regulated in a coordinated manner by the product of an ecdysteroid-induced early gene. The identification of this gene should provide relevant tools to the studies of differentiation of the adult integument. As demonstrated, anticipated secretion of Pb-specific proteins was not observed in Pw integuments incubated in the presence of 20E. This result may reflect a developmentally regulated activity of ecdysone receptors in the epidermis. Thus, for example, the inhibited secretion of Pw-specific epidermal proteins in Pb, but not in the proper Pw integuments incubated in the presence of 20E (exception for the Pw-specific 61 kDa protein, and for the 56 and 38 kDa proteins), could be explained in terms of the activity of ecdysone receptors restricted to the epidermis of Pb pupae. Alternatively, 20E could have activated synthesis of transcription factors by *early* genes in Pb-, but not in Pw-epidermis. Temporal regulation of transcription factors by 20E in the epidermis was demonstrated in *M. sexta* (Zhou et al., 1998), and other insects.

It should be pointed out that the secretory activity of Pb integuments incubated in vitro, quantified by liquid scintillation spectrometry, was significantly higher when compared to the younger Pw or to the older Pbl integuments. Like the change in protein secretion pattern, this high secretion by Pb integuments was correlated with the higher levels of endogenous ecdysteroids. However, 20E added to the culture media used to incubate Pw and Pbl integuments failed to promote a significant increase in total secretory activity, thus indicating that the secretion of the majority of the Pw and Pbl integumental proteins were not affected by the hormone, at least at the dosage and time of exposure to the hormone used.

The Pb and Pbl integuments can be distinguished from integuments of prior pupal stages by the secretion of characteristic low molecular weight proteins. These proteins were previously observed in cuticle extracts from honey bee pupae run on SDS-PAGE (Santos et al., 2001), indicating that they become incorporated into the cuticle. By injecting pupae with 20E, the expression of these low molecular proteins was prolonged, showing that an elevated ecdysteroid titer is necessary to maintain their expression. The secretion of these proteins by epidermis could be important for the differentiation of the adult cuticle. However, as epidermis is also a source of hemolymph proteins (Palli and Locke, 1987), it is possible that some of the secreted proteins characterized in the present study are also components of hemolymph. This aspect requires further investigation.

4.2. Synthesis and secretion of a 70 kDa subunit reactive to anti-proPO by pupal integuments

We identified an integumental 70 kDa subunit reactive to an antibody raised against a hemolymph proPO. Although the two isoforms from hemolymph and integument share immunological properties, only the former (in its native form) reacts with L-Dopa. The lack of activity with L-Dopa indicates that the integumental isoform is somehow different from hemolymph proPO.

Enzymes known to catalyse oxidation of L-Dopa are phenoloxidases (also called arthropod-specific tyrosinases, EC 1.14.18.1), and a laccase-type phenoloxidase (EC 1.10.3.1). Apparently, the activated prophenoloxidase found in honey bee hemolymph is a tyrosinase. It is possible that the integumental honey bee isoform showing cross-reactivity with anti-hemolymph proPO is a laccase, presenting very low specificity for L-Dopa. Laccases are found in insect integument and have a function in the synthesis of compounds that cross-link integument proteins to chitin (Sugumaran, 1988). Biochemical characterization of the isoforms from hemolymph and integument will be required to further assess their identity.

The 70 kDa subunit was detected throughout the entire pupal stage. Also, in hemolymph as well as in integument, the 70 kDa subunit was expressed independently of the level of endogenous ecdysteroids. Therefore, the constitutive expression of this honey bee protein does not make it a good candidate to exert a function in melanin synthesis for integument pigmentation, an event starting at the Pbl phase. Thus, the described activity in honey bee pupae may differ in function from the granular proPO found in *Manduca sexta* (Hiruma et al., 1985; Hiruma and Riddiford, 1988), a developmentally and hormonally regulated protein. A function of honey bee proPO in immune defense is more plausible, but it has to be investigated.

The detection of a 70 kDa band reactive to anti-proPO in integuments raised the question of whether *A. mellifera* epidermal cells in fact synthesize this product. Northern blot analysis of total RNA extracted from the epidermis of silkworm larvae failed to detect transcripts of proPO. On the basis of this negative result, Ashida and Brey (1995) concluded that proPO is not synthesized by the epidermis and inferred its transport from hemolymph – where abundant proPO mRNA was detected – to the cuticle. Later, Asano and Ashida (2001) provided evidence for the transport of proPO from hemolymph to the cuticle. The situation is different for the tobacco hornworm *M. sexta*, in which synthesis of epidermal proPO was demonstrated by labeling proteins in vivo and in vitro, and by immunoprecipitating them with a polyclonal antibody (Hiruma and Riddiford, 1988). The incubations of honey bee integument, followed by SDS-PAGE and fluorography of the newly synthesized proteins, strongly indicated that the 70 kDa subunit reactive to anti-proPO is produced by epidermal cells. However, we cannot completely exclude the possibility that attached hemocytes might be involved in its synthesis, although it seems unlikely that the strong band seen in the fluorographies is a product of adherent hemocytes.

In conclusion, the present study showed, for the first time, developmentally and hormonally regulated proteins, synthesized and secreted by epidermal cells from *A. mellifera*. Also, an antibody raised against an activated proPO detected in hemolymph, recognized a 70 kDa protein subunit produced in vitro by epidermal cells, thus suggesting that epidermis synthesizes a genuine proPO.

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Résumé – Sécrétion in vitro par le tégument d'*Apis mellifera* de protéines sous dépendance de l'ecdystéroïde et d'une sous-unité de 70 kDa réagissant au sérum anti-prophénoloxydase. La mélanisation et la sclérotisation sont des processus impliqués dans la différenciation du tégument de la nymphe de l'Abeille domestique (*Apis mellifera* L.) formé de la cuticule et des cellules épidermiques sous-jacentes. Les deux processus impliquent la synthèse et la sécrétion de protéines et d'enzymes épidermiques spécifiques, résultats des changements modulés par l'ecdystéroïde survenus dans l'activité des gènes spécifiques. Nous avons étudié le profil des bandes de la sécrétion de protéines épidermiques au cours du stade nymphal. Un système d'incubation in vitro pour le tégument d'abeilles a été utilisé pour cribler les protéines épidermiques synthétisées de novo et sécrétées en présence ou en l'absence de 20-hydroxyecdysone (20E). La synthèse in vitro et la sécrétion de prophénoloxydase (proPO) par l'épiderme ont été également étudiées. La proPO est un précurseur inactif de la phénoloxydase (PO), enzyme clé dans la synthèse de la mélanine et la pigmentation de la cuticule.

Des changements qualitatifs et quantitatifs ont été observés dans la sécrétion des protéines épidermiques au cours du stade nymphal. Le début de la pigmentation de la cuticule a été précédé par la

suppression de l'expression des protéines de haut poids moléculaire et par l'activation des protéines de faible poids moléculaire (Fig. 1). Ces changements coïncident avec une augmentation des taux d'ecdystéroïde endogène. Il a été en particulier montré que la protéine de 25 kDa ne s'exprimait que lorsque le taux d'ecdystéroïde endogène était élevé et les incubations in vitro ont montré que certaines protéines spécifiques des nymphes blanches (Pw) étaient régulées vers le bas par la 20E. Ces protéines sont donc sous la dépendance du taux d'hormones (Fig. 1). Une augmentation significative de la synthèse et de la sécrétion des protéines par l'épiderme est aussi corrélée à des taux élevés d'ecdystéroïde endogène. Néanmoins la 20E ajoutée aux incubations in vitro n'a pas modifié significativement la quantité de protéines sécrétées (Fig. 2).

Pour étudier si la proPO est synthétisée par les cellules épidermiques, un anticorps a été préparé contre une proPO activée trouvée dans l'hémolymphe à l'aide d'une électrophorèse sur gel de polyacrylamide (PAGE) et de L-Dopa comme substrat (Fig. 3A). La bande d'activité de la L-Dopa permet d'identifier les sous-unités de 70 kDa dans l'analyses par PAGE colorée à l'argent (Fig. 3B). L'anticorps anti-proPO a reconnu la protéine de 70 kDa et dans les extraits de téguments (Fig. 4). Le taux d'ecdystéroïde ne régule pas la protéine de 70 kDa au cours du développement. Son expression constitutive tout au long du stade nymphal et son indépendance par rapport au taux d'ecdystéroïde endogène rendent difficile de lier sa fonction à la pigmentation progressive de la cuticule au dernier stade nymphal. Des téguments de nymphes ont été mis à incuber in vitro et les protéines sécrétées ont été immunoprécipitées avec de l'anti-proPO. Cet anticorps a reconnu une sous-unité protéinique sécrétée de 70 kDa, suggérant que les cellules épidermiques synthétisent bien une véritable proPO.

***Apis mellifera* / tégument / épiderme / cuticule / protéine cuticulaire / prophénoloxydase**

Zusammenfassung – In vitro Sekretion von Ecdysteroid-abhängigen Proteinen bei *Apis mellifera* und von einer mit Anti-Prophenoloxidase Serum reagierenden 70 kDa Untereinheit im Integument. Melanisierung und Sklerotisierung sind Prozesse, die bei der Differenzierung des Integuments der Puppen auftreten. Das Integument besteht aus der Cuticula und den darunter liegenden Epidermiszellen. Beide Prozesse umfassen die Synthese und Sekretion von spezifischen epidermalen Proteinen und Enzymen durch Ecdysteroid-modulierte Änderungen in der Aktivität von spezifischen Genen. Wir untersuchten das Bandenmuster der epidermalen Proteinsekretion während der Puppenstadien. Ein in vitro Inkubationssystem für das Integument von Honigbienen wurde für das Screening von solchen epidermalen Proteinen benutzt, die de novo bei An- bzw. Abwesenheit von 20-Hydroxyecdyson (20E) synthetisiert und sezerniert wurden. Die in vitro Synthese und Sekretion von Prophenoloxidase (proPO) durch die Epidermis wurde ebenfalls untersucht. ProPO ist die inaktive Vorstufe von Phenoloxidase (PO), einem Schlüsselenzym für die Synthese von Melanin und für die Pigmentierung der Cuticula.

Es ergaben sich qualitative und quantitative Änderungen in der epidermalen Proteinsekretion während der Puppenphase. Vor Beginn der Pigmentierung der Cuticula erfolgte eine Unterdrückung der Bildung von Proteinen mit hohem Molekulargewicht und eine Aktivierung der Bildung von Proteinen mit niedrigem Molekulargewicht. Dies wurde mit SDS-PAGE/Fluorographien von neu synthetisiertem und sezerniertem Protein gezeigt (Abb. 1). Diese Änderungen fallen mit dem Anstieg des endogenen Ecdysteroidniveaus zusammen. Es wurde gezeigt, dass besonders die Bildung von einem 25 kDa Protein nur bei einem hohen Titer von endogenem Ecdysteroid auftrat. In vitro Inkubationen erwiesen, dass einige Pw-spezifische Proteine (61, 56 und 38 kDa) durch 20E vollständig nach unten reguliert wurden. Das zeigt die Abhängigkeit dieser Proteine vom Hormontiter (Abb. 1). Außerdem zeigte sich, dass ein signifikanter Anstieg der Proteinsynthese und Sekretion in der Epidermis mit einem hohen Niveau der endogenen Ecdysteroide korrelierte. Trotzdem veränderte die Zufügung von 20E zu den in vitro Inkubationen die Menge des sezernierten Proteins nicht signifikant (Abb. 2).

Zur Prüfung, ob Prophenoloxidase (proPO) von den Epidermzellen sezerniert wird, wurde ein Anitkörper gegen in der Hämolymphe entdecktes aktives proPO mit SDS-PAGE und L-Dopa als Substrat eingesetzt (Abb. 3A). Das L-Dopa aktive Band ließ 70 kDa Untereinheiten in der mit Silber gefärbten SDS-PAGE erkennen (Abb. 3B). Dieser Anti-proPO Antikörper erkannte das 70 kDa Protein im Western Blot von Hämolymphe (ein Beweis für die Spezifität dieses Antikörpers) und in Extrakten des Integuments (Abb. 4). Das 70 kDa Protein wird im wesentlichen sowohl in der Epidermis als auch in der Hämolymphe von unpigmentierten und pigmentierten Puppen gebildet, unabhängig vom endogenen Gehalt an Ecdysteroid (Abb. 4). Um zu untersuchen, ob diese Untereinheit ein Produkt der Epidermis ist, wurden Integumente von Puppen in vitro inkubiert und die sezernierten Proteine wurden als Immunopräzipitat mit anti-proPO ausgefällt. Dieser Antikörper erkannte eine sezernierte 70 kDa Protein Untereinheit (Abb. 5), was auf eine Synthese von proPO durch Epidermzellen hinweist.

***Apis mellifera* / Integument / Epidermis / Cuticula / cuticulare Proteine / Prophenoloxidase**

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