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Identification and developmental profiles of hexamerins in antenna and hemolymph of the honeybee, *Apis mellifera*

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Abstract

Four distinct hexamerin subunits (referred to as « hexamerins » in the following text) have been identified in the developing honeybee, *Apis mellifera*, by N-terminal protein sequencing. Hexamerins are abundant in the hemolymph of late larval and early pupal stages, and gradually decline during metamorphosis and adult development. Three hexamerins in the 70 kDa range have been found (Hex70a, Hex70b, Hex70c). In worker and drone, Hex70a is the only hexamerin present in large amount in later adult stages. Hex70b and c exhibit a similar developmental profile, disappearing in the drone just before adult emergence, and in the worker just after. Hex70b or Hex70c are still detectable in the adult queen. Hex80/110 likely exist in at least 3 different subunits, 1 of 110 kDa, and 2 of around 80 kDa, which all share a common N-terminus. They disappear during metamorphosis earlier than Hex70b and c. All these hexamerins have been found also in the antenna, suggesting their utilization in building up of antennal cuticle structures. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: Hexamerin; Storage protein; Arylphorin; Hemolymph; Antenna; *Apis mellifera* L

1. Introduction

Hexamerins are a family of hemocyanin-related insect hemolymph proteins composed of six subunits of about 70 to 85 kDa (Telfer and Kunkel, 1991). They were first found in Diptera (Munn and Greville, 1967), where they were also named larval-serum proteins (LSP), and Lepidoptera (Tojo et al., 1980), then in all insect species investigated so far (Burmester and Scheller, 1996, Burmester and Scheller, 1997). Hexamerins containing a high concentration of aromatic amino acids were named arylphorins (Telfer et al., 1983). In holometabolous insects, hexamerin biosynthesis is typically confined to the fat body and takes place either mainly or exclusively during the final larval stadium (Kanost et al., 1990). The fat body releases hexamerins into the hemolymph where they reach extraordinary concentrations up to 80% of the total hemolymph protein concentration (Scheller et al., 1990) just prior the metamorphosis (Telfer and Kunkel, 1991). In holometabolous insects, they are partially recaptured by receptor-mediated endocytosis in the fat body and stored in cytoplasmic protein granules (Telfer and Kunkel, 1991). Specific hexamerin receptors have been reported in Diptera (Burmester and Scheller, 1992, 1997) and in Lepidoptera (Wang and Haunerland, 1994). Hexamerins are thought to act mainly as storage proteins that provide amino acids for adult development. They may also serve as a component of the pupal and adult cuticle (Munn and Greville, 1971; Kanost et al., 1990; Peter and Scheller, 1991; Kaliafas et al., 1984). The protein granules are gradually, but not completely, broken down during the pupal stage; in *Helicoverpa zea*, for example, many granules have been detected in adult fat body and it has been suggested that hexamerins may also serve as an amino acid source for yolk protein production (Wang and Haunerland, 1991; Haunerland,

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1996). The presence of storage proteins of the hexamerin-type in adults has been confirmed in a variety of hemimetabolous and holometabolous insect species (for review, see Wheeler and Martinez, 1995).

In the overall goal of our study on the molecular bases of the functioning of the olfactory system of the honeybee, we have started to identify antennal proteins which might be associated with olfaction (Danty et al., 1998). In this paper, we describe the identification and the developmental pattern of some hemolymph honeybee proteins, with the main emphasis on the special interest to the hexamerins.

2. Material and methods

2.1. Animals

Apis mellifera L. (Hymenoptera: Apidea) were raised under natural conditions, and studied during the nymphal and adult development. After 3 days of embryonic development, the duration of post embryonic development, composed of larval and pupal stages, is around 18 days for the worker and 21 days for the drone (Jay, 1963).

For an exact determination of the stage of the worker pupae, the queen of a colony was restricted to one comb for several hours. Starting 14 days after egg laying, i.e. 7 days before the emergence of the adult (E-7), pupae were collected daily (from E-7 to E0).

Drone fifth instar larvae, prepupae and pupae since E-10 to E0, were collected from combs containing drone brood of varied ages. Their precise age was determined by the colors of the compound eyes and the thorax (Rembold et al., 1980).

Age-selected worker and drone adults were obtained by labeling emerging insects with a dot of paint on the thorax, and maintained in the natural conditions of the hive until they reach the required age.

Emerging queens were put in a cage with adult workers until they were 4 days old.

2.2. Protein extraction

Pupae and adults were anesthetized by cooling at -20°C for a few minutes. The dorsal vessel was punctured and hemolymph was collected with a syringe. Antennae were excised and were homogenized in liquid nitrogen and dissolved in PBS (10 mM sodium phosphate, 10 mM potassium phosphate, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4) containing EDTA 5 mM pH 7.4, PMSF 1 mM, chymostatin (Boehringer Mannheim) 1 μg/ml, antipain (Boehringer Mannheim) 1 μg/ml, leupeptin (Boehringer Mannheim) 1 μg/ml, pepstatin (Boehringer Mannheim) 1 μg/ml, N-tosyl L-phenylalanine chloromethyl ceton (Sigma) 1 μg/ml, N-tosyl L-alanine chloromethyl keton (Sigma) 1 μg/ml. Sediments were removed by centrifugation at 12 000 ***g*** for 5 min at 4°C. Soluble and insoluble proteins from the antennal extracts and from hemolymph were separated by ultracentrifugation at 140 000 ***g*** (Beckman TL5, rotor Ti 60) for 45 min at 4°C. The pellets were dissolved in PBS and stored at −80°C until use.

2.3. Electrophoresis, electroblotting and N-terminal sequencing

Protein analysis was performed by denaturing gel electrophoresis according to the procedure of Laemmli (1970). Five microliters of hemolymph equivalent or 20 antennae equivalent were subjected to analytical electrophoresis in an 8% polyacrylamide gel and separated for 2.5 h at 250 V. Proteins were stained with a 0.035% solution of a colloidal Coomassie Blue-R (Serva) in 12% trichloroacetic acid, 5% ethanol and destained in water. Electrophoretically separated proteins were electroblotted onto ProBlott membranes (Applied Biosystems Incorporation) according to the procedure of Rassmussen et al. (1991), for 18 h at 30 V, 37 mA. Proteins were visualized after incubation 1 min. in a 0.1% amidoblack, 0.5% acetic acid, 40% methanol solution, then destained in water. Bands of interest were cut and submitted to microsequencing. Automated Edman degradation of the N-terminal sequences was performed using an Applied Biosystem Procise Sequencer with reagents and the method of the manufacturer.

2.4. Western blotting

Hemolymph proteins were subjected to SDS polyacrylamide gel electrophoresis as described and transferred to nitrocellulose membranes for 2 h at 0.8 mA/cm^2. Non-specific binding sites were blocked for 2 h at room temperature with 5% non-fat dry milk in TTBS (10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.1% Tween-20). Hexamerins were detected by incubation for 2 h at room temperature overnight at 4°C with polyclonal anti-[*Drosophila melanogaster*] LSP-1 and LSP-2 antibodies (Burmester, unpublished), diluted 1:5000 in 5% milk/TTBS. The filters were washed thoroughly in TTBS (2 × 5 min, 2 × 20 min) and subsequently incubated with goat anti-rabbit IgGs conjugated with peroxidase (Promega), diluted 1:2500 in 5% milk/TTBS. After washing as above, the detection was carried out by using the ECL system (Amersham).

2.5. Sequence similarities and multiple alignment

Sequences were compared to known data base entries in BLAST 1.4.9 MP (Altschul et al., 1990): GenBank, PDB, SwissProt, SPUpdate and PIR.

The following N-terminal sequences of some hexamerins were used for the creation of a multiple

sequence alignment: *Locusta migratoria* Juvenile-hormone binding hexamerin (LmiJHBP; Braun and Wyatt, 1996), *Periplaneta americana* hexamerin (PamHexC12; Wu et al., 1996), *Blaberus discoidalis* hexamerin (BdiHex; Jamroz et al., 1996), *Leptinotarsa decemlineata* diapause protein 1 (LdeDp1; de Kort and Koopmanschap, 1994; Koopmanschap et al., 1995), *Camponotus festinatus* hexamerin 1 (CfeHex1; Martinez and Wheeler, 1994), *Camponotus festinatus* hexamerin 2 (CfeHex2; D. Wheeler, personal communication), *Bracon hebetor* hexamerin (BheHex; Quistad and Leisy, 1996), *Galleria mellonella* LHP82 (GmeLHP82; Memmel et al., 1994), *G. mellonella* arylphorin (GmeAryl; Memmel et al., 1992), *Bombyx mori* Sex-specific storage protein 1 (BmoSSP1; Sakurai et al., 1988), *B. mori* Sex-specific storage protein 2 (BmoSSP2; Fujii et al., 1989), *Manduca sexta* arylphorin α (MseAryla; Willott et al., 1989) and β (MseArylb; Willott et al., 1989), *M. sexta* methionine-rich storage protein (MseMRSP; Wang et al., 1993), *Trichoplusia ni* acidic juvenile-hormone-suppressible protein (TniAJHSP1; Jones et al., 1990), *T. ni* Basic juvenile-hormone-suppressible protein 1 (TniBJHSP1; Jones et al., 1993), *T. ni* basic juvenile-hormone-suppressible protein 2 (TniBJHSP2; Jones et al., 1993), *Drosophila melanogaster* larval serum protein 1β (DmeLSP1b; Massey et al., 1997) and 2 (DmeLSP2; Mousseron-Grall et al., 1997), *Calliphora vicina* arylphorin (CviAryl; Naumann and Scheller, 1991), *C. vicina* larval serum protein 2 (CviLSP2; Burmester et al., in press), *Anopheles gambiae* hexamerin 1.1 (AgaHex1.1; Zakharkin et al., 1997). These sequences were aligned by hand to the *Apis mellifera* hexamerins.

3. Results

3.1. Hexamerin identification by microsequencing and immunochemistry

A comparative analysis of hemolymph and antenna proteins of drone, queen and worker in pupae as well as in adults was performed by SDS-PAGE (Fig. 1). The major proteins from 60 to 120 kDa were characterized by N-terminal sequencing. They have been classified according to apparent molar masses and sequence similarities (Table 1).

Three proteins ranging from 65 to 75 kDa were identified in drone and worker pupae. According to their N-terminal sequence similarities to insect hexamerins (Fig. 2), they were named Hex70a, b and c from the highest to the lowest apparent molar mass. The sequenced N-terminal regions of Hex70a and Hex70b were 40% identical and 87% similar to each other, that of Hex70a and Hex70c 43% and 86%, respectively.

Database research revealed the highest degree of sequence similarity of Hex70a and Hex70b to the hexamerins of the braconoid wasp, *Bracon hebetor* (73% and 80%), the ant *Camponotus festinatus* hexamerins 1 (63% and 93%) and 2 (66% and 80%), the blowfly *Calliphora vicina* arylphorin (62% and 80%) and LSP2 (69% and 87%), and to the *Drosophila melanogaster* larval serum proteins LSP1β (65% and 80%) and LSP2 (65% and 87%).

To confirm the identity of these proteins as hexamerins, antibodies against the hexamerins LSP-1 and LSP-2 of *Drosophila melanogaster* (Burmester, unpublished) were tested for their ability to detect the hexamerins of *A. mellifera* in Western blots (Fig. 3). While the anti-*D. melanogaster*] LSP-1 antibodies stained both Hex70b and Hex70c, the anti-[*D. melanogaster*] LSP-2 antibodies only detected Hex70c. None of the available anti-hexamerin antibodies cross-reacted with Hex70a.

Three other proteins ranging from 75 to 90 kDa were identified in drone pupae (E-8) (Fig. 1A and Fig. 4A). One of them was N-terminus blocked and the 2 others had identical N-terminal sequences (Table 1). Although their apparent molecular masses were higher than that of Hex70a, b and c, their N-terminal sequences suggested that these proteins were also members of the hexamerin family (see below) and were, therefore, designated as Hex80a and Hex80b.

In further analysis, we decided to characterize another major hemolymph and antennal protein of about 110 kDa. This protein, referred to as Hex110 in the following text, was only present in hemolymph and antenna of pupae and larvae (Fig. 1A and Fig. 3). The N-terminal sequence of this protein was identical to those of Hex80a and b (Table 1). The highest degree of pairwise sequence similarity of any sequence in the data base to Hex110 showed the hexamerin of *B. hebetor* (47% identity, 55% similarity), while lower scores were found with other hexamerins. However, in Western blot analysis none of the available anti-hexamerin antibodies cross-reacted with the Hex80 and Hex110 proteins (Fig. 3).

3.2. Developmental profile

To establish the developmental profiles of these proteins, drone hemolymph proteins of different stages, last instar larvae, prepupae, pupae and adults, were tested with the anti-[*D. melanogaster*] LSP-1 antibodies (Fig. 5). While Hex70b and c were highly abundant from the last larval instar to E-1, they disappeared immediately before the emergence of the adult. As visible in the Coomassie Blue-R stained gel, Hex70a is still present throughout the larval and pupal stages, and in the adult honeybee too.

A comparative analysis of the developmental profiles of the hexamerins in the hemolymph and antenna of both drones and workers was performed (Fig. 4). In the drone, the developmental stages E-10 to E + 7 were analyzed in the hemolymph, and E-10 to E + 21 in the antenna

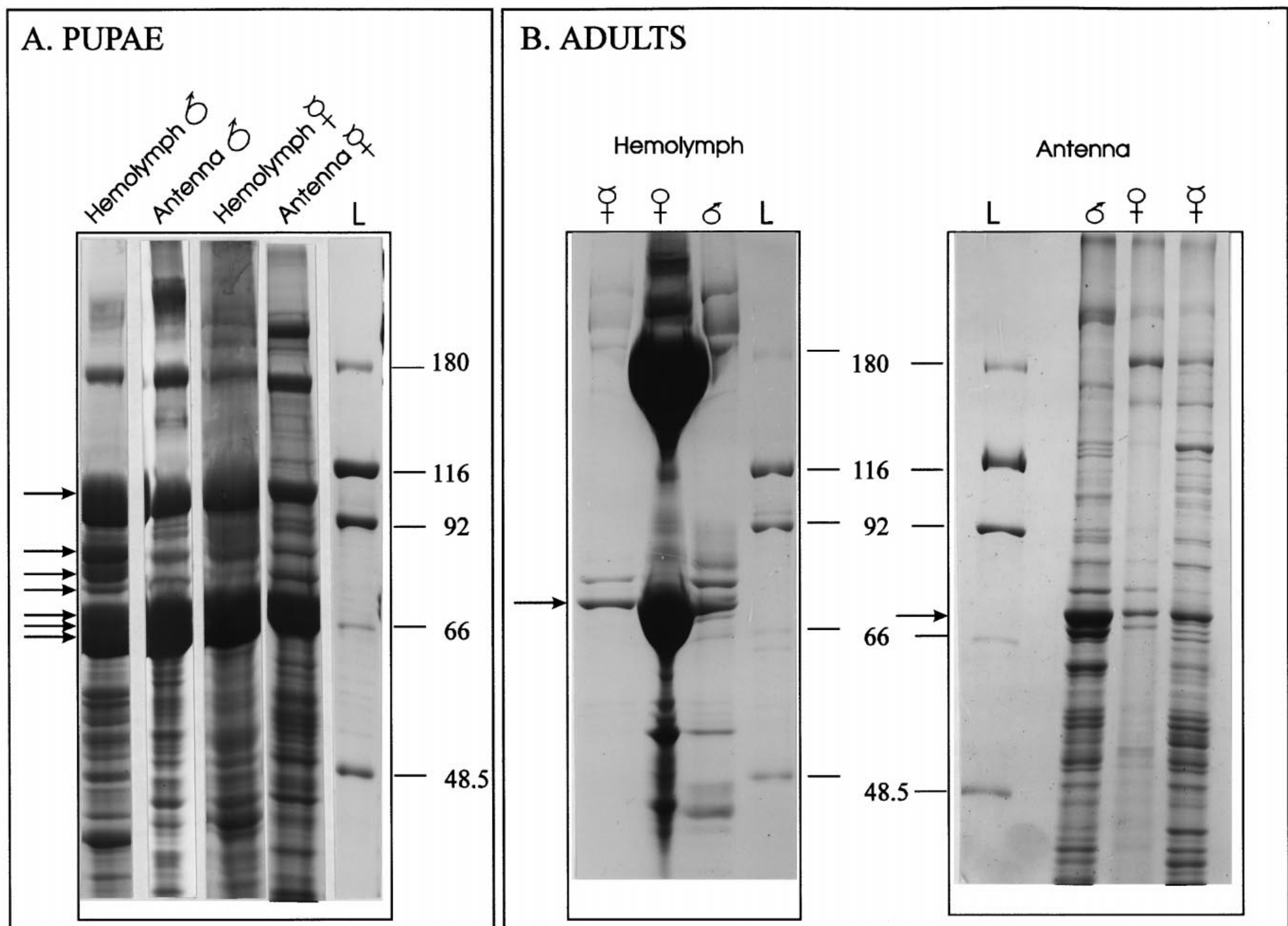

Fig. 1. Honeybee hemolymph and antennal proteins were analyzed by SDS-PAGE. A comparative analysis of proteins corresponding to 5 μl hemolymph and 20 antennae per lane was performed on an 8% polyacrylamide gel, which was Coomassie Blue-R stained. A: Drone and worker pupae (E-8 and E-7, respectively). B: Drone, queen and worker adults (E + 4). Arrows indicate bands selected for N-terminal microsequencing after electroblotting. L: Silver stained SDS Molecular Weight Mixture (Sigma). Apparent molecular weights are given in kDa.

(Fig. 4A). In both hemolymph and antenna, the 3 protein groups were highly concentrated at the beginning of pupation and then decreased gradually. As estimated from sequencing data, Hex70a, b and c were present in similar amounts at E-4, i.e. 5–10 μM.

In the hemolymph, the Hex70b and c amounts were maintained from the last instar larvae to E-6 pupae, then decreased and disappeared at E0, unlike Hex70a which was still present in the adult insects (Fig. 4A and Fig. 5). The abundance of Hex70a was about five times less at E0 than at E-4 and was maintained constant until E + 7 (Fig. 4A). Hex80s were specifically detected from E-10 to E-5 and their amount decreased dramatically between E-5 and E-4 (Fig. 4A). Hex110 was specifically detected from last instar larvae to E-6 pupae and then its amount decreased dramatically until its complete disappearance (Fig. 4A and Fig. 5A).

In drone antenna (Fig. 4A), Hex70a, b and c were present in the highest amount from E-10 to E-4 and their amounts decreased rapidly from E-3. Whatever the stages Hex80s were few abundant unlike in the hemolymph. Hex110 had, globally, the same developmental profile as in hemolymph.

Protein extracts from hemolymph and antenna of workers from E-7 to E + 19 were analyzed as described above (Fig. 4B). No striking difference was found in the developmental profile of the hexamerins. Hex70a, b, and c were present in the highest amount from E-7 to E-1 in hemolymph, and declined rapidly between E-1 and E0, while their amount progressively decreased from E-5 to E0 in antenna. The amount of Hex70a remained constant in adult hemolymph and antenna until depletion between E + 6 and E + 19. Hex110 gradually disappeared from E-7 to E-2 in hemolymph, and rapidly from E-7 to E-5 in antenna.

3.3. *Sex, caste and stage specificity of Hex70b and c*

The anti-[*D. melanogaster*] LSP-1 antibodies were utilized to investigate the sex- and stage specific pattern

Table 1
Characterization by microsequencing of honey bee hexamerin-like proteins in hemolymph and antenna of drones, workers and queens

Name	Origin	N-terminal sequence
	65–75 kDa proteins	
Hex70a	Hemolymph Drone E-4	EYYDTKT
	Hemolymph Drone E0	EYYDTKTADKDFLLK
	Hemolymph Worker E-4	EYYDTKTAD
	Hemolymph Worker E0	EYYDTKTADKDFLLK
	Hemolymph Queen E + 4	EYYDTKTADKDFLLKQKKVYNLLYRVAQ PALA?ITWYNEG
	Antenna Drone > E + 4	EYYDTKTADKDFLLKQKKVYNLL YRVAQPAL
Hex70b	Hemolymph Drone E-4	VPNKVADKTYVTRQK
	Hemolymph Worker E-4	VPNKVADKT
Hex70c	Hemolymph Drone E-4	AYYAGRHTADMFFLH
	Hemolymph Worker E-4	A???GRH
	75–90 kDa proteins	
Hex80a	Hemolymph Drone E-8	APNVKQRAADQDLLNKQQDV
Hex80b	Hemolymph Drone E-8	APNVKQRAADQDLLNKQQDV
Hex80c	Hemolymph Drone E-8	blocked N-ter
	110 kDa proteins	
Hex110	Hemolymph Drone E-8	APNVKQRAADQDLLNKQQDVIQLLQKI SQPIPNQELQNLG
	Hemolymph Worker E-7	APNVKQRAADQDLLNKQQDVIQLLQKI SQPIPNQE
	Antenna Drone E-8	APNVKQRAADQDLLNKQQDVIQLL
	Antenna Worker E-7	APNVKQRAADQDLLNKQQDV

of Hex70b and Hex70c (Fig. 6). In the pupal stage (E-6), these hexamerins were detected in both workers and drones. At the time of emergence, they had disappeared in drones, but not in workers. In 4 days old adults, neither workers nor drones possessed Hex70b and c. However, traces of at least one of these proteins could be still found in the adult queen. Therefore, there are at least 2 hexamerins present in the adult queen: Hex70a, Hex70b and/or Hex70c.

4. Discussion

In this study, we have identified abundant proteins in the developing honeybee, and traced their stage-, sex-, and caste-specific appearance. N-terminal sequence data strongly suggest that all these proteins belong to the family of hexameric storage proteins or hexamerins (Telfer and Kunkel, 1991). Ryan et al. (1984) already demonstrated the presence of proteins in the 75–80 kDa range in hemolymph of the 5th instar larvae of the honeybee, that specifically reacts with an anti-[*M. sexta*] arylphorin (hexamerin) serum. However, in those studies these proteins were not further characterized.

4.1. *The hexamerins of* A. mellifera

The hexamerins in the hemolymph and the antenna of *Apis mellifera* display a highly diversified picture. Taking into account the distinct reactivity of Hex70a, b, and c, and Hex80/110 with the α-LSP antibodies, and the observed N-terminal sequence differences, these proteins can be considered as similar, but different members of a family of hexamerin genes. We detected 4 different N-terminal sequences, suggesting the existence of at least 4 different hexamerin genes in *A. mellifera.*

The arrangement of the different subunits in the native hexamerin is not clear. In other insect species, hexamerin subunits associate in various heterogeneities (see Kanost et al., 1990; Telfer and Kunkel, 1991). Our studies did not tackle the question of subunit association. However, the common developmental profiles and antigenic properties of Hex70b and Hex70c may suggest that these proteins represent 2 different subunits of the native hexamers, while for the same reasons the Hex80/110 proteins may form another hexamerin. Hex70a displays a distinctive developmental profile, as this is the only hexamerin that is still present in the adult honey bee of all castes.

Hex110 likely corresponds to a major 105 kDa hemo-

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           1                                                 42
 DmeLSP1b  HQTHEVKIAD KAFLMKQKFL FEIVYRVEDP LM.FEDHIKQ GE
  CviAryl  ISKHEVKIAD KEFLAKQKFL FEIVYRVEDP LM.FEEWIKM GK
AgaHex1.1  STKFEAKYAD KEFLFKQKFF FEVLRNIHLP LK.YDEYIPY TK
  DmeLSP2  AKHLDSKVAD KDFLMKQKFM YQILQHIYQD DVFTTPFGGS YV
  CviLSP2  SKHIESKVAD KDFLVKQKFM LDILQHVYQD DVFVKKYDES YV
 MseAryla  QHHYKTSPVD AIFVEKQKKV FSLFKNVNQL DY.EAEYYKI GK
 MseArylb  KHSFKVKDVD AAFVERQKKV LDLFQDVDQV NP.NDEYYKI GK
  BmoSSP2  PSTIKSKNVD AVFVEKQKKI LSFFQDVSQL NT.DDEYYKI GK
  GmeAryl  HYDVETRKLD PSLLNIQTKV LSLLENWKQV NP.DDEYYKI GK
TniBJHSP1  VVIGKDNMVT MDIKMKELCI LKLLNHILQP TM.YDDIREV AR
  MseMRSP  VVIGKETLVN VDVKVRELCI LKLLNHILQP TI.YEDIREV AR
TniBJHSP2  PEIDDTTLVT MDIKQRQLVI LKLLNHVVEP LM.YKDLEEL GK
  BmoSSP1  MVFTKEPMVN LDMKMKELCI MKLLDHILQP TM.FEDIKEI AK
 GmeLHP82  PVKKLQRTVD QTVLDRQYKL LTLFFHPHEP IH.IKEQQEI AA
TniAJHSP1  RNNVPQKPAD PVFAKRQMDL MTLFFHVLEP NY.IEANKVI VN
   Hex70a  .EYYDTKTAD KDFLLKQKKV YNLLYRVAQP ALA?ITWYNE G?
   Hex70b  ...VPNKVAD KTYVTRQK?? ?????????? ????????? ??
   Hex70c  AYYAGRHTAD MFFLH????? ?????????? ????????? ??
Hex80/110  APNVKQRAAD QDLLNKQQDV IQLLQKISQP IP.NQELQNL G?
  CfeHex1  .TQVPV?TAD KTYLLKQKNI YELFWVVDQP T????????? ??
  CfeHex2  GLHITGKTAD LDFLHKQKKL YELLFFVKQN TLTDMEFHKI GR
   BheHex  DFYYTDVIAD QDFLLKQKKV FQLLYHVSQP DISNPELFQE GL
  LdeDP19  TAVLSNPVAD TNYLKREQQI LKLLYHVNQP S.TYPEHVEI GK
   BdiHex  STQDYRVVAD KTFLTRQRDF LRLLVRIEQP NY.YADQYEV GN
PamHexC12  DHKDYKQIAD KQFLAKQRDV LRLFHRVHQH NI.LNDQVEV GI
  LmiJHBP  ATAAPNPEGE KVFLTRQRDV LRLFVKIQQP AV.IPEHIEI IK
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Fig. 2. Multiple sequence alignment of the N-terminal protein sequences of some hexamerins. The degree of shading corresponds to the conservation of a particular amino acid at the corresponding position. See Materials and Methods for abbreviations. The sequences of the *Apis mellifera* hexamerins are boxed.

lymph protein previously identified in immature stages of *A. mellifera* (Shipman et al., 1987), which forms a native hexamer of 640 kDa (Schmidt et al., 1990). Moreover, this protein is recognized by polyclonal antibodies raised against the hexamerin 2 of the ant *C. festinatus* (Martinez and Wheeler, 1993). In spite of its high untypical molecular mass its N-terminal sequence clearly demonstrates that it is a member of the hexamerin superfamily. The closest known relative is the hexamerin of a braconoid wasp, *B. hebetor* (Quistad and Leisy, 1996), suggesting a hymenopteran-specific protein. However, at the moment there are too few sequence data to assign this protein to some particular hexamerin class (Beintema et al., 1994; Burmester and Scheller, 1996), or infer the phylogeny of it.

The N-terminal sequence identity of Hex110 to Hex80a and b strongly suggests that these proteins represent different isoforms of a single type of hexamerin subunit. They might result from some posttranslational modifications, e.g. glycosylation of Hex110 on one hand, or C terminal cleavage of the Hex80 proteins on the other. However, because we did not observe any degradation of Hex70b and c by Western blotting, such a possible degradation is very likely not due to the preparation procedure, except if Hex110 is particularly unstable. Moreover, we cannot rule out the possibility that, despite the observed N-terminal sequence identity, Hex80a, Hex80b, and Hex110 are encoded by different genes.

4.2. Stage-, sex- and caste-specific hexamerin pattern

While Hex80 and Hex110 disappear from the hemolymph before the end of metamorphosis, Hex70a is persistent in the hemolymph until later adult stages in all sexes and castes, although in lower amount. The situation concerning Hex70b or Hex70c is more complex: both rapidly disappear from the hemolymph of the drone a little later than Hex80 and Hex110. However, traces of Hex70b and Hex70c are still present in the worker at the time of emergence, but not in the 4 day old adult. In the queen, the hemolymph concentration of these proteins gradually declines as well (data not shown), but either Hex70b or Hex70c is still present in four day old adults. Taken together, the differences in hexamerin pattern suggest a sex- and caste-specific regulation of hexamerin synthesis and utilization throughout development.

The accumulation of hexamerins during the larval feeding stage has led to the suggestion that these proteins act as an amino acid reserve, which are thereafter util-

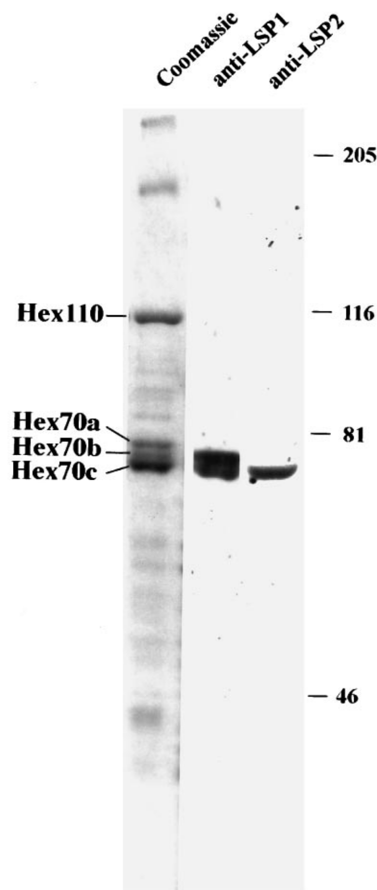

Fig. 3. Identification of hexamerins (Hex) in *Apis mellifera* by Western blotting. Drone pupal (E-7) proteins from 5 μl hemolymph were subjected to SDS-PAGE and blotted onto nitrocellulose. Lane 1: Coomassie Blue-R stained; lane 2: detection with anti-LSP-1 antibodies; lane 3: detection with anti-LSP-2 antibodies. On the right side, the apparent molecular weights are given in kDa according to protein standards.

ized for production of adult protein structures (Levenbook and Bauer, 1984; Kanost et al., 1990). Although initially identified as larval-specific proteins, more recent studies demonstrate the presence of hexamerins even in adult stages (Benes et al., 1990; Wyatt et al., 1992) and have been associated in some cases with a diapause (Miura et al., 1991; Koopmanschap et al., 1992, 1995). Sex-specific differences in hexamerin expression have been repeatedly demonstrated in different Lepidoptera (e.g. Mine et al., 1983; Ryan et al., 1984). Among the Hymenoptera, in the ant *Camponotus festinatus*, hexamerins were identified in last instar larvae, which also specifically accumulate in adult queens and queenless workers (Martinez and Wheeler, 1993). In this species, hexamerins have also been associated with colony founding by queens and larval rearing by workers (Martinez and Wheeler, 1994). Similar nutritional requirements might explain the longer persistence of Hex70b and Hex70c in both workers and queens of *A. mellifera* than in drones. In all sexes and castes, Hex70a is more abundant in larvae and pupae than in the adults, although it is the only hexamerin which is still present in detectable amounts even in older adults. This may indicate a particular role of this hexamerin in the adult phase, which sets it apart from the other hexamerins.

The differences in hexamerin utilization times clearly demonstrate the specificity of this process, which is, therefore, particular for each type of hexamerin. While some hexamerins are used for building up the adult cuticle, the main tissue responsible for hexamerin clearance and storage is the insect fat body (Telfer and Kunkel, 1991). Specific receptors responsible for hexamerin endocytosis have been identified in the Diptera (Haunerland, 1996; Burmester and Scheller, 1997). Evolutionary studies suggest that similar receptors exist in the Hymenoptera as well (Burmester and Scheller, 1996). Therefore, it is likely that the different affinities to the postulated honeybee hexamerin receptor discriminate the hexamerins during uptake and may be responsible for the specific disappearance of the hexamerins during development.

4.3. The function of the hexamerins in the antenna

The abundance of proteins of the hexamerin-type in the antennal tissue is rather surprising. The presence of the hexamerins in this tissue is likely not a specific feature of the antenna, and very probably hexamerins are not specifically involved in the odor ligand and odor receptor protein interaction. Most likely, hexamerins are used to build up antennal cuticular tissue, as shown e.g. for the arylphorin-type hexamerins in the blowfly, *Calliphora vicina* (Peter and Scheller, 1991). Our studies indicate some correspondence between the onset of the sclerotization of the antennal honeybee cuticle and the diminution of the hexamerins from the antenna of the developing honeybee. The beginning of the diminution of the hexamerins in the antenna (E-5) also corresponds to the beginning of the sclerotization of the head cuticle (Andersen et al., 1981), while the stabilization of the level of the hexamerins (E-1) corresponds to the end of this sclerotization process. In adults, maintenance of Hex70a might however be related to morphological modifications of the external structure of the *sensilla placodea*, which occur from E0 to E + 8 in workers (Arnold and Masson, 1981).

5. Conclusion

Like in other holometabolous insects (see Telfer and Kunkel, 1991), several distinct classes of hexamerins exist in the hemolymph of the honeybee. Although the developmental profiles differ among hexamerin classes as well as among castes, our findings are consistent with

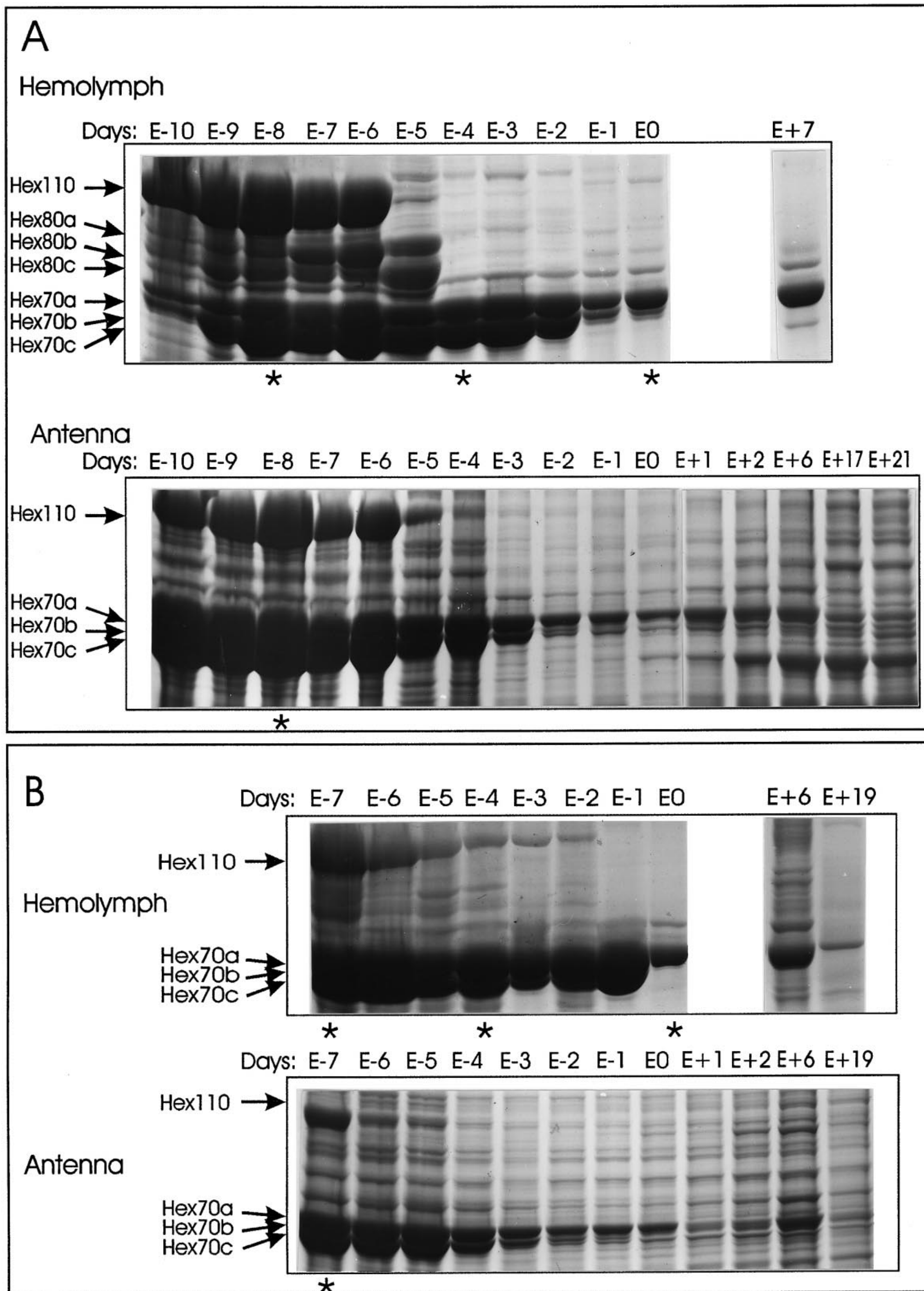

Fig. 4. Stage specific appearance of the hexamerins in hemolymph and antenna during development shown by SDS-PAGE. Proteins from 5 μl hemolymph and 20 antennae per run were separated in an 8% polyacrylamide gel, which was Coomassie Blue-R stained. A: Drone, B: Worker. The numbers refer to the age relative to the emergence (E0) of the adult. Asterisks indicate stages where bands were selected for N-terminal microsequencing after electroblotting.

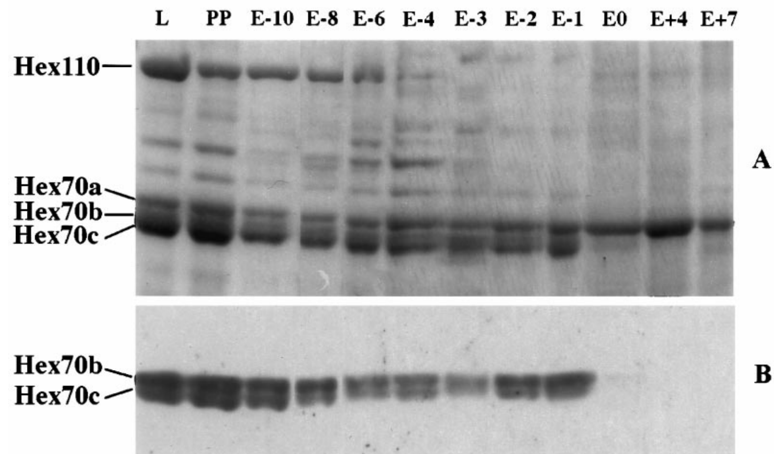

Fig. 5. Stage-specific appearance of Hex70a, b, c and Hex80/110 during drone development. Hemolymph proteins of the desired age were prepared and subjected to SDS-PAGE. The proteins were stained with Coomassie Blue-R stained. (A), or detected with anti-[*D. melanogaster*]LSP-1 antibodies by Western blotting (B). The numbers on the top of the figure refer to the age of the animal in respect to the emergence (E0) of the adult; L = last instar larvae; PP = prepupa.

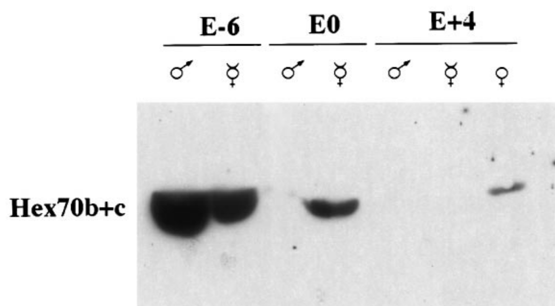

Fig. 6. Sex- and stage-specific appearance of Hex70b and c. Hemolymph of pupal (E-6) and freshly emerged (E0) drones and workers, and adult (E + 4) drones, workers and queens were separated by SDS-PAGE. Hex70b and c were detected with anti-[*D. melanogaster*]LSP-1 by Western blotting.

the assumption that hexamerins are mainly storage proteins. Their stage-specific utilization may indicate particular functions in certain stages of metamorphosis, e.g. providing high concentration of specific amino acids. In the adult bee, particularly in the queen, hexamerins may supply the material and energy required for the first days of the adult life. This may be a precautionary measure in case that food is not immediately available in sufficient quantity.

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