The B Proteins Secreted by the Tubular Accessory Sex Glands of the Male Mealworm Beetle, Tenebrio molitor, have Sequence Similarity to Moth Pheromone-binding Proteins‡

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B proteins represent one of the four major protein groups secreted by the tubular accessory glands of adult, male mealworm beetles. They are acidic proteins with an apparent molecular mass of 18.8 kDa. In this paper we present the deduced amino-acid sequences of two, almost identical B proteins, termed B1 and B2. The mature proteins are 118 amino acids long. They contain 11 (B2) or 12 (B1) possible phosphorylation sites and are rich in glutamic acid (16%). Lectin binding experiments indicate the presence of asparagine linked carbohydrate. The secondary structure of the B proteins is predicted to be almost completely α-helical. The B proteins show significant sequence resemblance to a group of pheromone- and odorant-binding proteins in moths and Drosophila, suggesting a role as carrier proteins for lipids.

INTRODUCTION

The tubular accessory sex glands (TAGs) of the adult, male mealworm beetle (Tenebrio molitor), secrete mainly four groups of proteins, which were termed A, B, C and D (Happ et al., 1977; Black et al., 1982). These highly abundant TAG products apparently form part of the seminal fluid; antisera against A, B and D group proteins recognize epitopes in the lumen of the spermatophore (Black et al., 1982; Paesen et al., 1992). In previous papers we reported the sequence of the C and D group proteins.

C proteins have a basic pI and an apparent molecular mass of 21.9 kDa. On Coomassie stained SDS-gels of TAG homogenates, they can be detected from the second day after adult ecdisis. The fact that they have a high avidity for heparin suggests a possible role in sperm capacitation (Paesen and Happ, 1994).

‡Sequences presented in this paper have been submitted to GenBank and are accessible under numbers M97916 and M97917.
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MATERIAL AND METHODS

Animals

Mealworms (*Tenebrio molitor*) were purchased from a commercial supplier, they were kept at room temperature and reared on Purina Chick Lab Chow. Males and females were separated in the pupal stage.

Protein purification and characterization

TAGs were excised from 10–20 days adult mealworms, rinsed in distilled water containing protease inhibitors (1 μg/ml Leupeptin, 0.5 μg/ml Antipain, 1 μg/ml Pepstatin A, 50 μg/ml PMSF, 1 mM EDTA, pH 8.0; all Sigma products), and collected on dry ice. The sample was homogenized in a 20 mM Tris–HCl buffer (pH 8.0), supplemented with the protease inhibitors mentioned above. The homogenate was centrifuged at 12,000 g (4°C, 3 × 5') and the supernatant was pushed through a 0.2 μm Ion Chromatography Acrodisc syringe filter (Gelman Sciences). Protein purification was by anion-exchange HPLC, followed by reversed phase HPLC (Fig. 1). SDS-PAGE (Laemmli, 1970), western blotting and 2D-gel electrophoresis according to O'Farrell (1975) were used to identify the purified protein.

N-terminal sequence analysis of the purified protein was performed in the protein analytical facility of the Medical Biochemistry Department at the University of Vermont, on an Applied Biosystems 475A Protein Sequencing System, employing a gas phase Edman degradation protocol with online HPLC identification of PTH derivatives of amino acids.

A panel of biotinylated lectins, provided in the Lectin-Link kit (Genzyme), were used to detect and identify asparagine-linked carbohydrate. Purified B protein was submitted to SDS-PAGE, blotted onto nitrocellulose paper and exposed to the lectins. Bound lectin was visualized by use of an avidin–alkaline phosphatase conjugate. A mixture of transferrin, α1-acid glycoprotein and ribonuclease B was used as a control sample.

cDNA library screening

A Lambda Zap II cDNA-library (Paesen et al., 1992), constructed with mRNA from adult TAGs, was screened with a rabbit polyclonal antiserum (anti-A/B; Black and Happ, 1985), according to Mierendorf et al. (1987). The pBluescript SK (−) phagemid of two positive clones (B1 and B2) was excised in vivo, using the R408 helper phage, as described by Short et al. (1988).

Sequencing

Sequencing was carried out by the Sanger dideoxy-mediated chain termination reaction (Sanger and Coulson, 1975), using Sequenase (UCB). The sequence of B1 was obtained by single- and double-stranded sequencing, using the up- and downstream primer sites (SK and KS) of the pBluescript polylinker region. An additional single-stranded sequencing reaction, using the T3 primer site, was carried out after the excision with SacI of 125 basepairs at the 5' end of the cDNA and

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**FIGURE 1.** Purification of the B proteins. (A) The soluble fraction of a TAG homogenate was submitted to anion-exchange HPLC, using a 7.5 mm DEAE-5PW Spherogel column (Beckman) and a 0.02 M Tris–HCl (pH 8.0) running buffer with a 0–0.5 M NaCl gradient (G). The absorbance was measured at a 210 nm wavelength (A210). The B-protein containing fraction (identified by western blotting and marked with the bar in the figure) was quickly concentrated, using Centricon-10 microconcentrators (Amicon). (B) The concentrated fraction was submitted to a reversed phase HPLC run (Vydac 250 mm C18 Reversed Phase Column, 0.1% trifluoroacetic acid (Pierce), 0–100% acetonitrile). The fraction indicated by the bar was collected and analyzed by western blotting, SDS-PAGE according to Laemmli (1970) and two-dimensional polyacrylamide gel electrophoresis following the method of O’Farrell (1975). (C) Analysis of the HPLC purified sample on a SDS (15–30%)–polyacrylamide gradient gel (lane 2). Lane 1 contains molecular mass standards (Biorad; a = 21,500 Da; b = 14,400 Da).
reliquation of the plasmid. Single stranded DNA was rescued and purified according to Short et al. (1988). Double stranded phagemid DNA was purified on Plasmid Quik columns (Stratagene) and alkali-denatured (Mierendorf and Pfeffer, 1987). Compressions were resolved by substituting diTTP for dGTP. The sequence of B2 was obtained by double-stranded sequencing, using the SK and KS primer sites.

Sequence data were analyzed using IBI Pustell and Genetics Computer Group (GCG; Devereux et al., 1984) sequence analysis software.

**Probe construction**

The B1 clone was grown to saturation in liquid LB. Plasmid DNA was isolated using PlasmidQuik columns (Stratagene). The insert was excised with the restriction endonuclease NotI, and separated from the plasmid arms by electrophoresis through a 1% agarose gel onto a piece of NA-45 DEAE-cellulose membrane (Schleicher & Schuell Inc.). The cDNA was recovered by submerging the membrane in a high salt buffer (1 M NaCl, 0.1 mM EDTA, 20 mM Tris, pH 8), precipitated with ethanol and redissolved in distilled water. It was then used as a template for labeling by random primed incorporation of digoxigenin-labeled deoxyuridine-triphosphate (Boehringer Mannheim).

**Northern blotting**

mRNA was isolated from TAGs from adult beetles, using the Micro-FastTrack isolation system (Invitrogen). 2 µg RNA samples were subjected to electrophoresis on formaldehyde containing agarose (0.8%) gels, according to Sambrook et al. (1989). The RNA was transferred to a nylon membrane (Hybond N+, Amersham) using 20 x SSC as transfer buffer and hybridized with the digoxigenin-labeled probes. Nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were used for visualization of bound anti-digoxygenin alkaline phosphatase conjugate.

**RESULTS**

**B protein purification (Fig. 1)**

After two HPLC runs, the protein fraction that reacted with the anti-A/B antiserum was submitted to 1-dimensional SDS-PAGE, western blotting and 2D-gel electrophoresis. The fraction apparently contains 2 proteins of slightly different molecular weight and with slightly different pIs. Based upon their electrophoretic mobility, their abundance, and their reaction with the anti-A/B antiserum, these proteins could be identified as B group proteins (Black et al., 1982).

We did not attempt to separate the two proteins, but submitted the complete fraction to amino-terminal sequence determination, resulting in the following N-terminus: (I/L)TXXDL(Q/E)LLR.

The purified B proteins are bound by the *Datura stramonium* agglutinin, indicating the presence of asparagine-linked carbohydrate (Fig. 2). The lectin specifically recognizes *N*-acetylgalactosamine oligomers, *N*-acetyllactosamine and oligosaccharides containing repeating *N*-acetyllactosamine sequences (Merkle and Cummings, 1987). The B proteins did not contain the specific ligands for the other lectins used in the study (Concanavalin A, *Ricinus communis* agglutinin, *Phaseolus vulgaris* erythrolectin and wheat germ agglutinin, all of which bind to *N*-linked sugars).

**cDNA sequences**

The nucleic acid sequence of the B1 clone and its inferred amino-acid sequence are shown in Fig. 3. The B2 cDNA (not shown) lacks the first 4 bases, but has 6 extra bases at the 3' end (of the coding strand). Several additional clones were sequenced; all were identical to either B1 or B2. In the region the two clones have in common, differences are observed at 7 positions, 5 of which result in changes in the amino acid sequence. The cDNAs do not contain a polyadenylation signal.

**Deduced amino-acid sequences**

Although there are no start codons in the cDNAs, their deduced amino-acid sequences do contain the *N*-terminal sequence of the HPLC-purified proteins (Fig. 3). The *N*-terminus is preceded by a 12 amino-acid sequence, most probably the C-terminal part of the signal sequence. A signal peptidase site immediately
FIGURE 3. cDNA (b1) and deduced amino-acid sequence (B1) of the B1 protein. The nucleotides and amino-acids in which B2 differs from B1 are also presented (the lines indicated by b2 and B2, respectively). The signal sequence is underlined. The arrow indicates the most probable signal peptidase cleavage site. The sequence obtained by N-terminal sequencing of the purified protein is overlined. The dots indicate possible phosphorylation sites (the open circle corresponds with a putative phosphorylation site in B1 that is absent in B2).

following this sequence (indicated by the arrow in the figure), is in total agreement with predictions according to the method of Von Heijne (1986). Stretches of amino acids are repeated in the N-terminal half of the molecule (Fig. 4), suggesting that a DNA duplication might have occurred in the course of the protein's evolution.

The mature proteins are mainly hydrophilic, with only one significant hydrophobic stretch, corresponding with the sequence MQLLCIFKALEIVA (positions 55–68). According to Robson-Garnier predictions, about 85% of the protein assumes an α-helical secondary structure. The method of Chou–Fasman predicts more than 70% helix.

The calculated molecular masses of the deduced B1 and B2 proteins (without signal sequences) are 13,363 and 13,262 Da, respectively. The acidic pIs (calculated to be 4.19 and 4.26) are due to the relatively high content of aspartic acid (10%) and glutamic acid (16%). The high content of hydroxyl amino acids (13.5%) explains the substantial number of consensus phosphorylation sites for protein kinases [(S/T)-X-X-(E/D), (S/T)-X-(R/K) and (R/K)-X-X-S] (Weber, 1979; Kishimoto et al., 1985; Glass et al., 1986; Kuenzel et al., 1987; Pinna, 1990). B1 has 12 consensus sites, B2 11.

Although the B proteins are bound by the Datura stramonium agglutinin, they do not contain the N-glycosylation signal N-X-(S/T) (Oikawa et al., 1987).

TENEBRIO ACCESSORY GLAND B PROTEIN

FIGURE 5. Northern blotting showing relative content of B-protein mRNA (B) in TAGs of 1, 3 and 6 day adult mealworms beetles. Standards were from Gibco-BRL (A = 1.28 kb; B = 0.78 kb; C = 0.53 kb; D = 0.40 kb).

Northern blotting

The northern blot in Fig. 5 shows that the amount of mRNA coding for B proteins is relatively low in young adult males (1 day after emergence), but increases later on in the development. This is completely in accordance with the dramatic increase in B protein production after day two of the adult development, as determined by crossed immunoelectrophoresis (Black et al., 1982).

Comparison to sequences in the data base

The B proteins show highly significant sequence resemblance to a family of proteins expressed in the olfactory tissues of the moths Manduca sexta and Antherea polyphemus, and the fruitfly Drosophila melanogaster (Fig. 6). Some of the moth proteins are male-specific and have been demonstrated to bind female pheromone (Vogt et al., 1988; Gyoergyi et al., 1988; Raming et al., 1989). The function of the other proteins has not been proven yet. However, by virtue of their sequence similarity to the pheromone-binding proteins and because of their presence in the olfactory tissue of the antennae, it is assumed that they bind non-pheromonal odorants (Vogt et al., 1991; Pikielny et al., 1994; McKenna et al., 1994). The antennal proteins contain six conserved cysteines, four of which are also found in the B proteins.

DISCUSSION

On Coomassie-stained 2-dimensional polyacrylamide gels, no more than 2 B proteins can be distinguished, one of which has a somewhat higher molecular weight and a somewhat more acidic pl than the other. The more acidic B protein therefore possibly corresponds with B1 (calculated molecular mass = 13.36 kDa, pl 4.2), and the other B protein might be B2 (13.26 kDa, pl 4.3). The N-terminal sequencing results also seem to indicate that the two proteins in the HPLC-purified sample are in fact B1 and B2. After the first Edman degradation cycle, two, equally convincing peaks were obtained, one corresponding with isoleucine (the first residue in B1), the other with leucine (the first residue in B2). The 7th amino acid was identified as a glutamine (in accordance with the B2 sequence), or a glutamic acid (in accordance with B1).

As is also the case for the C and D proteins (Paesen et al., 1992; Paesen and Happ, 1994), there is a considerable discrepancy between the calculated molecular mass of the cDNA translation products (13.3 kDa) and the apparent molecular mass of the B proteins on SDS–polyacrylamide gels (about 18.8 kDa; Black et al., 1982). The binding of the Datura stramonium agglutinin to the purified B proteins indicates that at least a fraction of the discrepancy may be due to the covalent addition of carbohydrate.

In previous papers (Black et al., 1982; Black and Happ, 1985), it was suggested that A and B group proteins are closely related to each other. The two protein groups share antigenic determinants, are close in molecular weight and pl, appear at the same point in the development and seem to accumulate at similar rates. The anti-AB antiserum reacts equally well with A and B proteins on western blots. It is therefore surprising, that, while B protein cDNAs were easily detected in the expression library, we never found A-protein cDNAs (i.e. cDNAs of which the putative translation products have A-protein characteristics) using the anti-AB antiserum. Perhaps the A group proteins are no more than modified or degraded B proteins, and should not be considered a separate class of secretion products.

B group proteins form part of the seminal fluid, where they remain soluble (Black and Happ, 1985). Insect seminal fluid proteins are reportedly involved in sperm protection, maturation or activation, or they may modulate the physiology and behavior of the female after copulation (Leopold, 1976; Mane et al., 1983; Schmidt et al., 1989; Chen, 1991). At this point, we do not know the exact function of the B proteins. However, the sequence similarity to the antennal proteins in Manduca sexta, Antherea polyphemus and Drosophila melanogaster suggests that B proteins may be lipid carriers. Indeed, the proposed function of the antennal proteins is to solubilize the very hydrophobic odorants or pheromones (which enter through holes in the cuticula) in the aqueous lymph that fills the sensory hairs. The binding proteins furthermore transport the lipids towards mem-
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**FIGURE 6.** Alignment of the *Tenebrio* B1 and B2 proteins (*B1prot* and *B2prot* respectively) with pheromone-binding proteins and pheromone-binding related proteins expressed in insect antennae. Signal sequences are not shown. The alignment was obtained by using the pileup (gapweight = 1.0, gaplength weight = 1.0) and prettyplot (threshold = 1) commands of the GCG software. Dots indicate conserved cysteines, circles indicate cysteines conserved in the antennal proteins but absent in the *Tenebrio* sex gland proteins. *Pbp_Antp*: *Antheraea polyphemus* pheromone-binding protein (*Raming et al., 1989*); *Pbp_Manse*: *Manduca sexta* pheromone-binding protein (*Gyoergy et al., 1988*); *Obp1_Manse*, *Obp2_Manse*: *Manduca sexta* general odorant binding proteins 1 and 2 (*Vogt et al., 1991*). *Pbhpos-E_Drome*, *Pbhpos-F_Drome*: *Drosophila melanogaster* pheromone-binding protein homologues E and F (*McKenna et al., 1994*). *Pbhpr1_Drome*, *Pbhpr2_Drome*: *Drosophila melanogaster* pheromone-binding related proteins 1 and 2 (*Pikelney et al., 1994*).
brane receptors on the olfactory neurons (Vogt et al., 1988).

Lipids and fatty acids in the seminal fluid of insects are sometimes male-specific and in some cases mediate the reproductive behavior of the female after copulation. The defensive chemical cantharidin, for example, cannot be synthesized by female meloid beetles, but is produced by the adult males and transferred during copulation (Sierra et al., 1976; Carrel et al., 1993). In the silkmoth Hyalophora cecropia, juvenile hormone is transferred from the male accessory gland to the female bursa copulatrix during mating (Shirk et al., 1980) and in the field cricket Teleogryllus commodus the spermatophore provides the female with arachidonic acid (Stanley-Samuelson and Loher, 1983). Of particular interest are the male-specific aggregation pheromones found in the ejaculatory bulb of Drosophila species. Some of these pheromones are transferred to the female during mating and subsequently released from the female to the surrounding surfaces (Brieger and Butterworth, 1970; Bartelt et al., 1983; Vander Meer et al., 1986; Schaner et al., 1989). It is not unlikely that similar, highly hydrophobic substances are also present in the aqueous seminal fluid of the mealworm beetle, and that binding proteins are required to keep them in solution.

REFERENCES


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