Lipophorin of the larval honeybee, *Apis mellifera* L.

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Abstract Most insects have a major lipoprotein species in the blood (hemolymph) that serves to transport fat from the midgut to the storage depots in fat body cells and from the fat body to peripheral tissues. The generic name lipophorin is used for this lipoprotein. In larvae of the honeybee, *Apis mellifera,* a lipophorin has been found with properties that correlate well with those of the only other lipophorin reported for an immature insect, that of the tobacco hornworm, *Manduca sexta.* The honeybee lipophorin (M_r = 530,000) has a density of 1.13 g/ml, contains approximately 41% lipid and 59% protein, and contains two apoproteins, apoLp-I, M_r = 250,000 and apoLp-II, M_r = 80,000, both of which are glycosylated. The lipids consist predominantly of polar lipids, of which phospholipids and diacylglycerols represent 60% of the total. When the intact lipophorin is treated with trypsin, apoLp-I is rapidly proteolyzed, while apoLp-II is resistant, indicating a difference in exposure of the two apoproteins to the aqueous environment. Honeybee apoLp-II cross-reacts with antibodies to *M. sexta* apoLp-II, but not to anti-*M. sexta* apoLp-I. No cross-reactivity of honeybee apoLp-I to anti-*M. sexta* apoLp-I was observed. — **Robbs, S. L., R. O. Ryan, J. O. Schmidt, P. S. Keim, and J. H. Law.** Lipophorin of the larval honeybee, *Apis mellifera* L. *J. Lipid Res.* 1985. **26:** 241–247.

Supplementary key words apoproteins • high density lipoprotein • immunology

Lipophorins are the principal lipoproteins of the hemolymph of insects (1). They function to transport fats and other hydrophobic materials between absorption sites at the midgut, depots in the fat body cells, muscle, and other tissues (2). In adult locusts a peptide hormone, the adipokinetic hormone, induces lipid loading of the lipophorin particle, which then increases in size and decreases in density (3–8). Lipophorin thus fluctuates between a traditional high density (HDL) class and a low density (LDL) form, and during loading associates reversibly with a small polypeptide (4, 6, 8, 9).

Lipophorins have been isolated from a number of insect classes, including moths (10–12), cockroaches (13, 14), and locusts (14, 15). Only in the moth, *Manduca sexta,* have larval and adult lipophorins been compared (9, 11, 12). The larval form is simpler, as it contains only two apoproteins, apolipophorin-I (apoLp-I), M_r = 245,000, and apolipophorin-II (apoLp-II), M_r = 78,000 and about 40% lipids, in which diacylglycerols and phospholipids predominate (11). The larval lipoprotein has a density of 1.15 g/ml (9, 11). In the adult, the density of the major form is 1.11 g/ml and small amounts of a second, less dense form of density 1.06 g/ml are also present (9). These particles have a third, smaller apoprotein, apolipophorin III, (apoLp-III, M_r = 17,000) associated with them (9, 16). Following injection of synthetic locust adipokinetic hormone, a dramatic shift to the lower density form takes place, with simultaneous association of large amounts of apoLp-III (9). Hemolymph of larvae contains very little apoLp-III and larval *M. sexta* lipophorin does not change in density. These differences may reflect different functions of lipophorin in the larva and in the adult. Lipophorin is probably principally involved in moving fat from its absorption site in the midgut to storage depots in the fat body during larval life, while in the adult it serves primarily for mobilizing fat from the fat body and transporting it to muscle for flight and other adult functions.

In order to broaden our base of understanding of lipophorin structure and function, we have now isolated lipophorin from the larval honeybee *Apis mellifera,* and we report its properties here. The honeybee is an economically important insect representing the order Hymenoptera, which has been little studied with respect to hemolymph proteins.

MATERIALS AND METHODS

Animals

Fifth instar larvae were obtained from normally managed colonies of *Apis mellifera ligustica* Spin. Frames of honeycomb with the larvae were freed of adult bees,

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; apoLp-I, apolipophorin I; apoLp-II, apolipophorin II; apoLp-III, apolipophorin III; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; FITC-con A, fluorescein isothiocyanate-conjugated concanavalin A.

placed in humid protective transporting boxes, and taken to the laboratory where individual larvae were carefully removed. *Manduca sexta* eggs were generously supplied by Drs. J. P. Reinecke and J. Buckner, USDA, Fargo, ND. Larvae were reared according to Kramer et al. (17).

Purification scheme

Honeybee larvae (18) and *M. sexta* larvae (12) were bled as described previously. The collected hemolymph (blood) was placed in 0.10 M sodium phosphate, pH 7.0, containing 0.15 M NaCl, 0.05% ethylenediaminetetraacetic acid (PBS), which was made 5.0 mM in glutathione and 2 mM in diisopropylphosphorofluoridate. After removal of hemocytes by centrifugation at 5,000 *g* for 10 min at 4°C, the sample was brought to 44% in KBr, overlayered with 0.9% NaCl, and centrifuged at 50,000 rpm in a VTi 50 rotor for 4 hr at 10°C (12). The lipophorin, which forms a sharp band at density 1.13 g/ml, was removed by injecting a needle directly into the centrifuge tube beneath the lipophorin band and withdrawing the portion of solution containing the yellow lipophorin. The KBr was then removed by dialysis overnight against PBS.

Polyacrylamide gel electrophoresis (PAGE)

Native gel electrophoresis was performed on 4–20% acrylamide gradient gel slabs. The resolving gel (in 0.375 M Tris-HCl, pH 8.8) was overlayered with a 2.5% stacking gel containing 0.125 M Tris-HCl, pH 6.8. Electrophoresis was performed at 4°C for 4,000 volt-hr at a constant voltage of 140. Sodium dodecyl sulfate (SDS)-PAGE was performed according to the procedure of Laemmli (19). Except where indicated, a 4–10% acrylamide gradient separating gel and 2.5% stacking gel were employed.

Gel filtration

The behavior of purified honeybee lipophorin on gel permeation chromatography was determined using a 2.5 × 90 cm column of Bio-Gel A-1.5. A molecular weight estimate was made by comparison of the relative mobility of honeybee lipophorin to that of known standards (Bio-Rad), using a plot of log molecular weight versus relative mobility. Inclusion volume of the column was determined with vitamin B_{12}.

Apoprotein molecular weights

Molecular weight estimates for apoLp-I and II were made by SDS-PAGE from linear regression analysis of plots of log molecular weight versus relative migration for known standards (Bio-Rad SDS-PAGE standards) plus ferritin and thyroglobulin (Pharmacia).

Density, lipid:protein ratio, and lipid analysis

The densities of lipophorin samples were determined from the refractive index of KBr on a Bausch and Lomb Abbe-3L refractometer following density gradient ultracentrifugation. The lipid:protein ratio of honeybee lipophorin was determined as follows. A protein determination was made according to the procedure of Bradford (20) using honeybee arylphorin (18) as standard. A lipid extract (21) was performed on this sample. The solvent was removed from the extract and the lipid was quantitated gravimetrically. The relative abundance of individual lipid classes was also determined on lipid extracts of lipophorin. Phospholipids were ashed by the method of Ames and Dubin (22) and the phosphate was quantitated colorimetrically (23). Cholesterol and cholesteryl ester were initially separated by thin-layer chromatography. The lipid-containing areas were scraped, the silica was eluted, and the cholesteryl ester fraction was saponified in 5% KOH in methanol (w/v). The cholesterol was then quantitated colorimetrically according to the procedure of Bowman and Wolf (24). Hydrocarbon was quantitated gravimetrically following adsorption of polar lipids by chromatography on a column (2 ml bed volume) of Bio-Sil A (Bio-Rad) silicic acid. Other esterified lipids and free fatty acids were separated by TLC and quantitated by gas–liquid chromatography. Prior to extraction, standards consisting of 150 μg of 1,2-dipentadecanoin and 100 μg each of monopentadecanoin, tripentadecanoin, and heptadecanoic acid, were added to the sample. Lipid classes were then separated by thin-layer chromatography on Adsorbosil-Plus silica gel G plates (Applied Sciences) employing a solvent system of hexane–diethyl ether–formic acid 80:20:1. Monoacylglycerols were subsequently separated from phospholipids by a second TLC step employing a solvent system of diethyl ether–hexane–methanol–formic acid 70:20:10:1. Areas of the plate representing different lipid classes were scraped and the silica was eluted with diethyl ether. The samples were then saponified in 5% KOH in methanol (w/v) by heating to 60°C for 1 hr in a closed vessel. The samples were methylated by addition of 14% BF_3 in methanol (w/v). Following extraction, the fatty acid methyl esters were chromatographed on a Shimadzu Model GC-mini 1 gas chromatograph equipped with a flame ionization detector. Samples were separated on a J-XR 3% Chrom-Q 100–120 mesh column and integrated with a C-R1A chromatopac integrator.

Carbohydrate

The presence of covalently bound carbohydrate in lipophorin apoproteins was detected by periodate-Schiff staining of an SDS-PAGE gel slab (25). Further characterization of the carbohydrate moieties was accomplished by the use of fluorescein isothiocyanate-conjugated concanavalin A (FITC-con A). Sodium dodecyl sulfate-PAGE slab gels were stained with this reagent according to Furlan, Perret, and Beck (26).

Separation of apoproteins

Apoproteins of honeybee lipophorin were dissociated in 6 M guanidinium chloride and isolated by gel permeation chromatography on a 1.0 × 69 cm Sepharose 6B column equilibrated in 6 M guanidinium chloride, 50 mM phosphate buffer, pH 7.0. After addition of guanidinium chloride to 5 mg of lipophorin, the solution was heated to 50°C for 30 min. Column eluent was monitored at 280 nm and protein peaks were combined, dialyzed against distilled water, and lyophilized. A second procedure involving electrophoretic elution of apoproteins from SDS-PAGE gels was also employed. Bands of protein were cut out of the gel, placed in dialysis tubing containing 25 mM Tris, 192 mM glycine, 0.1% SDS, and electrophoresed at 20 mA for 12 hr on a BRL model HG horizontal gel apparatus. The eluted protein was concentrated by ultrafiltration using an Amicon UM 10 membrane.

Amino acid analysis

Duplicate samples were hydrolyzed in 6 N HCl at 110°C in vacuo for 24, 48, and 72 hr. Cysteine and cystine were determined as cysteic acid after performic acid oxidation (27). Analyses were performed on a Dionex D-300 amino acid analyzer using the standard column and three-buffer system suggested by the manufacturer. Peaks were directly integrated on a Hewlett-Packard 3388A integrator.

Limited trypsinization

Native honeybee lipophorin was subjected to limited trypsinization. One hundred μg of lipophorin in PBS was incubated for various times with trypsin (Worthington) at a concentration of 1:100 (trypsin:lipophorin) for times ranging from 0 to 30 min. Tubes containing only lipophorin were used as controls. The reaction was initiated by the addition of trypsin and stopped by addition of SDS-PAGE sample treatment buffer and boiling. Samples were subsequently analyzed by SDS-PAGE using a 4–15% gradient gel slab.

Immunology

Antibodies prepared according to Shapiro, Keim, and Law (12) against *Manduca sexta* native lipophorin and separated apolipoproteins I and II were utilized in this study. The antisera were used to probe honeybee lipophorin apoproteins that had been separated by SDS-PAGE and transferred electrophoretically to nitrocellulose (28, 29). Bound antibody was detected by incubation with ^{125}I-labeled *Staphylococcus* protein A followed by radioautography. Protein A (Sigma) was iodinated with Iodobeads (Pierce) and Na^{125}I (New England Nuclear) as specified by Markwell (30). *Manduca sexta* lipophorin prepared as described by Shapiro et al. (12) was used as a positive control in immunoblotting experiments.

RESULTS

The major lipoprotein of the hemolymph of larval honeybees was isolated by a single step density gradient ultracentrifugal separation. A readily observable yellow band corresponding to a density of 1.13 g/ml was floated above the remainder of the hemolymph proteins. Upon collection of this material and dialysis to remove KBr, the sample was subjected to native and denaturing polyacrylamide electrophoresis. Under native conditions (**Fig. 1**) the lipoprotein migrated as a single homogenous particle. Electrophoresis in the presence of SDS revealed the presence of two apoproteins (**Fig. 2**), roughly corresponding in molecular weight to the *M. sexta* apoLp-I and apoLp-II.

Lipid analysis

The ratio of protein to lipid was determined by lipid extraction of a known amount of lipophorin. After removal of solvent the lipid extract was quantitated gravimetrically; it comprised 41% of the particle weight. The density of the intact particle was determined to be 1.13 g/ml. The results of these analyses are shown in **Table 1.** Phospholipid and diacylglycerol dominated the lipid composition of this lipoprotein with much lesser amounts

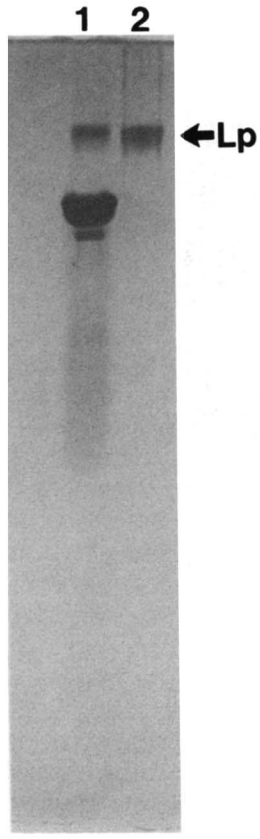

Fig. 1 Native PAGE of 1) crude honeybee larval hemolymph, and 2) 40 μg of purified honeybee larval lipophorin (Lp).

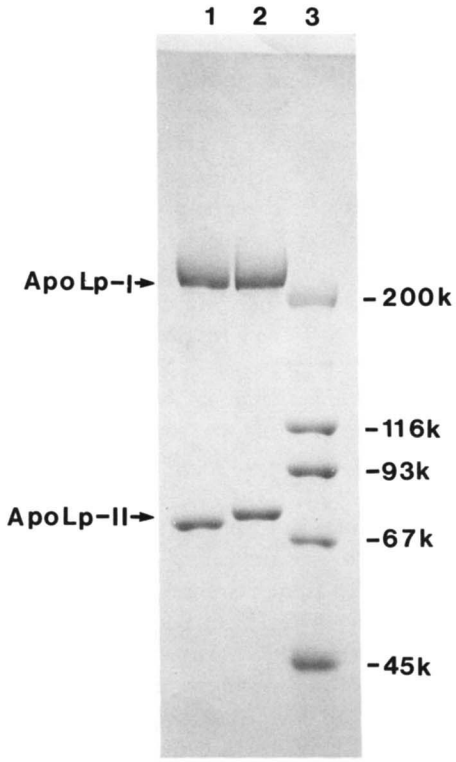

Fig. 2 SDS-PAGE analysis of honeybee lipophorin: 1) *M. sexta* lipophorin (10 μg); 2) honeybee larval lipophorin (10 μg); and 3) molecular weight standards.

of cholesterol, hydrocarbon, triacylglycerol, monoacylglycerol, and free fatty acid.

Molecular weight determinations

The molecular weight of the native lipoprotein particle was estimated by gel permeation chromatography. Using linear regression analysis of the data points, a plot of log molecular weight versus relative mobility was constructed from known standards. The honeybee lipoprotein yielded a molecular weight estimate of 530,000. The molecular weights of the apolipoproteins were estimated by SDS-PAGE by comparison with the migration with known standards. Values of 250,000 and 80,000 for apoLp-I and apoLp-II, respectively, were obtained from plots of log molecular weight versus relative migration of known standards.

TABLE 1. Relative percentage composition of lipid classes in honeybee lipophorin[a]

Lipid Class	Percentage[b]
Phospholipid[c]	31.3 ± 4.0
Monoacylglycerol	1.7 ± 0.2
Diacylglycerol	32.4 ± 1.2
Cholesterol[d]	14.7 ± 0.2
Free fatty acid	5.4 ± 0.1
Triacylglycerol	9.5 ± 1.0
Cholesteryl ester[d]	trace
Hydrocarbon[e]	4.8
Total	99.8

[a]Except where indicated, lipid classes were quantitated by gas–liquid chromatography as fatty acid methyl esters.
[b]Mean ± standard deviation (n = 3).
[c]Determined by the procedure of Ames and Dubin (22) and Chen et al. (23).
[d]Determined by the procedure of Bowman and Wolf (24).
[e]Determined gravimetrically.

Carbohydrate

Both the large and small apoproteins were found to contain linked carbohydrate residues as determined by periodate-Schiff staining of apoproteins separated on SDS-PAGE. Furthermore, the observed binding of these apoproteins by FITC-con A indicated the presence of mannose-containing oligosaccharide chains.

Separation of apoproteins

Dissociation of the lipoprotein particle and separation of the apoproteins was accomplished by gel permeation chromatography in the presence of 6 M guanidine hydrochloride or electrophoretic elution from SDS-PAGE gels. The purity of separated apoproteins was assessed by SDS-PAGE (**Fig. 3**). While apoLp-II was soluble after removal of guanidine hydrochloride, apoLp-I was not.

Amino acid analysis

The amino acid analysis of isolated apoLp-I and apoLp-II is shown in **Table 2.** Prominent features of the

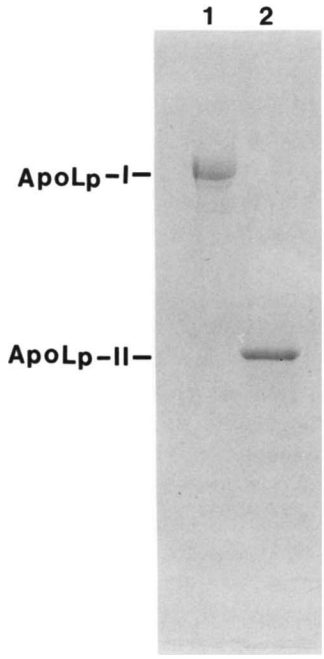

Fig. 3 SDS-PAGE of isolated honeybee lipophorin apoproteins: 1) apoLp-I; 2) apoLp-II.

TABLE 2. Amino acid composition of honeybee lipophorin apoproteins

Amino Acid	ApoLp-I[a]	ApoLp-II
	mol %	
Aspartate	11.4	10.1
Threonine	5.6	4.8
Serine	11.1	12.0
Glutamate	11.4	12.5
Proline	4.4	5.2
Glycine	8.5	10.0
Alanine	6.6	8.3
Cystine[b]	0.8	1.2
Valine	6.8	6.2
Methionine	1.4	1.4
Isoleucine	4.2	3.2
Leucine	8.2	7.14
Tyrosine	3.3	2.3
Phenylalanine	3.1	2.6
Histidine	3.0	3.6
Lysine	7.5	5.2
Arginine	2.9	4.1
Tryptophan	n.d.[c]	n.d.[c]

[a]Data derived from analyses of duplicate samples hydrolyzed for 24 hr, 48 hr, and 72 hr in 6 N HCl in vacuo, 110°C.
[b]Duplicate samples were performic acid-oxidized, then hydrolyzed for 24 hr in 6 N HCl in vacuo.
[c]Not determined.

compositions include the relatively high serine levels and low cysteine content in both apoLp-I and apoLp-II. Furthermore, the analyses reveal similarities in the compositions of apoLp-I and apoLp-II. This is also the case for the only other lipophorin for which apoprotein compositions are available (12).

Trypsin treatment

Limited trypsinization of honeybee lipophorin was performed for various times with a 1:100 concentration of trypsin to lipophorin. The results of this experiment are depicted in **Fig. 4.** SDS-PAGE of the trypsinized lipoprotein showed that apoLp-I is more susceptible to proteolytic cleavage than apoLp-II. The apoLp-I band in the control (lane 1) can be seen to be completely degraded by limited trypsin digestion, whereas apoLp-II remained resistant to trypsin cleavage for a much longer time. The decrease in intensity of staining of apoLp-I corresponded with the appearance of several lower molecular weight bands.

Immunological comparisons

Three antibody preparations against *M. sexta* lipophorin components were available. The first was obtained by injecting whole *M. sexta* lipophorin into rabbits. As shown earlier (12), these antibodies (anti-*M. sexta* lipophorin) react well with intact lipophorin and with apoLp-I, but weakly with apoLp-II, presumably because apoLp-II in the intact particle is shielded from the external environment and thus does not serve as an effective antigen when it is present in the intact particle. In addition, we had antibodies raised to the separated *M. sexta* apoproteins (anti-apoLp-I and anti-apoLp-II) both of which reacted strongly with their specific antigens (12).

When anti-*M. sexta* lipophorin was tested against honeybee lipophorin in radial immunodiffusion, no precipitin lines were observed. By immunoblotting, (**Fig. 5**) anti-*M. sexta* apoLp-I likewise showed no cross-reactivity with the honeybee apoLp-I. Similarly, honeybee apoLp-II did not cross react with this antiserum. With anti-*M. sexta* apoLp-II, however, cross-reactivity with honeybee apoLp-II was observed; this antibody did not cross-react with honeybee apoLp-I.

DISCUSSION

Hemolymph from fifth instar larvae of the honeybee *Apis mellifera* contains a single high density lipoprotein that is comparable to lipophorins of other insect species. Its properties correspond most closely to those of the only

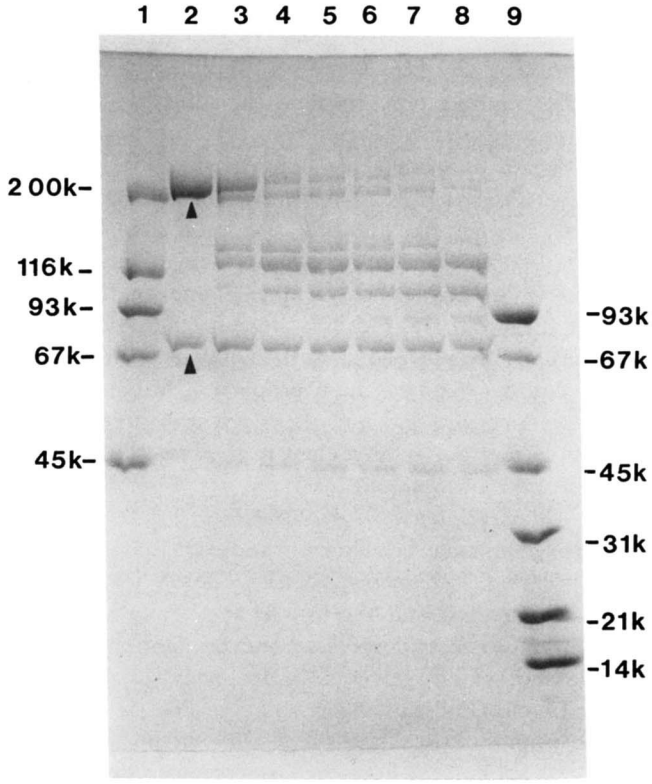

Fig. 4 SDS-PAGE analysis of honeybee lipophorin following incubation with trypsin for 0 (2), 2.5 (3), 5 (4), 10 (5), 15 (6), 20 (7), and 30 (8) min. Lanes 1 and 9, molecular weight standards. Arrows indicate apoLp-I and apoLp-II.

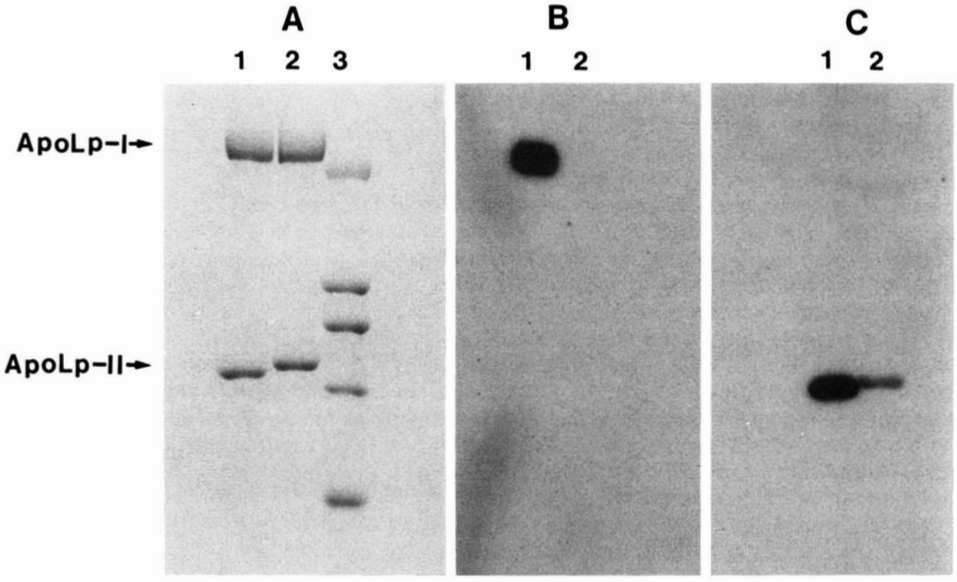

Fig. 5 A) SDS-PAGE of 1) *M. sexta* lipophorin (8 μg), 2) honeybee lipophorin (8 μg), and 3) molecular weight standards. The gel was stained with Coomassie blue. B) Radioautograph of proteins separated by SDS-PAGE, transferred electrophoretically to nitrocellulose, and incubated with *M. sexta* anti-apoLp-I and ^{125}I-labeled *Staphylococcus aureus* protein A, respectively: 1) *M. sexta* lipophorin (4 μg); 2) honeybee Lp (8 μg). C) As in B except *M. sexta* anti-apoLp-II was used.

other larval lipophorin that has been extensively studied, that of *M. sexta*. These two larval lipophorins have similar densities, are composed of two apoproteins of comparable sizes, and contain 40% lipids with similar lipid class composition. In contrast, adult *M. sexta* contains two lower density lipophorins (9). In behavior similar to *M. sexta* lipophorin, the honeybee lipophorin can be extensively digested with trypsin without changing its solubility properties, and, as also in the case of the *M. sexta* lipophorin, the large apoprotein is much more susceptible to proteolysis than the smaller. We have argued earlier (11, 12) that this suggests that apoLp-II is at least partially shielded from the aqueous environment. This, plus the relatively high proportion of polar lipids, especially phospholipid and diacylglycerol, argues for a different structural arrangement in the insect lipoprotein than in those of mammals (31).

One difference between the lipophorins of *M. sexta* and the honeybee is the behavior of the intact lipoprotein on polyacrylamide gel electrophoresis. The *M. sexta* protein does not enter gels well and usually appears as a smeared band or multiple bands. The honeybee protein, on the other hand, enters gels well and appears as a sharply defined band (Fig. 1).

The immunological cross-reactivity of the lipophorin from *M. sexta* and the honeybee is of special interest. The only cross-reactivity was observed between antibodies to isolated *M. sexta* apoLp-II and the honeybee apoLp-II. This might suggest that apoLp-II is a more conserved polypeptide between these rather remote species. The lack of cross-reactivity of the intact honeybee lipophorin with anti-*M. sexta* lipophorin reflects the lack of similarity of the large apoproteins, since the apoLp-II of the intact particle is shielded from antibody reactions (12). Cross-reactivity between anti-*M. sexta* apoLp-I and the corresponding honeybee apoprotein is negligible, confirming the lesser similarity of the large apoproteins.

Thus, larval lipophorins from two dissimilar insect species share most properties, including size, density, and composition, all of which differ from adult lipophorins of two adult insect species, *M. sexta* (9) and *L. migratoria* (4–8). The adult lipophorins are less dense, larger, and have a much higher ratio of lipid to protein. In addition, these lipoproteins contain a third apoprotein, apolipophorin III, which appears to associate reversibly during the processes of lipid loading and unloading, normal events associated with transport of diacylglycerols from fat body depots to flight muscle where diacylglycerols provide the fuel to propel the flying adult. The differences between larval and adult lipophorins are, therefore, probably reflections of the different functions of the lipoproteins in larvae and in adults.

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