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Purification and Partial Characterization of a Lutein-binding Protein from the Midgut of the Silkworm *Bombyx mori**

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A lutein-binding protein was purified from fifth instar larval midgut of *Bombyx mori* by a combination of ammonium sulfate fractionation and three chromatographic procedures, gel filtration, chromatofocusing, and anion exchange chromatography. The protein has a pI of 5.4 and an apparent molecular mass of 35,000 Da, as determined by a linear gradient SDS-polyacrylamide gel electrophoresis. The lutein-protein complex is water-soluble and more stable than the carotenoid or protein alone. The carotenoid moiety was identified by thin layer chromatography, light absorption spectroscopy, and high performance liquid chromatography as all-*trans*-lutein. Lutein is specifically and stoichiometrically bound to the protein, with a ratio of 3 mol of lutein per mol of protein. Binding of lutein (absorption maximum in hexane at 454 nm) to the apoprotein results in a marked red spectral shift of about 38 nm, giving rise to absorption maxima at 432, 462, and 492 nm in 20 mM Tris-HCl, pH 7.0. The lutein-protein complex is characterized by fine spectral structure indicating that lutein is in a relatively rigid environment. This protein is distributed in equal amounts throughout the midgut and in all developmental stages of the larval *B. mori*.

Carotenoids cannot be synthesized by animals or insects and must be acquired from exogenous sources. In many insects, absorption of dietary carotenoids is selective with a preference for carotenes in Orthoptera and Phasmida, and for xanthophylls (oxygenated carotenes) in Lepidoptera, as in the case for the silkworm, *Bombyx mori*, the subject of this study (1, 2). Once absorbed, carotenoids are either irreversibly or reversibly modified. Irreversible modification includes (*a*) decomposition of the carbon skeleton into smaller units or (*b*) the addition of new functional groups, *e.g.* hydroxylation. Reversible modification includes (*a*) esterification of the hydroxy carotenoids with long chain fatty acids, as observed in fat body (3, 4), or (*b*) conjugation of the carotenoids with proteins forming carotenoid-protein complexes (carotenoproteins) which are water-soluble and more stable than the carotenoids alone (5–7). Such carotenoproteins occur mainly in invertebrates and plants (8).

In *B. mori*, a major fate of the absorbed carotenoids is incorporation into the cocoon, which imparts various colors to the cocoon depending on the type of carotenoid. The mechanisms by which carotenoids are transported from the lumen of the midgut to the hemolymph lipoprotein, lipophorin, and from lipophorin into the silk gland, where the cocoon is produced, are unknown. Cocoons of the wild-type *B. mori* are yellow in color due to presence of lutein. There are three known mutants in *B. mori* that produce white cocoons, and genetic analysis has revealed that two mutations are in midgut-expressed proteins, whereas the third mutation is in a silk gland-specific protein (9). Currently, we are using these mutants as a model system to examine, at the molecular level, the pathways by which dietary carotenoids are absorbed and transported to their final destination, and as part of this study a specific luetin-binding protein has been isolated and partially characterized from the *B. mori* midgut.

EXPERIMENTAL PROCEDURES

Materials—Benzamidine hydrochloride, aprotinin, leupeptin, pepstatin A, glutathione, dithiothreitol, butylated hydroxy toluene, and thin layer chromatography (TLC) plates (Whatman 60A) were purchased from Sigma; Spherogel TSK DEAE-5PW was purchased from Beckman; Sephacryl S-200, Sephadex G-75 superfine, polybuffer 74, and polybuffer exchanger PBE 94 were purchased from Pharmacia Biotech. YMC C-18 (250 × 4.6 mm) was purchased from Wilmington, NC.

Animals—Silkworms of the polyvoltine race N_4 (a gift from Dr. Y. Yamashita, Nagoya University) were reared at 25–27 °C on an artificial diet prepared from mulberry leaves (Yakult Corp., Tokyo, Japan). Third- and fourth-day fifth instar larvae of both sexes were used as sources of the lutein-binding protein.

Purification Procedure—The silkworms were anesthetized on ice for 30 min and then the midguts were dissected. The midgut contents were removed and the tissue washed three times with ice-cold buffer A (20 mM Tris-HCl, pH 7.0, containing 150 mM NaCl, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM diisopropyl fluorophosphate, and 0.5 mM benzamidine). The midguts were either frozen at −70 °C or immediately used for preparation of solubilized cytosolic proteins. Using a Polytron homogenizer, midguts were homogenized in buffer A, followed by centrifugation at 10,000 × *g* for 30 min at 4 °C, and the supernatant was then subjected to an ultracentrifugation at 100,000 × *g* for 1 h at 4 °C in a Ti 60 rotor. To remove lipophorin ($d < 1.12$ g/ml), which itself contains carotenoids and is bound to the surface of the midgut, the midgut supernatant was adjusted to a density of 1.31 g/ml with solid KBr and subjected to density gradient ultracentrifugation at 50,000 rpm for 16 h at 4 °C (10), and the gradient was fractionated from the top of the tube into 1-ml fractions using a fraction collector. Lipophorin-free fractions, with a density ≥1.2 g/ml, were pooled and dialyzed overnight at 4 °C against buffer A.

All subsequent purification steps were carried out at 4 °C with minimum illumination. Sufficient solid ammonium sulfate was added slowly, with continuous stirring, to the lipophorin-free supernatant to achieve 25% saturation. The suspension was maintained in an ice water bath for 30 min, followed by centrifugation at 10,000 × *g* at 4 °C for 30 min, and the pellet was discarded. Using the same procedure, solid ammonium sulfate was added to the supernatant to obtain, sequentially, 10% increments of ammonium sulfate fractionation. The precipitated proteins obtained between 25–35, 35–45, 45–55, 55–65, 65–75, 75–85, and 85–100% ammonium sulfate saturation were resuspended in a minimum amount of buffer B (20 mM Tris-HCl buffer, pH 7.0, containing 200 mM NaCl, 1 mM EDTA, 0.5 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, 0.25 mg/liter pepstatin A, 0.25 mg/liter aprotinin, and 0.1 mg/liter leupeptin). Protein and carotenoid concentrations were determined for each fraction.

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Proteins precipitated in the range of 45–55% ammonium sulfate saturation were used to purify the lutein-binding protein. This sample was loaded onto a Sephacryl S-200 column (2 × 78 cm) packed following the manufacturer's instructions and pre-equilibrated with buffer A. The column was eluted with the same buffer at a flow rate of 0.23 ml/min, and 3-ml fractions were collected and monitored at 280 and 460 nm for protein and carotenoids, respectively. Fractions containing the lutein-binding protein were pooled and concentrated by precipitation with solid ammonium sulfate (85%, w/v). The precipitate was resuspended in a minimum amount of buffer B and loaded onto a Sephadex G-75 superfine column (2.5 × 65 cm) pre-equilibrated with buffer A. The column was eluted with buffer A at a rate of 0.3 ml/min; 2.5-ml fractions were collected and monitored for protein and for carotenoids. Fractions containing the lutein-binding protein were concentrated by ultrafiltration using an Amicon model 52 with a YM10 ultrafiltration membrane and then diluted with 25 mM histidine-HCl buffer, pH 6.3, and reconcentrated.

The concentrated sample was loaded on a PBE 94 chromatofocusing column (0.5 × 18 cm) pre-equilibrated with 25 mM histidine-HCl buffer, pH 6.3, and prewashed with 5 ml of polybuffer PBE 74, pH 5. The column was developed with polybuffer PBE 74, and 1-ml fractions were collected. The pH of each fraction was measured, and all fractions were monitored at 280 and 460 nm. In order to remove the polybuffer PBE 74, solid ammonium sulfate (85%, w/v) was added to the lutein-binding protein fractions, and after 1 h on a shaker at 4 °C, the precipitate was collected by centrifugation in a microcentrifuge tube at 15,000 × *g* for 30 min in the cold room. The yellow precipitate was washed 3 times with ice-cold 85% ammonium sulfate buffer and then frozen at −70 °C for further analysis.

The peak obtained from the chromatofocusing column was dialyzed overnight at 4 °C against 10 mM BisTris[1]-HCl buffer, pH 7.0, and loaded on a HPLC Spherogel TSK DEAE column (7.5 mm × 7.5 cm) pre-equilibrated with the same buffer, and fractions were eluted using a 100 mM NaCl linear gradient in the same buffer and 0.5-ml fractions collected. All fractions were monitored for 280 and 460 nm.

Purification was monitored spectrophotometrically using a Diode array spectrophotometer. Fold purification achieved was determined as the ratio of the carotenoid absorbance at 460 nm to protein absorbance at 280 nm.

Analysis of Carotenoids—Carotenoid-containing samples were incubated with an equal volume of ice-cold ethanol for 10 min, followed by two extractions with equal volumes of hexane. The samples were briefly centrifuged to facilitate phase separation, and the organic extracts were pooled and evaporated under argon gas. Carotenoids were analyzed using isocratic HPLC in which 25 μl were injected onto a YMC C18 column (250 × 4.6 mm; S-5 mm; 120A) equilibrated with 90% methanol, 10% tetrahydofuran containing 0.25 g/liter butylated hydroxy toluene. Eluted components were identified using either a Milton Roy dual wavelength detector or a Hewlett Packard photodiode array detector. Using the dual detector, the chromatograms were monitored at 452 and 280 nm, and with the photodiode array the spectrum was scanned from 200 to 550 nm. Carotenoid standards were run under the same conditions. Lutein concentrations were determined from standard curves. Extracted carotenoids, carotenoid standards, and standards of triglycerides, diglycerides, monoglycerides, free fatty acids, and cholesterol were also applied to TLC plates and developed with hexane/acetone (19:1, v/v).

Immunology—Antibodies were raised by intramuscular injection of the protein-adjuvant mixture (Ribi Immunochem Research, Hamilton, MT) into a New Zealand White male rabbit. The antibody was stored at 4 °C in the presence of 0.02% azide.

Tissue specificity of the lutein-binding protein was determined on the following samples from hormonal sac and eggs from day 2 adults and from day 3 fifth instar larvae: whole midgut, midgut divided into three parts (anterior, middle, and posterior), hemolymph, fat body, testis, ovaries, and silk gland. For each sample, 20 μg of protein was separated on a 4–15% linear gradient SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted using goat anti-rabbit IgG and an alkaline phosphatase color development system (Bio-Rad).

To determine the developmental appearance of the lutein-binding protein in the midgut, 40 μg of midgut homogenates from larvae on day 3 of the first through the fifth instar were separated on 4–15% linear gradient SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted.

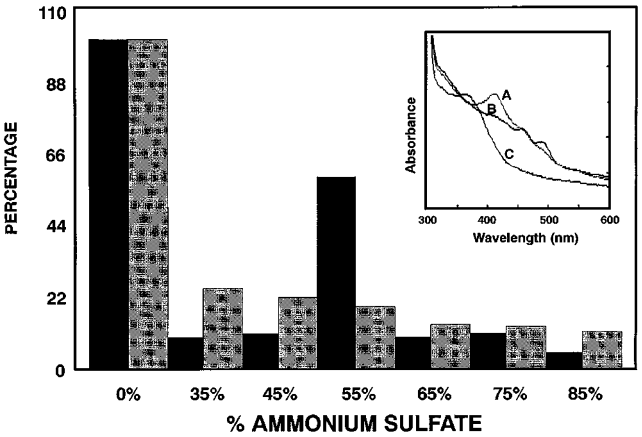

FIG. 1. **Ammonium sulfate fractionation.** The lipophorin-free cytosolic fraction obtained from midgut homogenates of fifth instar *B. mori* were subjected to ammonium sulfate fractionation. Data represent percent protein and percent carotenoid content in each precipitated fraction. **Inset**, *A*, absorption spectrum of the 35–45% precipitated proteins; *B*, absorption spectrum of the 45–55% precipitated proteins; and *C*, absorption spectrum of the 75–85% precipitated proteins. *Solid bar*, percent carotenoids; *shaded bar* percent protein.

Analyses—Protein concentrations were determined using the BCA method following the manufacturer's procedure with bovine serum albumin as a standard (11). Apparent molecular weights were determined using either a gradient (4–15%) or linear (12%) SDS-PAGE prepared as described by Laemmli (12) in the presence of low and high molecular weight markers obtained from Sigma. All gels were stained with Coomassie Brilliant Blue R-250. Protein bands on some gels were photometrically scanned using a Beckman Gel Scanner which determined the purity as well as the amount of the protein present.

In order to test for glycosylation, 15 μg of the partially purified protein (obtained from the chromatofocusing step) were run on two 12% SDS-PAGE. The protein on one gel was transferred to nitrocellulose and incubated in a solution containing 100 μg of fluorescein-labeled concanavalin A in 50 ml of 20 mM HEPES buffer, pH 7.5, containing 150 mM NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ for 1 h at room temperature, rinsed in the same buffer, and observed under UV illumination (Calbiochem). The second gel was incubated in 25% 2-propanol, 10% acetic acid for 6 h at room temperature, followed by another incubation in the same solution containing 0.2% thymol for 90 min. Then the gel was immersed in 80% sulfuric acid, 20% ethanol until the gel clarified and glycoproteins appeared as pink-red bands (13).

RESULTS AND DISCUSSION

Distribution of Carotenoids in B. mori—In the larvae of *B. mori*, the only tissues that contain significant amounts of carotenoids are the midgut, the hemolymph, and the silk glands. During larval development, the fat body does not contain any detectable carotenoids until late in the fifth instar and the spinning stage, when carotenoids are relocated to the fat body, which becomes yellowish in color.

Midgut carotenoids account for 32.2 and 35.5% of the total carotenoids present in the larvae on the 3rd and 4th days of fifth instar, respectively, whereas on the 1st and 2nd days the midgut carotenoids account for only 11.7 and 20.6%, respectively, of the total carotenoids. For this reason, only 3rd- and 4th-day fifth instar insects were used to isolate the carotenoid-binding protein.

Purification of the Lutein-binding Protein—Lipophorin, the major hemolymph carrier of lipids in insects, is rich in carotenoids (14–16). In *B. mori*, about 40% of the total carotenoids found in the larvae are present in lipophorin. In order to remove lipophorin which was absorbed to the midgut, the midgut cytosolic fraction was subjected to a density gradient ultracentrifugation. Ammonium sulfate fractionation of the lipophorin-free cytosolic homogenates demonstrated the presence of carotenoids in all fractions (Fig. 1). Light absorption spectra of

[1] The abbreviations used are: BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

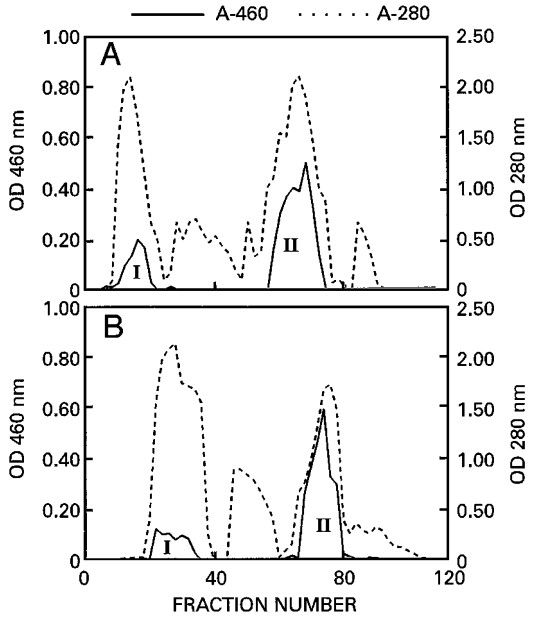

FIG. 2. **Elution profile of the lutein-binding protein on gel filtration.** *A*, the 45–55% ammonium sulfate precipitated proteins were loaded onto a Sephacryl S-200 column and eluted with 20 mM Tris-HCl buffer, pH 7.0. Fractions were monitored at 280 nm for protein (- - -) and at 460 nm for carotenoids (—). *B*, fraction II obtained from the S-200 column was concentrated and loaded onto a G-75 superfine column and eluted with Tris-HCl buffer, pH 7.0. Fractions were monitored at 280 nm for protein (- - -) and at 460 nm for carotenoids (—).

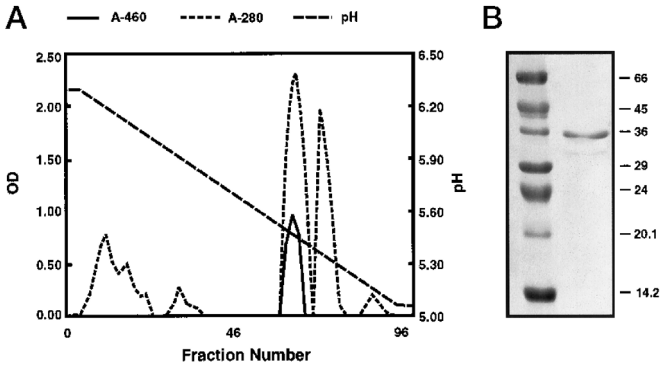

FIG. 3. **Elution profile of the lutein-binding protein on chromatofocusing.** *A*, the sample containing the lutein-binding protein obtained from the G-75 column was loaded onto a chromatofocusing column (PBE 94) and eluted with polybuffer PBE 74, pH 5. Fractions were monitored at 280 nm for protein (- - -), at 460 nm for carotenoids (—) and for pH (- - -). *B*, 12% SDS-PAGE gel of the lutein-binding protein obtained from the chromatofocusing column stained with Coomassie Brilliant Blue; *left lane*, molecular weight marker; *right lane*, semi-purified protein.

the three ammonium sulfate fractions that contained the bulk of the carotenoids are depicted in the *inset* to Fig. 1. All fractions displayed a relatively high absorption spectra with discernible peaks in the visible region between 310 and 500 nm. The presence of these peaks suggests that the carotenoids are bound to proteins. For example, proteins precipitated between 35–45% and 75–85% exhibited distinguishable single peaks, characterized by some spectral fine structures at 410 and 368 nm and accounted for 11 and 5% carotenoids, respectively (Fig. 1, *inset*, *A* and *C*). The majority of the midgut carotenoids (60%) were present in the fraction precipitating between 45 and 55%. This fraction exhibited a light absorption spectrum with two discernible peaks at 458 and 480 nm and a ratio of carotenoid absorbance at 460 nm to protein absorbance at 280 nm of

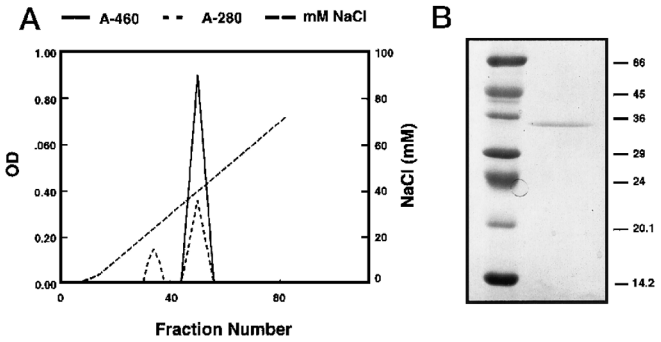

FIG. 4. **Elution profile of the lutein-binding protein on a HPLC Spherogel TSK DEAE column.** *A*, the protein obtained from the chromatofocusing step was loaded onto a DEAE and eluted in a 100 mM NaCl gradient in Tri-HCl, pH 7.0. Fractions were monitored for protein at 280 nm (- - -) and for carotenoids at 460 nm (—). *B*, 4–15% linear gradient SDS-PAGE gel of the purified protein stained with Coomassie Brilliant Blue. *Left lane*, molecular weight marker; *right lane*, lutein-binding protein.

TABLE I
Summary of the purification of the lutein-binding protein from B. mori larval midgut

Step	A_{460}/A_{280}	Purification
		-fold
Lipophorin-free supernatant	0.0071	1.0
45–55% ammonium sulfate fraction	0.0189	2.6
Sephacryl fraction	0.043	6.1
Sephadex G-75 fraction	0.143	20.0
Chromatofocusing fraction	0.625	87.5
DEAE fraction	2.5	350.0

0.0189 (Fig. 1, *inset*, *B*). Thus, this fraction was used as the source of lutein-binding protein.

The elution patterns of the lutein-binding protein on gel filtration chromatography are depicted in Fig. 2. On Sephacryl S-200, two peaks with absorption spectra at 460 nm were observed (Fig. 2*A*). Fraction I, with an apparent molecular mass greater than 125 kDa, accounted for less than 25% of the total carotenoids and was not further characterized. The second peak (II) contained the bulk of the carotenoids (70%) and was characterized by absorption maxima at 430, 458, and 490 nm and a ratio of 0.043 for the absorbance at 460 to 280 nm. The pooled fractions from this peak were precipitated with 85% ammonium sulfate, reconstituted with buffer B, and applied to a G-75 superfine Sephadex column. The elution profile indicated the presence of one major carotenoid-containing peak with a ratio of 0.143 for the absorbance at 460 to 280 nm (Fig. 2*B*).

When the material from the G-75 column was loaded onto a PBE 94 isoelectric focusing column, only one peak at 460 nm was obtained, with a ratio of 0.625 for the absorbance at 460 to 280 nm and a pI value of 5.4 (Fig. 3*A*). SDS-PAGE analysis of the protein obtained from chromatofocusing step revealed the presence of two bands of 35 and 31 kDa (Fig. 3*B*).

Final purification of the lutein-protein was achieved using HPLC and a TSK-DEAE column (Fig. 4*A*). Only the high molecular weight protein (35 kDa) which was eluted at 40 mM NaCl contained carotenoids (Fig. 4*B*) and exhibited a ratio of 2.5 for the absorbance at 460 to 280 nm. This represents a 350-fold purification from the lipophorin-free supernatant (Table I).

Spectral Characterization of the Lutein-binding Protein and Prosthetic Group Identification—The absorption spectrum of the purified lutein-binding protein is characterized by three absorbance maxima in the visible region at 432, 460, and 492 nm (Fig. 5*A*), and the position of the three maxima was the

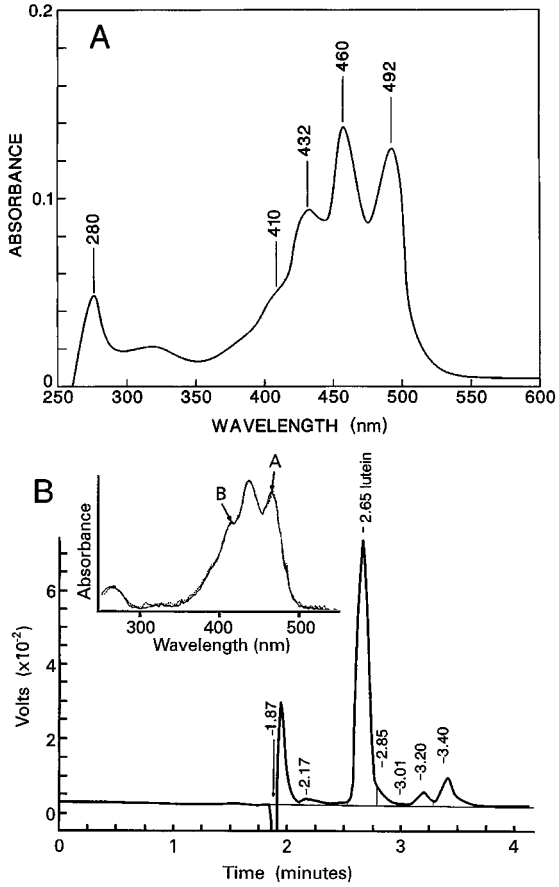

FIG. 5. *A*, ultraviolet-visible absorption spectrum of the purified lutein-binding protein. *B*, isocratic HPLC analysis of the carotenoids extracted from the purified lutein-binding protein. Extracted carotenoids were analyzed on a YMC C18 column. Retention times and peak areas are recorded. *Inset*, chromatogram of the extracted lutein (*A*) and the lutein standard using the photodiode array detector (*B*).

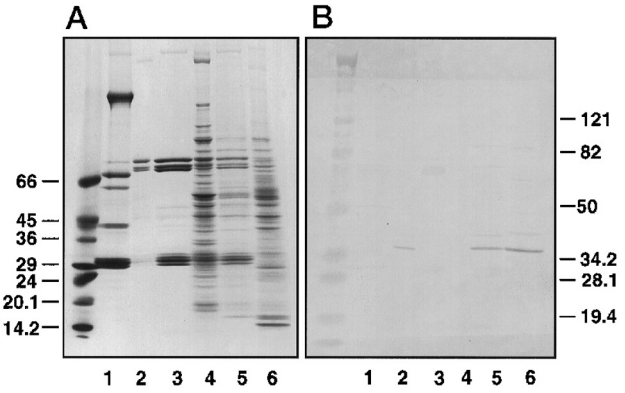

FIG. 6. **Tissue distribution of the lutein-binding protein.** Samples (20 μg) from hemolymph (*lane 1*), fat body (*lane 2*), eggs (*lane 3*), hormonal sac (*lane 4*), testis (*lane 5*), and midgut homogenates (*lane 6*) were separated on a 4–15% SDS-PAGE gel. *Gel A* was stained with Coomassie Brilliant Blue. *Gel B* was transferred to nitrocellulose and immunoblotted using rabbit anti-lutein-binding protein antibodies.

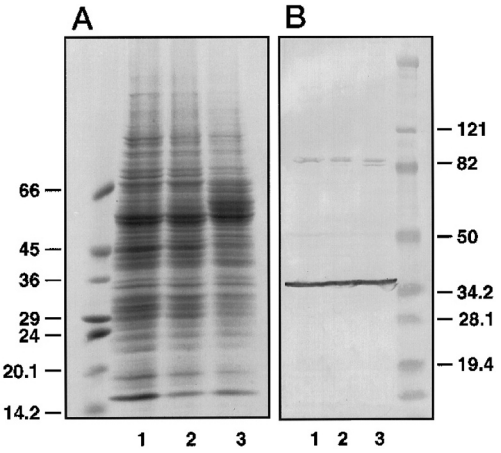

FIG. 7. **Distribution of the lutein-binding protein within the midgut of larval *Bombyx mori*.** Twenty μg of midgut homogenate protein from the anterior (*lane 1*), middle (*lane 2*), and posterior (*lane 3*) portion of the midgut were separated on a 4–15% linear gradient SDS-PAGE gel. *Gel A* was stained with Coomassie Brilliant Blue. *Gel B* was transferred to nitrocellulose and immunoblotted using rabbit anti-lutein-binding protein antibodies.

same in all preparations and was independent of the ionic strength of the medium and pH within the range of 5 and 8. There is considerable variation in the absorption maxima for carotenoproteins, depending on the color of the carotenoprotein. The majority of invertebrate carotenoproteins exhibit absorption maxima between 560 and 680 nm, characteristic of blue or green coloration, but absorption maxima between 490 and 530 nm (red or purple) and 370 to 410 nm (yellow and blue) have also been reported (17–21).

The carotenoids extracted from the purified protein were examined by thin layer chromatography and only one carotenoid was identified which cochromatographed with the lutein standard (no other lipids were detected, data not shown). The absorption spectrum of the extracted carotenoid was also identical to that of lutein standard (Fig. 5, *B*, *inset*). Isocratic HPLC analysis of the extracted carotenoid confirmed the presence of only all-*trans*-lutein, with a ratio of 3 mol of lutein per mol of protein (Fig. 5*B*).

The absorbance maxima of the lutein-binding protein represent a significant red shift of 22–38 nm compared with the spectrum of lutein in hexane or acetone, respectively. This major distinctive spectral shift in the complex is attributed to the polarizing effect of the apoprotein on the conjugated double bonds of the polyene chains of the carotenoid, as observed for the crustacyanins by resonance Raman spectroscopy (19). The polarization effect could be involved in binding, as well as in the spectral shift. It is possible that the C-3 and C-3′ hydroxy groups of lutein are involved in holding the carotenoid in the binding cavity of the protein and that other charged groups in the protein interact with the polyene system and affect the electronic configuration, resulting in a lower energy complex with a higher spectral shift. In α-crustacyanin, Weesie *et al.* (22) have shown that a charge redistribution mechanism is the only cause for the bathochromic shift observed for astaxanthin. Britton *et al.* (1) have reported that the presence of an oxo group in the carotenoid is important for binding to the protein and for production of a spectral shift through enolization, Schiff base formation, or hydrogen bonding to the amino acids of the protein. Recombination of the apoproteins of the lobster, *Homarus gammarus*, crustacyanin and of the star fish, *Asterias rubens*, asteriarubin with different carotenoids indicated that C-4 oxo groups in both rings are essential for the production of the large bathochromic shift (1). This is not the case with ovorubin, in which some of the largest shifts were seen with carotenoids containing only one oxo group, for example adonixanthin (119 nm) and 3-hydroxycheinenone (120 nm) and even zeaxanthin (24 nm) and crustaxanthin (33 nm) which have no oxo group at all. The magnitude of the spectral shift observed for the *B. mori* lutein-binding protein is similar to that observed in recombination studies of ovorubin using zeaxanthin and crustaxanthin, indicating that significant spectral shifts are obtained despite the absence of the 4-oxo groups.

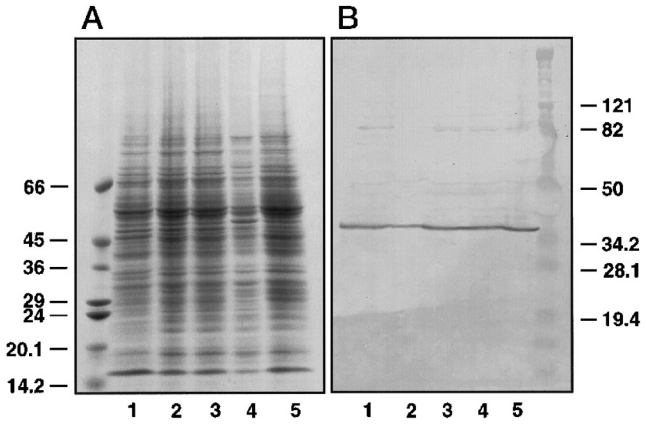

FIG. 8. **Developmental distribution of the lutein-binding protein in larval *Bombyx mori*.** Forty μg of midgut homogenates from day 3 of the first instar (*lane 1*), second instar (*lane 2*), third instar (*lane 3*), fourth instar (*lane 4*), and fifth instar (*lane 5*) were separated on a 4–15% SDS-PAGE gel. *Gel A* was stained with Coomassie Brilliant Blue. *Gel B* was transferred to nitrocellulose and immunoblotted using rabbit anti-lutein-binding protein antibodies.

The absorption spectrum of the lutein-binding protein is not only characterized by a significant bathochromic shift, but by fine structure, indicating that the carotenoids are in a relatively rigid environment (Fig. 5*A*). This observation favors the distortion/freezing mechanism in which the binding of the carotenoid to the protein results in twisting of the polyene system of the carotenoid (17). In previous recombination studies, it was observed that carotenoids with two 4-oxo-β-rings exhibited a considerable increase in spectral fine structure on binding to protein, but that in the case of carotenoids with only one C-4 oxo group the increase in fine structure was very small, and that the binding of carotenoids with two hydroxy groups and no oxo functions caused almost complete loss of vibronic structure (1). In contrast, the *B. mori* lutein-binding protein exhibited fine spectral structure, even though it has two hydroxy groups and does not have any oxo groups. These spectral structural differences could be attributed to the nature of the binding of the lutein to the apoprotein.

Characterization of the Lutein-binding Protein—Figs. 6 and 7 present data on the tissue specificity of the lutein-binding protein. Western analysis of the different tissue proteins indicated that the lutein-binding protein is present in the midgut, fat body, and testis tissues, and in ovary and silk gland tissues (data not shown). This protein is not found in the hemolymph, hormonal sac, or eggs (Fig. 6*B*). The lutein-binding protein was equally distributed throughout the length of the midgut (Fig. 7*B*), and the midgut of all larval instars have the lutein-binding protein in equal amounts (Fig. 8*B*), suggesting that this protein is involved in lutein uptake throughout the larval development stages.

The acidic nature of the lutein-binding protein (pI = 5.4) is not unique to this protein, as several studies have reported carotenoid-protein complexes to be acidic in nature and rich in aspartic acid (8, 23). In invertebrates, carotenoid-protein complexes have pI values between 3.6 and 5 (23–25). In plants, Bryant *et al.* (26) reported the purification of carotenoproteins from carrot roots with pI values of 3.6 and 3.8.

Most carotenoproteins identified to date are very large complexes with molecular masses ranging from 100 to 1000 kDa (8, 26, 27) and are made up of more than two subunits ranging in size between 6 and 43 kDa (2). The molecular mass of 35 kDa for the midgut lutein-binding protein of *B. mori* is small in comparison to these large complexes and is similar in mass to the tocopherol-binding protein isolated from rat liver (28). Both the lutein- and tocopherol-binding proteins are about twice as large as the superfamily of lipid-binding proteins which includes fatty acid- and retinol-binding proteins (29).

The absence of carbohydrates in the lutein-binding protein was demonstrated by the failure to obtain a positive stain with thymol-sulfuric acid, as well as the absence of a fluorescent band in the presence of fluorescein-labeled concanavalin A. Our results are in agreement with the α-crustacyanin and related crustacyanin carapace carotenoproteins that lack glycosylation (25). Both sugar and lipids have been found more frequently in carotenoid-protein complexes from ovaries and eggs (8).

Purification and characterization of this lutein-binding protein is the first step in characterizing the pathways by which dietary carotenoids are absorbed and transported in insects. To our knowledge this lutein-binding protein represents the first example of a carotenoid-binding protein which may be involved in the intracellular transport and/or absorption of carotenoids.

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