Journal Title: Archives of Insect Biochemistry and Physiology

Volume: 20
Issue: 4
Month/Year: 1992
Pages: 315-331

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Article Title: Storage proteins of the larval root weevil Diaprepes abbreviatus (Coleoptera, Curculionidae)-riboflavin binding and subunit isolation

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Archives of Insect Biochemistry and Physiology 20:315–331 (1992)

Storage Proteins of the Larval Root Weevil *Diaprepes abbreviatus* (Coleoptera: Curculionidae): Riboflavin Binding and Subunit Isolation

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Proteins present at high concentrations in hemolymph of the larval weevil *Diaprepes abbreviatus* were previously shown to bind a synthetic coumarin, 7-amino-3-phenyl coumarin (coumarin-10). One of the two native proteins previously identified (protein I) is now shown to separate into two distinct bands (proteins Ia and Ib) using native gradient pore-limiting electrophoresis. The high concentration of proteins Ia, Ib, and II in larval hemolymph, their disappearance from hemolymph upon pupation, and an apparent hexameric structure shown by chemical crosslinking identify them as hexameric storage proteins (hexamerins). At least one chromatographic form of Ib isolated by anion exchange HPLC is now shown to bind riboflavin (Rb). Binding was also demonstrated by quenching of Rb fluorescence by a partially isolated mixture of the storage proteins. Lipophorin did not quench Rb fluorescence. Rb was heat-extracted from whole hemolymph and identified by its fluorescence spectra and by reverse phase HPLC with fluorescence detection. The two subunits shared by the three holoproteins have been isolated by sequential density gradient ultracentrifugation, gel permeation HPLC, and reverse phase HPLC. All three holoproteins shared the α subunit (M, 75,000), while the β subunit (M, 71,000) was lacking from one of the three. Repeated passage through an anion exchange column yielded two of the three proteins (Ib and II) in homogeneous form. Chemical crosslinking with dimethylsuberimidate indicated a hexameric structure for the holoproteins. All subunits and holoproteins stained as high mannose glycoproteins when probed with biotinylated concanavalin A on PVDF membranes. The α subunit was high in Met, His, and Thr, and the β subunit was high in Lys. Both were high in Pro and had approximately 16% Phe + Tyr. Sequences of the 20 N-terminal amino

Acknowledgments: We wish to thank Ada Vazquez, Curtis Murphy, and Delores Lomberk for invaluable technical assistance, and Randall Smith for production of graphics.

Received February 25, 1992; accepted April 23, 1992.

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acid residues of each subunit showed 45–60% homology between subunits. These coleopteran proteins also showed some sequential homology but no immunological cross-reactivity with storage proteins from the lepidopterans *Galleria mellonella* and *Heliothis virescens*.

Key words: coleopteran storage proteins, hexamerins, riboflavin binding, fluorescence quenching

INTRODUCTION

Among the roles established for insect hemolymph proteins, those concerning toxicology and nutrition are perhaps least understood. While hemolymph proteins were shown to bind insecticides nearly two decades ago, only recently have the binding proteins (lipophorins from the lepidopterans *Manduca sexta* [1] and *Heliothis zea* [2] and an arylphorin from *H. zea* [2]) been specifically identified. Since lipophorins are well known for their transport of lipids and lipid derivatives [3], they are also excellent candidates for binding and transport of other hydrophobic compounds such as insecticides and toxic phytochemicals. However, the transport potential of hemolymph storage proteins (e.g., arylphorins) is much less apparent.

Insect storage proteins are included in the class of proteins recently named hexamerins [4]. Work on hexamerins originated with, and has since concentrated on, the orders Diptera and Lepidoptera [5], progressing from isolation and characterization through genomic sequencing. Comparative studies have now begun to define broad phylogenetic relationships between arylphorins and arthropod hemocyanins [6,7]. Ligand binding studies have also been expanded to include binding of Rb*, introducing a potential nutritional role for hexamerins [6]. There is, however, little information to date on hexamerins of Coleoptera, the largest and one of the most economically important of insect orders.

We have recently employed a synthetic coumarin, 7-amino-3-phenyl coumarin, to study xenobiotic and natural product uptake and binding by hemolymph proteins in the citrus root weevil *Diaprepes abbreviatus*, in vivo and in vitro. The use of a synthetic coumarin tracer was appropriate in view of the diverse pyranocoumarins found in citrus roots and the utility of the highly fluorescent tracer [8]. When fed to larvae, 5% of ingested 7-amino-3-phenyl coumarin bound to lipophorin while the remainder bound to other proteins present in hemolymph at very high concentrations [9]. These latter proteins were partially purified and their binding characteristics examined [9]. In the present report, the holoproteins are further characterized and identified as a group of three hexamerins. Two of the proteins share common subunits—a

*Abbreviations used: BSA = bovine serum albumen; CHAPS = 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; DFP = diisopropyl fluorophosphate; DMS = dimethyl suberimidate; Em = emission wavelength; Ex = excitation wavelength; GP-HPLC = gel permeation HPLC; IEF = isoelectric focusing; N-PAGE = native polyacrylamide gel electrophoresis; PAS = periodic acid-schiff reagent; PTH = phenylthiohydantoin; PTC = phenylthiocarbamide; Rb = riboflavin; RP-HPLC = reverse phase HPLC; SDS = sodium dodecyl sulfate; TFA = trifluoroacetic acid.

75,000 M_r α subunit and a closely related 71,000 M_r β subunit—while one holoprotein contains only the α subunit. We now show that at least one of the three holoproteins (Ib) binds Rb, in parallel with hexamerins of *Hyalophora cecropia* [6] and *Heliothis virescens* [10].

MATERIALS AND METHODS

Anion Exchange HPLC of Native Proteins

Fresh hemolymph was collected as described [11] from larvae of approximately 5-month-old *Diaprepes abbreviatus* of 400–600 mg weight. Whole hemolymph was first fractionated on a 75 × 7.5 mm TSK DEAE-5PW HPLC column (TosoHaas, Philadelphia, PA) in pH 7.8, 50 mM Tris buffer with a 0–0.25 M NaCl gradient from 0–15 min at 0.5 ml/min, separating lipophorin from the storage proteins. Storage protein fractions were pooled (13–20-min elution times; 4 ml total), concentrated to 1 ml in a Centricon 30 ultrafiltration device (Millipore, Bedford, MA), diluted to 2 ml in pH 8.8, 50 mM Tris buffer, reconcentrated, rediluted, and finally concentrated to 1 ml. One hundred microliters were then injected onto the above column equilibrated in pH 8.8, 50 mM Tris buffer and eluted with a 0–0.25 M NaCl gradient from 0–15 min at 0.5 ml/min in the same buffer.

Riboflavin Analyses

To positively identify Rb in hemolymph, a total of 300 μl of hemolymph was collected from 3 larvae, diluted to 1 ml in water, and heated to 80°C for 15 min. Precipitated protein was pelleted for 2 min at 12,000*g* in a Beckman microcentrifuge (Beckman, Fullerton, CA) and the supernatant was loaded onto a C-18 SepPak (Millipore) equilibrated in 5 mM ammonium acetate buffer (pH 5)/methanol (72:28). After rinsing with equilibration buffer, Rb was eluted in ammonium acetate buffer/methanol (40:60). Eluted sample was chromatographed on an ODS Hypersil column (200 × 4.6 mm; 20 × 4.6 mm guard column; Hewlett-Packard, Palo Alto, CA) in ammonium acetate buffer/methanol (72:28). Standard Rb was also eluted from a SepPak and chromatographed. Rb was detected with a dual-monochromator fluorescence detector (GTI/Spectro-Vision FD300, Concord, MA; Ex = 469 nm, Em = 532 nm) (method adapted from technical bulletin "Thiamine and Riboflavin Vitamin Analysis," Waters Corp., Milford, MA). Fluorescence spectra of a Rb standard and supernatant from heated hemolymph (as above) were acquired on an SLM Instruments (Urbana, IL) SPF500C spectrofluorimeter.

Concentrations of Rb from anion exchange-eluted protein fractions (see Figs. 2, 4) were determined by heating the fractions in a boiling water bath for 15 min, cooling on ice for 5 min, pelleting precipitated protein in an Eppendorf microcentrifuge (Brinkmann Instruments, Westbury, NY) at 12,000*g* for 10 min, and quantitating fluorescence intensity of the supernatant on an Aminco-Bowman spectrofluorimeter model 521 (SLM). Fluorescence intensity (Ex = 445 nm, Em = 520 nm) was converted to concentration by interpolating from a Rb standard curve. Protein concentrations in anion exchange fractions were determined by the bicinchoninic acid method [12] with BSA as a standard (Pierce, Rockford, IL).

For fluorescence quenching experiments, 100 μl of a Rb stock solution (1 μg/ml in 30 mM, pH 6.8 sodium phosphate buffer) was added to an increasing quantity of storage proteins or lipophorin, then brought to 0.5 ml final volume. The storage proteins were isolated as a group on anion exchange HPLC and dialyzed overnight in Tris buffer (50 mM, pH 7.8). Lipophorin was isolated by ultracentrifugal flotation in a NaBr density gradient [13] and dialyzed in the same manner. Fluorescence intensity of the final Rb-protein mixture was read at Ex = 445 nm, Em = 520 nm.

Electrophoresis and Electroblotting

Both N-PAGE and SDS-PAGE [9] utilized the Laemmli buffer system (minus SDS for N-PAGE) [14]. Gradient pore-limiting N-PAGE was run on 14 × 16 cm 4–15% gels for 24 or 48 h at a tank temperature of 9°C, while nongradient N-PAGE was run at room temperature. Bands were stained with Coomassie-methanol [11] or with a colloidal Coomassie brilliant blue system [15] (Integrated Separation Systems, Hyde Park, MA). All SDS-PAGE standards were from Bio-Rad (Richmond, CA); high M_r standards consisted of myosin (200,000 M_r), β-galactosidase (116,000), phosphorylase B (94,000), BSA (66,000), and ovalbumen (45,000); low M_r standards consisted of phosphorylase B, bovine serum albumen, ovalbumen, and carbonic anhydrase (31,000); soybean trypsin inhibitor (21,000) and lysozyme (14,000) ran at the front. Wet gels were scanned for Coomassie stain density on a Shimadzu (Columbia, MD) CS-9000 scanner with a 633-nm laser light source.

General staining for glycoproteins after separation by SDS-PAGE was done using the PAS method of Konat et al. [16]. High mannose glycoproteins were detected, after SDS-PAGE, by blotting from minigels to PVDF membranes (Durapore Immobilon, Millipore, Bedford, MA) on a semi-dry electroblotter (ISS, Hyde Park, MA) for 2 h at 1.5 mA/cm^2 at room temperature using the recommended transfer buffer (20 mM Tris/150 mM glycine/1.3 mM SDS/20% methanol, pH 8). The membrane was blocked with BSA and probed with concanavalin A reacted with alkaline phosphatase [17].

Isolation of Subunits

For subunit isolation, KBr was added to fresh hemolymph to 44.3% (w/v), 2.2 ml of this mixture were overlayered with the same volume of 0.9% (w/v) NaCl, and samples were centrifuged 16 h to form an equilibrium density gradient [11]. Lipophorin floated above the sedimenting proteins. The yellow band containing most of the sedimenting protein was collected, diluted threefold with 50 mM $NaHPO_4$/0.1 M Na_2SO_4/0.004% NaN_3 buffer, and 100-μl samples were injected onto a TSK G3000SW-XL GP-HPLC column (TosoHaas) equilibrated with the same buffer and delivered at a flow rate of 1 ml/min by a Spectra Physics 8800 pump. Elution was monitored at 280 nm and the major peak (480,000 M_r [9]) was collected.

RP-HPLC followed the general guidelines of Nugent et al. [18] and Burton et al. [19]. Specifically, guanidine HCl (478 mg; U.S. Biochemicals, Cleveland, OH), 6.15 mg of CHAPS (Boehringer Mannheim, Indianapolis, IN), water, 1 μl of TFA (sequencing grade; Sigma, St. Louis, MO) and 100 μl of isopropanol (HPLC grade; B&J, Muskegon, MI) were mixed to 500 μl, added to a 500-μl

sample from GP-HPLC fractions (above), and brought to a total of 1 ml to give 5 M guanidine HCl, 10 mM CHAPS, and 0.1% TFA in 10% isopropanol. After filtering through a Millex-HV 0.45 μm filter (Millipore), 500 μl samples were kept on ice until injection onto a 1,000 Å PLRP-S polymeric (polystyrene/divinylbenzene) reverse phase column (Polymer Laboratories, Amherst, MA) and equilibrated at 60°C in 80% mobile phase A (10 mM CHAPS/0.1% TFA in 10% isopropanol)/20% mobile phase B (10 mM CHAPS/0.1% TFA in 90% isopropanol). Polypeptides were eluted with a gradient of 20–40% B over 5 min, 40–70% B over 15 min. Fractions of approximately 300 μl each were collected, diluted to 500 μl with 50% isopropanol, then microdialyzed in separate wells of a BRL (Bethesda, MD) apparatus for 5 h against 50% isopropanol. Pooled peak fractions were dried under vacuum at 40°C in a Speed-Vac Evaporator (Savant, Farmingdale, NY).

Crosslinking

The method of Davies and Stark [20] for chemical crosslinking of protein subunits was modified for use with our system. Protein prepared by density gradient centrifugation was diluted to a concentration of 1.0 absorbance units at 280 nm (1 cm pathlength) in 0.1 M Tris buffer at pH 8.8. To 100 μl of this solution were added 0–10 μl of DMS (5 μg/μl) dissolved in the same buffer shortly before addition to protein. The mixture was incubated 1 h at room temperature, then diluted in 100 μl of SDS-PAGE sample buffer and boiled for 5 min. Samples were run on large SDS-PAGE 3–8% gradient gels, 60 μl/lane. Approximate M_rs were estimated by linear regression, extrapolating from positions of standards.

Amino Acid Analysis and Sequencing

Protein sequencing and amino acid analyses were performed at the Biotechnology Core Facility (Protein Sequencing/Amino Acid Analysis Unit) at the University of Arizona, Tucson, AZ. Samples were analyzed for amino acid composition using an Applied Biosystems (Foster City, CA) Model 420A Derivatizer/130A Separation/920A Data Analyzer with automatic hydrolysis (vapor phase at 160°C for 1 h 40 min) using precolumn PTC-amino-acid analysis. Samples were sequenced using an Applied Biosystems 477A Protein/Peptide Sequencer (Edman chemistry) interfaced with a 120A HPLC (C-18 PTH, reverse phase chromatography) Analyzer to determine PTH amino acids.

RESULTS

Characteristics of Larval Storage Proteins

When whole hemolymph was electrophoresed on 4–10% gradient native minigels for 45 min or on large nongradient native gels for 7 h, two major bands (I and II) were readily distinguished. Subdivisions of band I were sometimes evident, though not resolved. However, band I did resolve into two distinct bands labeled Ia and Ib (Fig. 1) on pore-limiting gradient N-PAGE (10°C for 24–48 h). When electroeluted from a native gel and run on SDS-PAGE, the band I proteins yielded two subunits designated α (M_r = 75,000) and β (M_r = 71,000); band II contained only the α subunit [9]. Omission of β-mercaptoethanol

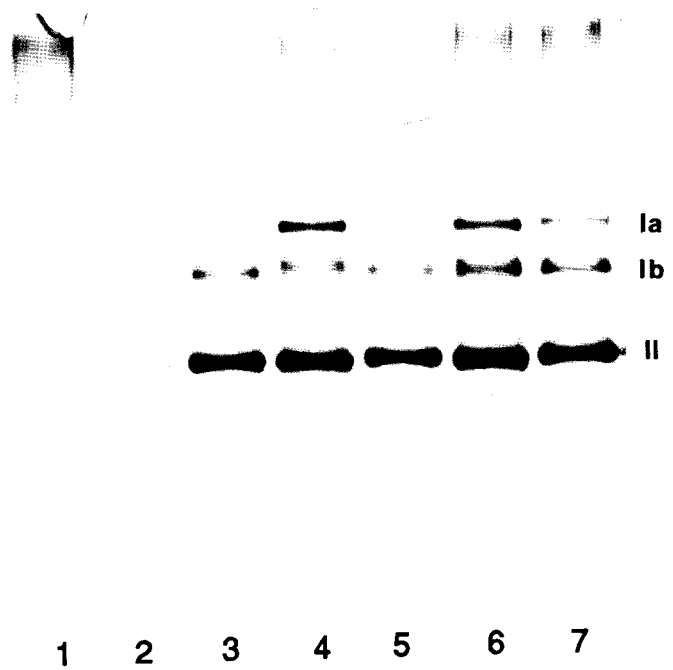

Fig. 1. Native pore-limiting PAGE of larval hemolymph. Hemolymph was collected from indicated numbers of insects, pooled, and diluted tenfold into phosphate-buffered saline containing DFP and PTC. Twenty microliters of the diluted hemolymph was further diluted to 400 μl with 200 μl of sample buffer and 180 μl water, and 20 μl (1 μl equivalent of hemolymph/lane) was applied to each lane of a 4–18% 15 × 20 × 0.15 cm gel. Electrophoresis was run for 18 h at 200 V, and the gel was stained in the methanol-Coomassie stain system. Hemolymph in **lane 1** was from adults (150 mg mean weight, n = 7), **lane 2** from pupae (308 mg mean weight, n = 2), **lanes 3–7** from larvae of 720 mg, 420 mg, 530 mg, 445 mg, and 179 mg mean weight, respectively (n = 3).

from the SDS-PAGE system had no effect on apparent molecular weight, indicating that subunits of band I or band II were not disulfide linked. As estimated by scanning densitometry of Coomassie-stained gels, native band II consistently comprised 65–70% of the total absorbance among the three bands, while the density of band Ia relative to Ib varied unpredictably (Fig. 1). Variation in band density did not correlate with larval weight or developmental state relative to the larval-larval molt. All three bands decreased in pupae and were undetectable in adults (Fig. 1). Based on this evidence, we concluded that these proteins behaved as true larval storage proteins.

Binding of Riboflavin by Storage Proteins

Since Rb has been shown to bind to hemolymph proteins [6,10], we heat-denatured whole hemolymph, pelleted the denatured protein by centrifugation, performed solid phase extraction of the supernatant, and analyzed the extract by reverse phase HPLC with fluorescence detection. Rb was clearly identified, since the elution time of the fluorescence peak at specific excitation and emission wavelengths was identical to that of the Rb standard (results not shown). Both excitation and emission fluorescence spectra of the extract

were identical to those of a Rb standard: spectral peaks were at Ex = 451 and 469 nm; Em = 529 nm.

Binding of Rb was indicated by quenching of Rb fluorescence [6; Miller and Silhacek, unpublished observations]. Pooled storage protein fractions (Fig. 2, peaks B,C) were isolated from lipophorin (peak A) on anion exchange HPLC. Lipophorin was purified by density gradient ultracentrifugation. Increasing amounts of storage proteins or lipophorin were then added to a Rb solution (0.2 μg/ml final concentration). Rb fluorescence was readily quenched by the storage protein fraction (Fig. 3), indicating protein binding. However, addition of the lipophorin fraction to Rb resulted in little or no quenching.

To further characterize the protein(s) that bound Rb, Rb content of protein fractions eluted from anion exchange HPLC was analyzed by fluorescence spectroscopy. When chromatographed at pH 7.8, three protein peaks were partially resolved (Fig. 2). Lipophorin was found exclusively in peak A. Although Rb was distributed across all three peaks, peak B contained about 80% of the total. When chromatographed at pH 8.8, the three major peaks were better resolved and Rb was redistributed (Fig. 4). Almost 90% of the total Rb was now equally distributed between peak A and the last peak, D/E; only 10% was associated with the second peak, B/C.

These results indicated a change in column affinity for at least a portion of the Rb-binding proteins with change in pH. We used this phenomenon to isolate one of the Rb-binding proteins. Whole hemolymph was chromatographed at pH 7.8; peaks B and C (see Fig. 2) were pooled (eliminating lipophorin, in peak A), concentrated in a Centricon 30, and rechromatographed at pH 8.8 (Fig. 5). The protein component moving into the vacated peak A position (9% of the total protein) was identified as protein Ib; it was associated with 61% of the total Rb in the sample. The remaining proteins (91%) were eluted in two peaks, B/C and D/E; the B/C peak was associated with the remainder of the Rb (39%). Analysis of fractions from the leading edge of peak A revealed approximately 1 μg of Rb/mg of protein. Peak B/C (a mixture of proteins Ia, Ib, and II) had only 0.1 μg Rb/mg protein and peak D/E (protein II) contained no Rb.

Subunit Isolation and Characterization

To isolate subunits, protein bands I and II were first copurified by flotation of lipophorin in an ultracentrifugal density gradient followed by GP-HPLC of the sedimenting proteins [9]. The protein sedimenting in the gradient was bright yellow, and 90% of it was band I/II protein. To resolve the α from the β subunit, samples direct from the GP-HPLC step were dissolved in solvent containing CHAPS, guanidine HCl, and isopropanol and applied to a polymeric reverse phase column. This yielded two peaks, at 10.9 min and 11.7 min (Fig. 6). SDS-PAGE illustrates the isolation from hemolymph through density gradient centrifugation, GP-HPLC, and RP-HPLC. Figure 7B shows that the 10.9-min peak contained only the α subunit (lane 1), while the 11.7-min peak contained the β subunit (lane 2).

Both subunits, separated by SDS-PAGE, stained positive for carbohydrate by the PAS method [unreported results], and both stained positive for mannose residue content with concanavalin A/alkaline phosphatase after blotting from an SDS-PAGE gel (Fig. 7C).

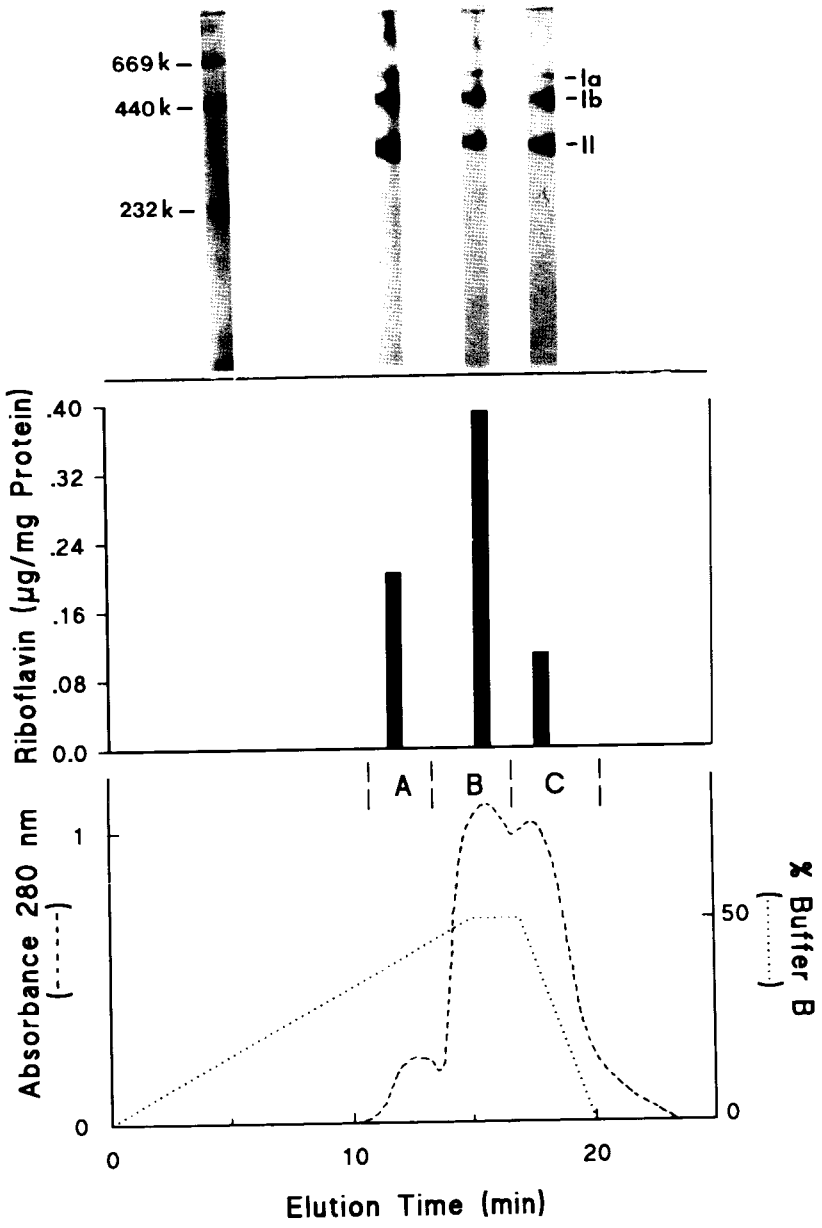

Fig. 2. Anion-exchange HPLC elution profile, pH 7.8 **(bottom)** with N-PAGE of proteins Ia, Ib, and II **(top)** and Rb content **(middle)** of fractions A, B, and C. Hemolymph (200 µl) was collected in 50 µl of PTC-saturated water, 100 µl was injected onto a column equilibrated in buffer A (50 mM NaCl) at 0.5 ml/min. Standards indicated by M_r **(left lane, top)** are Pharmacia (Piscataway, NJ) high molecular weight standards; native M_rs cannot be estimated from migration distances.

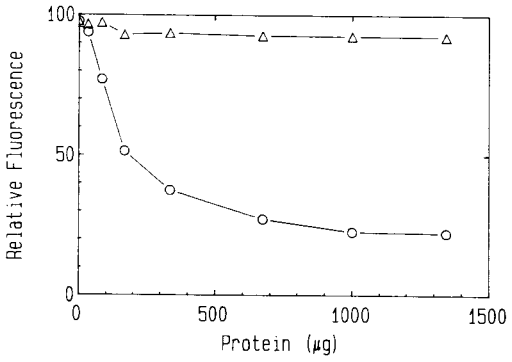

Fig. 3. Fluorescence quenching of Rb by hemolymph proteins. Increasing quantities of lipophorin (△—△) or storage proteins (○—○) isolated as described (in Materials and Methods) were added to an aqueous Rb solution to a total volume of 0.5 ml (final Rb was 0.53 µM) and fluorescence intensity was read at Ex = 445 nm and Em = 520. Points represent the means of triplicate determinations.

To relate subunit size to holoprotein size and subunit composition, protein isolated through the KBr density gradient centrifugation step was crosslinked with DMS and run on SDS-PAGE (Fig. 8). Probably due to the difference in molecular weight between the α and β subunits, doublet bands were distinct at the dimer level of crosslinking (M_r = 150,000). Higher molecular weight subunit polymers were rather disperse, with some apparent subdivision of banding. However, the appearance of six major groups of bands (lanes 5,6), with maximum M_rs of less than 600,000, indicates a hexameric structure for the holoproteins.

The amino acid compositions and N-terminal sequences of subunits isolated by RP-HPLC were determined. The two subunits were very similar in amino acid composition, with some distinct differences (Table 1). Both were high in aromatic residue content (Tyr + Phe contents of 16%; Trp was not analyzed). Both were high in Pro (5.6% and 5.4% for α and β subunits, respectively). The basic residue contents, excluding Asn and Gln, were similar: 10.6% in the α subunit and 12.1% in the β subunit. However, the α subunit was higher in His (6.0% vs. 3.4%), whereas the β subunit was higher in Arg (5.2% in the β subunit vs. 2.8% in the α subunit). The α subunit contained over twice the Thr and Met of the β subunit.

N-terminal sequences were easily determined to 20 residues (Fig. 9), although some uncertainty in the sequence of the β subunit may have resulted from a slight contamination by the α subunit following RP-HPLC. N-terminal sequence homology between subunit types was at least 45%, and probably closer to 60%.

DISCUSSION

The present results demonstrate that hemolymph proteins Ia, Ib, and II of *D. abbreviatus* are hexameric larval storage proteins, since hemolymph concentrations are high during the larval instars, concentrations decline precipi-

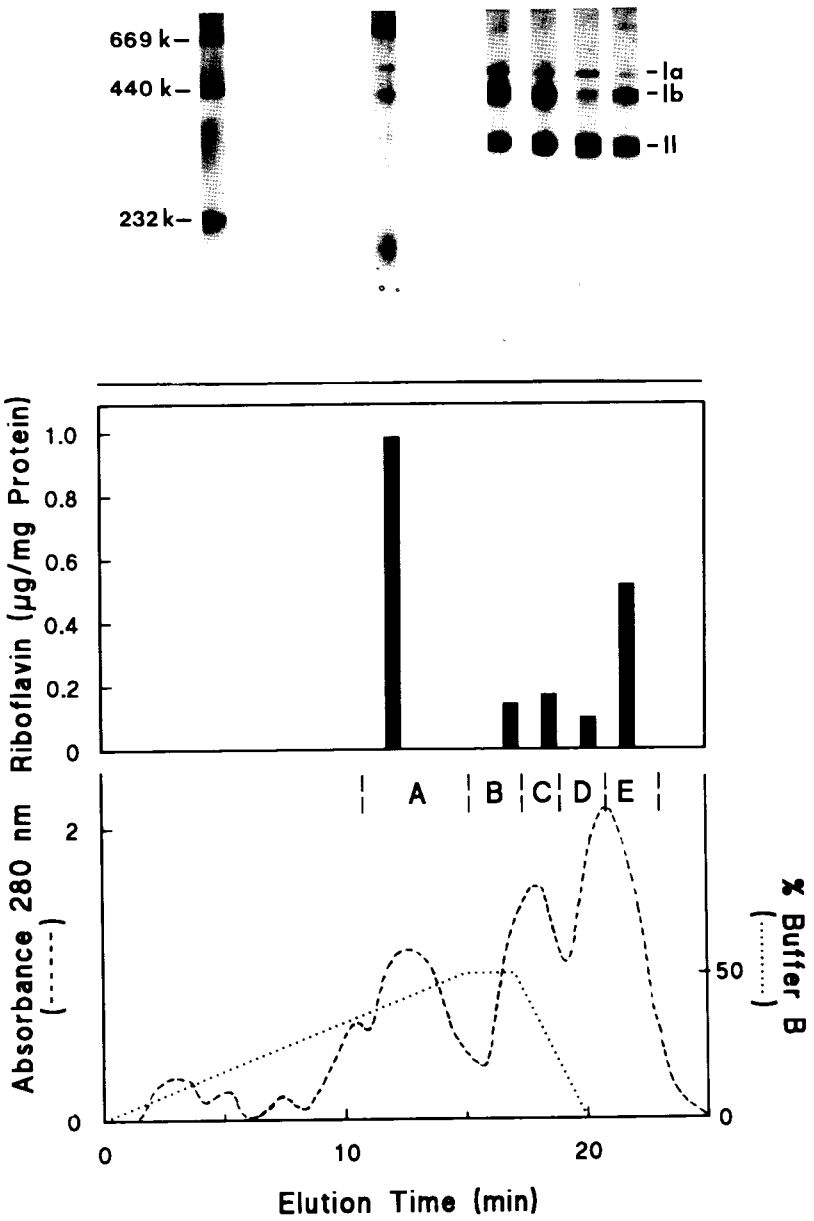

Fig. 4. Anion-exchange HPLC elution profile at pH 8.8 **(bottom)** with corresponding N-PAGE of proteins Ia, Ib, and II **(top)** and Rb content **(middle)** of fractions A–E. Buffer A = 50 mM Tris, pH 8.8; buffer B = 50 mM Tris, pH 8.8, 0.5 M NaCl. Flow rate was 0.5 ml/min.

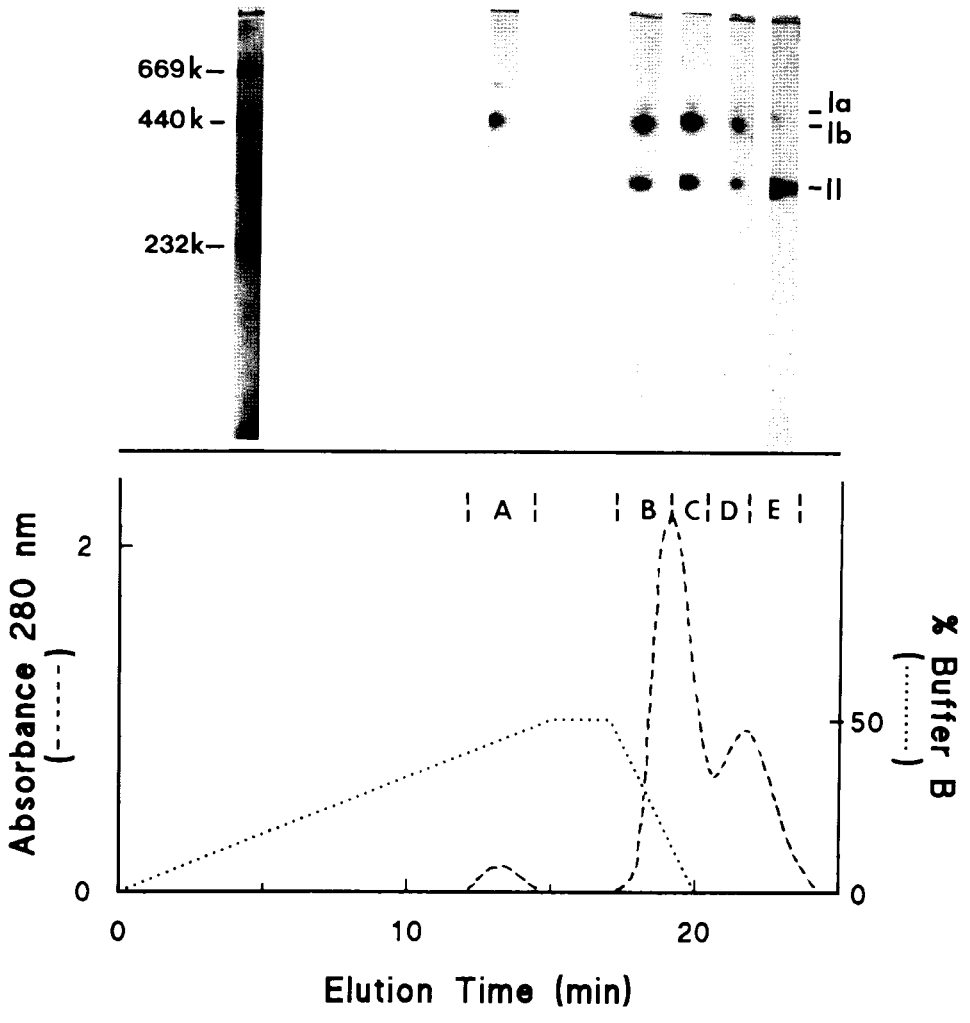

Fig. 5. Rechromatography at pH 8.8 of peaks B and C eluted at pH 7.8 (as in Fig. 2): elution profile **(bottom)** and N-PAGE of proteins Ia, Ib, and II **(top)**. Hemolymph was fractionated at pH 7.8 (see Fig. 2) and peaks B and C were pooled to total 4 ml. After concentrating and reconstituting twice with pH 8.8 buffer (50 mM Tris, pH 8.8), 100 μl of sample was injected onto the anion exchange HPLC column and eluted at 0.5 ml/min with 0.25 M NaCl gradient in the latter buffer.

tously during the pupal stage, and the proteins are apparently hexamers. Each of the two types of subunit that compose the holoproteins contains approximately 16% Tyr + Phe, an aromatic amino acid content slightly lower than that of the arylphorins [4,5]; both subunits are glycosylated. An analysis strictly by content of aromatic vs. methionine residues [4] indicates a relationship to hexamerins of Diptera and Lepidoptera: the α subunit identifies with calliphorin-like hexamerins, while the β subunit is similar in contents to both lepidopteran arylphorins and the "second hexamerins" of Diptera [4].

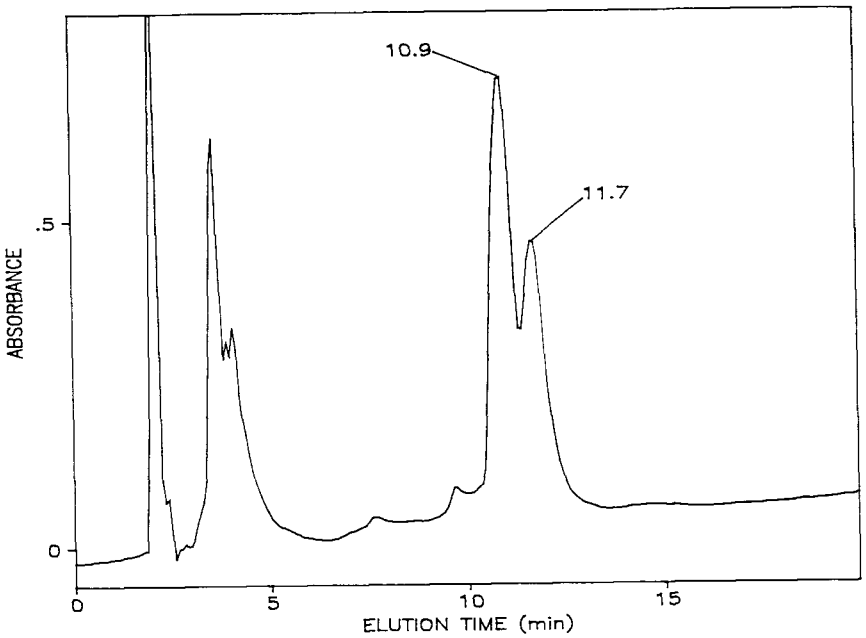

Fig. 6. Reverse phase HPLC. Peak fractions collected from GP-HPLC one day previously were added to premixed solvent, filtered, and 100 μl was injected onto the column.

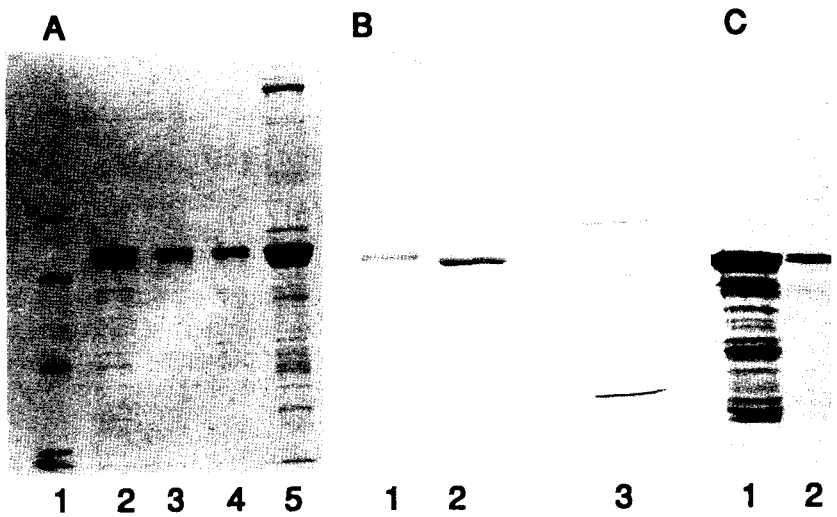

Fig. 7. **A:** SDS-PAGE of hemolymph and fractions from density gradient centrifugation and GP-HPLC. **Lane 1:** Low M_r standards. **Lane 2:** Density gradient fraction. **Lanes 3,4:** GP-HPLC fractions. **Lane 5:** Whole hemolymph. **B:** SDS-PAGE of pooled fractions (see Fig. 6) from peaks at 10.9 min **(lane 1)** and 11.7 min **(lane 2)** and low M_r standards **(lane 3)**. **C:** Alkaline phosphatase/concanavalin A staining of blotted proteins from SDS-PAGE. **Lane 1:** Whole hemolymph. **Lane 2:** Density gradient-purified protein. All samples were run on 7% minigels.

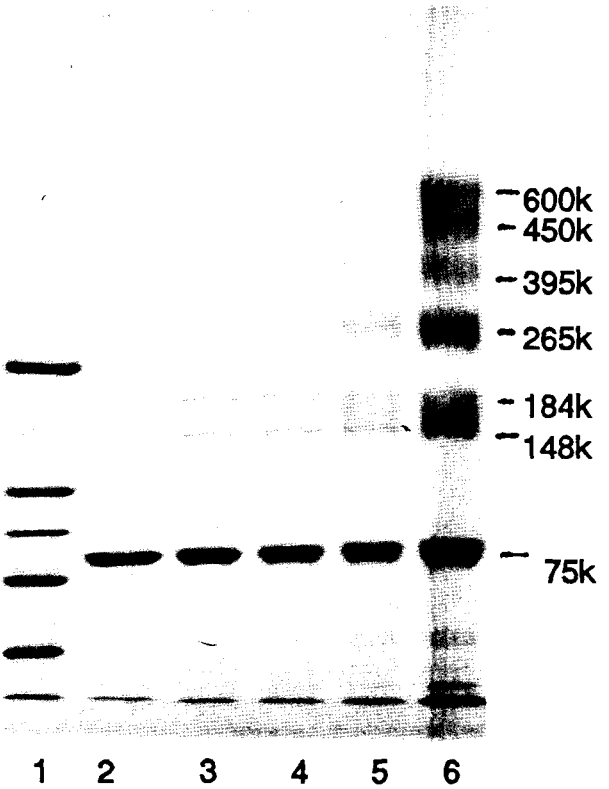

Fig. 8. SDS-PAGE of proteins from a density gradient centrifugation, crosslinked with DMS. **Lane 1:** High M_r standards. **Lane 2:** Untreated proteins. **Lanes 3–6:** Proteins crosslinked with 5, 10, 25, or 50 μg of DMS, respectively. M_rs given are rough estimates from extrapolation of standard M_rs and positions.

Developmentally, the *D. abbreviatus* proteins differ from the well-known lepidopteran storage proteins in that high concentrations are maintained throughout larval life, and no sudden increase in concentration occurs during the last larval instars. This may relate to the development of *D. abbreviatus* larvae, which have an indeterminate number of molts. Though their weight typically plateaus at 500–700 mg, molting continues until unknown stimuli (possibly soil moisture content [21]) prompt the larval-pupal molt. Furthermore, these are the only proteins besides lipophorin present at high concentrations (> 1 mg/ml) in the larval hemolymph of *D. abbreviatus*.

D. abbreviatus proteins I and II were previously shown to bind coumarin-10 in vivo and in vitro [9]. Here we clearly demonstrated that most (61%) Rb eluted from anion exchange HPLC with isolated protein Ib and that protein II (the homopolymer of α subunit) bound little or no Rb. Lipophorin, isolated by density gradient centrifugation or anion exchange HPLC, contained negligible amounts of Rb and did not significantly quench Rb fluorescence (i.e., bind Rb). As with coumarin-10 binding, *D. abbreviatus* lipophorin seems to play a minor role in Rb binding. We have not yet been able to isolate proteins Ia and

TABLE 1. Amino Acid Composition of α and β Subunits Isolated by RP-HPLC*

Amino acid	α-Subunit Residues/mol ± S.D.	α-Subunit Mol %	β-Subunit Residues/mol ± S.D.	β-Subunit Mol %
ASX	72 ± 2	11.2	77 ± 3	12.7
GLX	73 ± 3	11.3	77 ± 1	12.7
SER	36 ± 1	5.6	39 ± 1	6.5
GLY	41 ± 1	6.3	42 ± 2	7.0
HIS	39 ± 1	6.0	20 ± 1	3.4
ARG	18 ± 1	2.8	32 ± 1	5.2
THR	66 ± 2	10.3	27 ± 1	4.5
ALA	25 ± 1	3.9	25 ± 2	4.1
PRO	36 ± 1	5.6	33 ± 1	5.4
TYR	55 ± 2	8.5	56 ± 4	9.2
VAL	40 ± 2	6.3	32 ± 2	5.3
MET	24 ± 1	3.7	11 ± 1	1.7
ILE	21 ± 1	3.2	27 ± 1	4.4
LEU	40 ± 2	6.2	44 ± 1	7.3
PHE	47 ± 1	7.3	43 ± 1	7.1
LYS	12 ± 0	1.8	21 ± 3	3.5

*Mean ± S.D. of quadruplicates.

Ib as two distinct holoproteins, so it is not clear whether Ia can also bind Rb, if differently eluting forms of Ib maintain differing affinity and capacity for Rb binding, or if Rb binding in itself may distinguish these two forms. Definition of binding characteristics may be closely linked to clear definition of dynamic interactions among subunits and among holoproteins, a difficult task to date.

Rb binding by storage proteins of Lepidoptera has been described in at least two previous studies [6,10]. No immunological crossreactivity was observed on Western blots of *D. abbreviatus* proteins reacted with polyclonal antisera against arylphorins, female-predominant, or 85k proteins from *Galleria mellonella*, or 82k protein from *Heliothis zea* [Silhacek, unpublished results]. However, the N-terminal subunit sequences reported here show partial homology (between 15% and 35%) with the Rb-binding proteins of *H. virescens* (82k protein) and *G. mellonella* (85k protein) (Miller and Silhacek, unpublished data). Taken together with observations on binding of insecticides [2] and phytochemical analogs [9] by hemolymph storage proteins, these studies indicate that hemolymph storage proteins may serve to bind plant allelochemicals and xenobiotics following ingestion and absorption.

Although storage proteins are known for their distinct and unique developmental roles in insects, it is tempting to draw parallels with mammalian serum albumin when considering absorption and transport functions. Serum albumins have been studied for at least fifty years regarding their roles in transport of pharmacological, toxicological, and nutritional agents in mammals [22]. Perhaps the sheer deversity of insect hemolymph proteins and the recency of detailed knowledge and classification has precluded study of parallel roles. Rather, penetration of insecticides through cuticle, enzymatic metabolism and excretion, and final effects at target organs have borne the emphasis in insect toxicology; dynamics of digestive absorption and transport to target sites have

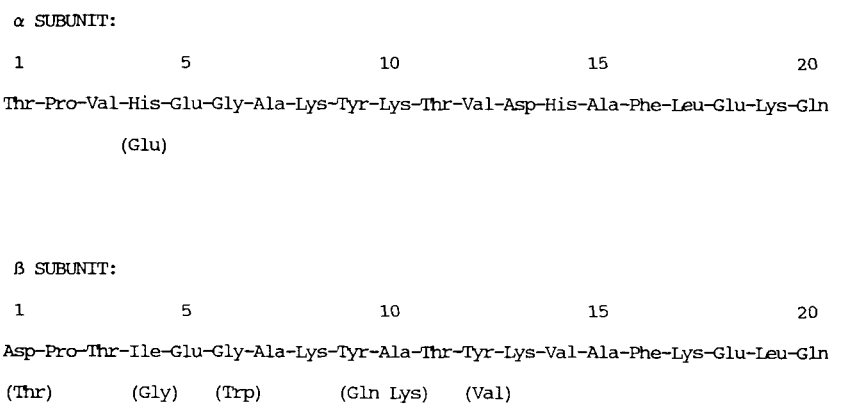

Fig. 9. N-terminal sequences of α and β subunits isolated by RP-HPLC. Amino acids in parentheses indicate possible ambiguities in the sequence.

been largely neglected [8]. If serum albumins are indicative, hemolymph proteins may be crucial in both detoxicative and intoxicative roles.

As described here, isolation of the native proteins to homogeneity proved difficult, requiring repetitive steps: repetitive anion exchange HPLC at two different pHs proved most useful, at least for purification of proteins Ib and II. Paradoxically, while single-step HPLC methods (cation and anion exchange, hydrophobic interaction, and gel permeation) incompletely resolved the holoproteins from each, N-PAGE seemed to cleanly resolve them [9]. Unfortunately, that separation was deceptive: although electroeluted band II protein was homogeneous, band I protein resolved into both band I and II proteins when rerun on N-PAGE. This may indicate that several heterohexameric combinations of the two subunits may exist, and/or that subunits may dynamically recombine, or that holoproteins may reversibly aggregate. Dynamic subunit recombination in the arylphorin class of storage proteins was first suggested by the spontaneous dissociation of *Calliphora* arylphorin at high pH [23,24].

Following initial attempts to isolate the three holoproteins, we ignored the potential complexities of subunit reassociation in favor of isolating individual subunits by RP-HPLC. Isolations were sufficient for amino acid sequencing, but may not have been absolute (Fig. 6). With minor improvement, especially in choice of detergent, our RP-HPLC method may prove to be an excellent general method for isolating subunits from storage proteins, especially since the subunits of a storage protein are often very similar in molecular weight, sequence, and amino acid composition (e.g., see [7,25] regarding *M. sexta* arylphorin). Furthermore, as suggested by the very small difference in molecular weight of *D. abbreviatus* subunits, other storage proteins may contain heterologous subunits despite apparent homogeneity on SDS-PAGE. This possibility has been suggested [5] in at least one other case: multiple clones of *Sarcophaga peregrina* arylphorin were discovered, despite apparent homogeneity of gene products on SDS-PAGE [26].

Interestingly, amino acid sequence comparison of subunits from *M. sexta* arylphorin with those of *Panulirus interruptus* (spiny lobster) hemocyanin have

shown maximum sequence homology at regions of known intersubunit contact in the hemocyanin [7]. These homologies may indicate an evolutionary significance for intersubunit dynamics in the hexamerins, and a functional significance beyond that of passive storage for amino acid residues. For example, in the *Hyalophora cecropia* hemolymph flavoprotein (a non-arylphorin), disruption of disulfide bonds by N-ethylmaleimide released bound Rb [6]. As with many hexamerins, the *D. abbreviatus* proteins dissociate in SDS-PAGE systems with or without added disulfide-reducing agents such as β-mercaptoethanol. As Telfer and Massey [6] noted, however, bonds other than covalent disulfides may be crucial to forming interchain associations, and similar observations may eventually account for the intersubunit dynamics noted here.

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