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Catecholamine-Containing Proteins from the Pharate Pupal Cuticle of the Tobacco Hornworm, *Manduca sexta*

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Three catecholamine-containing proteins from tanning, pharate pupal, abdominal cuticle of the tobacco hornworm, *Manduca sexta*, have been purified to apparent homogeneity and characterized. These proteins have apparent molecular masses of 32, 41 and 48 kDa and were shown by liquid chromatography–electrochemical analysis, after heating in 1 M acetic acid at 110°C, to release *N*-β-alanylnorepinephrine (NBANE). NBANE is a hydrolysis product of *N*-β-alanyldopamine (NBAD) that is bonded covalently at the β-side-chain carbon to amino acid residues of cuticular proteins. Amino acid compositional analysis revealed that MS-PCP32 (32 kDa) has a high content of alanine (25.6%), valine (13.1%), proline (10.8%) and glycine (10%), a low level of phenylalanine, and no detectable tyrosine or methionine. In contrast, MS-PCP41 (41 kDa) had a much higher content of glycine (31.2%) and substantial levels of serine (9.2%), proline (10.2%) and glutamate/glutamine (10.6%), whereas tyrosine and phenylalanine were not detected. Two of the three purified proteins showed apparent similarities to each other in N-terminal amino acid sequences, and to several other known insect cuticular proteins. Proteins MS-PCP41 and MS-PCP48 had the characteristic GGX triplet repeat, which is found in a variety of cuticular proteins and may be important for protein folding appropriate for cuticular functions. Therefore, a diversity of cuticular proteins with different amino acid sequences and properties apparently are secreted into the presumptive pupal exocuticle. These then can form adducts and possible cross-links with NBAD through its quinonoid intermediates during cuticular sclerotization. Published by Elsevier Science Ltd.

Cuticular proteins Cuticle Catecholamines Catechols Diphenols Amino acid sequence Sclerotization Pharate pupae *Manduca sexta* Tobacco hornworm Exoskeleton Tanning *N*-β-Alanyldopamine *N*-β-Alanylnorepinephrine

INTRODUCTION

Insect cuticle plays an important role both as an exoskeleton and as a barrier that protects the insect from adverse environmental conditions. This structure is composed primarily of proteins and chitin with lesser quantities of catechols, lipids, minerals and water (Hepburn, 1985; Kramer *et al.*, 1988). The architecture in which these compounds are arranged to form a rigid sclerotized structure is poorly known. Chitin is organized into microfibrils that are embedded in a protein matrix (Neville, 1975) secreted by the underlying epidermis (Doctor *et al.*, 1985; Riddiford *et al.*, 1986; Sass *et al.*, 1993). Progress in the isolation and characterization of cuticular proteins has been accelerated greatly in recent years by improvements in extraction, purification and amino acid sequencing procedures, providing a much better understanding of their diversity and properties (Willis, 1987; Andersen *et al.*, 1995). For example, proteins have been purified from the cuticle of *Drosophila melanogaster* (Fristrom *et al.*, 1978), *Locusta migratoria* (Hojrup *et al.*, 1986; Andersen, 1988; Talbo *et al.*, 1991; Andersen *et al.*, 1993) and *Hyalophora cecropia* (Willis, 1989), and their properties and primary structures characterized. Substantial progress also has been made in studying cuticular proteins with regard to their expression as markers of developmental programs (Chihara *et al.*, 1982; Kiely and Riddiford, 1985; Lemoine and Delachambre, 1986; Skelly and Howells, 1988; Rabossi *et al.*, 1991; Sridhara, 1994).

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Understanding the functional role(s) of these proteins depends not only on the elucidation of their primary, secondary and tertiary structures, but also on their posttranslational modifications and interactions with other cuticular components, such as glycosylation (Silvert *et al.*, 1984; Cox and Willis, 1987; Talbo *et al.*, 1991). Cuticular sclerotization also results in protein modifications when nucleophilic side-chains of the amino acids react with quinonoid tanning agents (Hopkins and Kramer, 1992; Okot-Kotber *et al.*, 1994). To better understand this type of posttranslational modification, we recently have purified two high molecular weight proteins and determined that they contain covalently bound catechols (Okot-Kotber *et al.*, 1994). The results showed that quinonoid metabolites of *N*-acylcatecholamines can form adducts and possibly cross-links during sclerotization. This finding supported earlier solid-state NMR evidence for bonding between catecholamines and histidyl residues in proteins of sclerotized cuticle of *Manduca sexta* (Schaefer *et al.*, 1987; Christensen *et al.*, 1991). We have now purified and partially characterized three additional proteins from the cuticle of *M. sexta* pharate pupae, which have relatively intermediate molecular masses and also contain covalently bound catecholamine residues.

MATERIALS AND METHODS

Chemicals

N-β-Alanylnorepinephrine (NBANE) was synthesized according to the procedure of Rembold *et al.* (1978). α-Methyldopa (αMD) and norepinephrine (NE) were purchased from Sigma (St. Louis, MO). Molecular weight markers were from BioRad (Hercules, CA). Unless otherwise stated, other chemicals and solvents were obtained from either Sigma, Fisher Scientific (Springfield, NJ), or Pierce (Rockford, IL).

Insect rearing

Tobacco hornworms (*Manduca sexta*) were reared at 27°C and 16:8 h light/dark photoperiod on a semi-artificial diet until the pharate pupal stage (late brown bar stage) when cuticle was collected for protein extraction (Okot-Kotber *et al.*, 1994).

Cuticle preparation

Pharate pupae were anesthetized with CO_2 and then frozen in either dry ice or in a freezer at −20°C. Then the cuticles were dissected and cleaned meticulously of adhering tissues in Ringer's solution containing protease inhibitors, phenol oxidase inhibitors, and an antioxidant as previously described (Okot-Kotber *et al.*, 1994).

Protein extraction

Cuticular proteins were extracted using low pH buffered 8 M guanidinium chloride fortified with protease and phenol oxidase inhibitors and reducing agents, which was found to be the most effective solvent for cuticle protein extraction (Okot-Kotber *et al.*, 1994). Briefly, the extraction was accomplished by grinding the cuticle in a glass tissue homogenizer in ice and stirring the homogenate overnight at 4°C. The homogenate was centrifuged, the supernatant saved, and the pellet re-extracted several times. The supernatants were pooled and centrifuged at 27,000 ***g*** for 1 h at 8°C to remove particulates. The final supernatant was diluted with 0.8 M acetic acid so that the guanidinium chloride concentration was compatible with the Amicon Centriprep ultrafiltration device according to the manufacturer's instructions. The retentate obtained after ultrafiltration was diluted with 0.8 M acetic acid and ultrafiltered several times to ensure that the non-covalently bound catechols, guanidinium chloride and low molecular weight molecules found in the cuticle were eliminated. Most of the final retentate was diluted in 0.05% trifluoroacetic acid (TFA) for liquid chromatography (LC), and a small portion in 1 M acetic acid was used for acid hydrolysis.

Protein purification

The initial step in the protein purification was accomplished by LC with either size exclusion chromatography (SEC) using a semipreparative BIOSEP SEC S3000 column (300 mm × 7.6 mm, 7.8 μm, Phenomenex) with 0.05% trifluoroacetic acid (TFA) as mobile phase (Okot-Kotber *et al.*, 1994), or by reversed phase LC using a semipreparative column, SEL-ECTOSIL RP 5 μm C8 300A (250 mm × 10 mm, Phenomenex). In the latter procedure, the column was equilibrated with 0.05% TFA (solvent A). The solubility of the cuticular protein extract was improved further by adding 8 M urea and 0.05% TFA (final concentrations). The sample then was centrifuged, and the supernatant was applied to the column and eluted using a stepwise gradient (30, 70 and 100%) of 50% isopropanol in 0.05% TFA (solvent B) at 15, 60 and 75 min, respectively, at a flow rate of 0.5 ml/min. The eluent was monitored at 280 nm. Several sample aliquots were chromatographed and those peaks with the same elution times were collected, pooled, frozen at −80°C, and concentrated in a SpeedVac or lyophilized. Aliquots were then taken from the pooled samples and subjected to SDS–PAGE, as described below, to determine those fractions containing the proteins of interest. The fractions then were subjected to a second round of reversed phase chromatography using the same mobile phases described above, but employing a slightly modified stepwise gradient (30, 70 and 100% B at 15, 55 and 60 min, respectively). Peaks were collected and processed as described above. Depending on the SDS–PAGE analysis of purity, appropriate fractions were rechromatographed when necessary to achieve homogeneity of the desired proteins. Purified proteins were lyophilized and reconstituted in 0.8 M acetic acid for subsequent analyses.

Electrophoresis

Protein fractions were subjected to SDS–PAGE essentially as described by Laemmli (1970) and modified by Ames (1974). To check for purity, the fractions were loaded onto discontinuous slab polyacrylamide gels (1 mm thick) with a 4–12% linear gradient gel for separating gels and 3% stacking gels. Broad range molecular mass marker proteins (6.5–200 kDa) were loaded and run along with samples to compare mobilities. Electrophoresis was performed overnight using a Bio-Rad Protean II apparatus at 70 V and 8°C. After electrophoresis, gels were stained with Coomassie brilliant blue R-250 and subsequently destained in methanol:water:acetic acid (5:4:1) for several hours and finally in methanol:water:acetic acid (3:6:1) before being photographed.

Determination of protein size and concentration

Apparent molecular masses of proteins were estimated by SDS–PAGE using a 4–15% gradient gel (Laemmli, 1970). The standard marker proteins were myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa).

Concentrations of proteins were determined using the Bio-Rad protein assay dye reagent procedure based on the method of Bradford (1976). Bovine serum albumin (Pierce) was used as the standard protein.

Mild acid hydrolysis of proteins and catechol determination

Solutions containing 100 μg of crude or purified proteins were lyophilized, reconstituted in 100 μl of 1.0 M acetic acid, and heated, together with αMD as an internal standard, at 110°C for 10 min under nitrogen to hydrolyze weakly bonded catechols. Hydrolysates were cooled and centrifuged at 13,000 ***g*** for 15 min to remove any precipitate. The supernatants then were recovered and analyzed for catechols by LC with electrochemical detection (LC–EC) as described by Morgan *et al.* (1987) and Okot-Kotber *et al.* (1994). LC–EC was performed by loading samples onto an ODS 5 μm spherical particle C18 reversed phase column followed by elution with an isocratic mobile phase consisting either of 26% acetonitrile, SDS, sodium phosphate and EDTA, or 13% methanol, sodium octyl sulphate, sodium phosphate and EDTA (Morgan *et al.*, 1987).

Amino acid composition and N-terminal amino acid sequencing

Aliquots of LC-purified proteins were analyzed for either amino acid composition or N-terminal amino acid sequences (Okot-Kotber *et al.*, 1994). Amino acid composition was determined at the University of Missouri–Columbia Experiment Station Chemical Laboratories. The sequencing was achieved by automated Edman degradation on an Applied Biosystem sequencer at the Biotechnology Microchemical Core Facility, Kansas State University, Manhattan. The sequences were then aligned for comparison with other proteins using the FASTP search program (Lipman and Pearson, 1985) by Dr Judith Willis, University of Georgia, Athens.

RESULTS

Protein purification

Two high molecular weight pharate pupal cuticular proteins have been purified previously by SEC (Okot-Kotber *et al.*, 1994). However, several of the major proteins in the 6–50 kDa molecular mass range were resolved poorly. Therefore, in this study, these fractions were collected and subjected to reversed phase LC. As an alternative method of purification, the crude extract was directly loaded onto a semipreparative C8 reversed phase column as a first step. This procedure resolved about 40 protein peaks (Fig. 1) and, of these, 13 were considered to be major based on their relative absorbances at 280 nm. Because preliminary results showed that the proteins present in these peaks released catecholamines, especially NBANE, after mild acid hydrolysis, we focused our attention on three of the major fractions, denoted "a", "b" and "c" in Fig. 1, which we were able to purify to homogeneity. We were unable to purify any of the other fractions to homogeneity. Fractions "a", "b" and "c" were purified further by reversed phase LC as described in the Materials and Methods section. When the major protein in each fraction was rechromatographed, it yielded a single large peak as shown for the peak "a" fraction [Fig. 2(A)]. This preparation was judged to be homogeneous as revealed by a single band after staining with Coomassie brilliant blue [Fig. 2(B)]. The apparent molecular mass of the peak "a" protein was estimated to be 32 kDa, and this protein is denoted as MS-PCP32, where MS-PCP represents "***Manduca Sexta*** **P**upal **C**uticle **P**rotein" and the number designates the molecular mass in kDa (Andersen *et al.*, 1995).

The major protein in fraction "b" was purified by a procedure similar to that used for MS-PCP32. The chromatogram of fraction "b" revealed a single major protein peak and five minor ones [Fig. 3(A)]. The protein of interest was collected from the center of the major peak. An aliquot subjected to SDS–PAGE gave a single band after Coomassie brilliant blue staining [Fig. 3(B)]. The apparent molecular mass of this protein was estimated to be 48 kDa, and the protein is denoted as MS-PCP48.

The peak "c" fraction (Fig. 1) was subjected similarly to reversed phase LC, and the chromatogram shows a main peak with an elution time of about 48 min and several other major and minor peaks [Fig. 4(A)]. A narrow band of the main peak was collected as indicated by a bar at approx. 48 min. The aliquot was subjected to SDS–PAGE and showed apparent homogeneity as revealed by a single band following Coomassie brilliant blue staining [Fig. 4(B)]. The apparent molecular mass of this protein

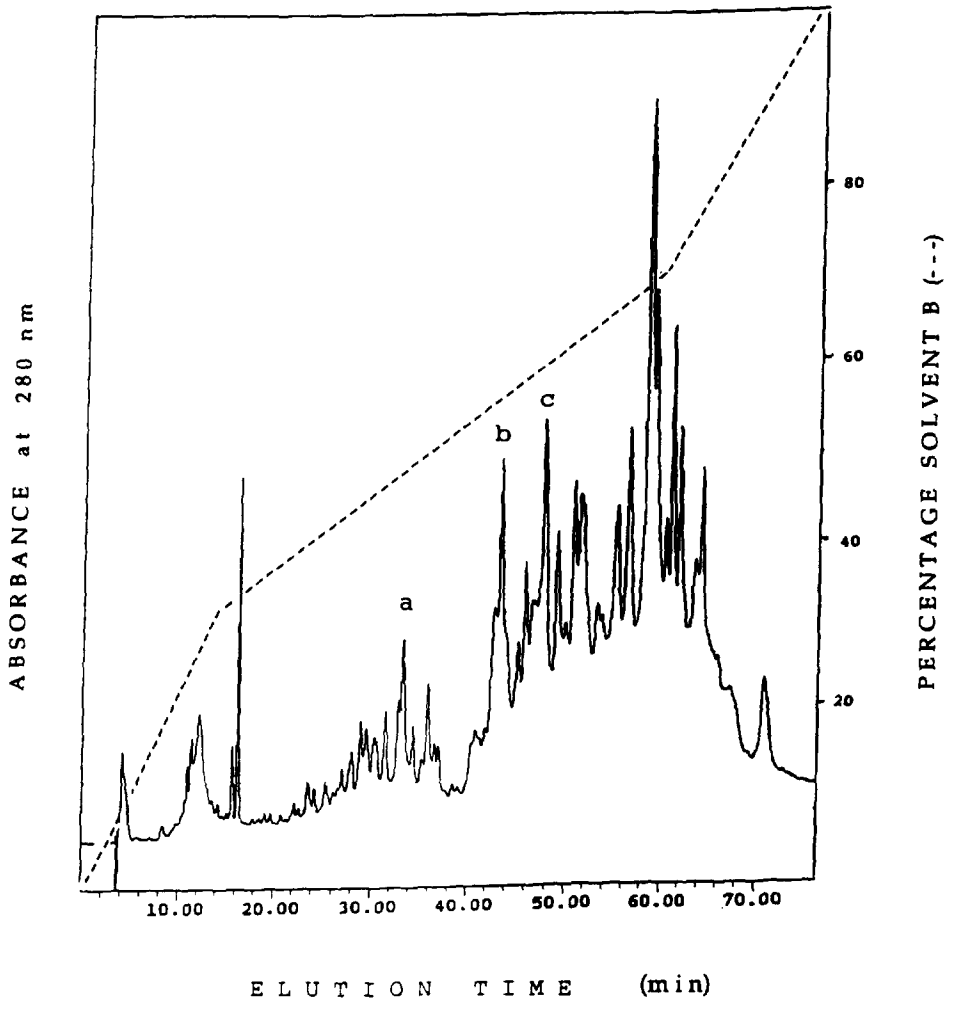

FIGURE 1. Semipreparative reversed phase LC chromatogram of a cuticular protein extract from *M. sexta* pharate pupal cuticle. The extract was solubilized in 0.05% TFA containing 8 M urea and loaded onto a 5 μm C8 250 mm × 10 mm column, equilibrated with 0.05% TFA (solvent A), and eluted with a stepwise gradient of 50% isopropanol in 0.05% TFA (solvent B) as described in Materials and Methods. Peaks collected for further purification are denoted as "a", "b" and "c".

was estimated by SDS–PAGE analysis to be 41 kDa, and the protein is designated as MS-PCP41.

Catecholamine analysis

LC–EC analysis of hydrolysates revealed that the crude cuticle protein extract and each of the purified protein fractions (MS-PCP32, MS-PCP41 and MS-PCP48) contained covalently bound NBAD as indicated by the release of its hydrolysis product NBANE (Table 1). MS-PCP41 released the highest amount of NBANE followed by MS-PCP48. Although MS-PCP32 also released NBANE, its level could not be determined quantitatively because of interference by an unknown compound. As expected, crude cuticle protein extracts also released NBANE as a hydrolysis product of covalently bound NBAD, but the concentration was lower than that found in MS-PCP41 and MS-PCP48. The molar ratios of catecholamine to the protein were relatively similar (Table 1).

Amino acid composition

The amino acid compositions, with the exception of cysteine and tryptophan, were determined for MS-PCP32 and MS-PCP41 (Table 2). The amino acid composition of MS-PCP48 was not determined because insufficient sample was available. Aspartate and glutamate levels are combined with their corresponding amides. MS-PCP32 appeared to be the most hydrophobic because of a very high content of alanine which constituted 26% of the total amino acids, whereas two other hydrophobic amino acids, valine and proline, accounted for 13% and 11%, respectively. Glycine was the most abundant hydrophilic amino acid in MS-PCP32 (10%), whereas aspartate and glutamate, together with their amides, accounted for 13%. The basic amino acids, histidine, lysine and arginine, constituted 14%, with histidine accounting for nearly half of this total. Aromatic amino acids were very low; tyrosine was not detected, and phenylalanine was less than 1%. MS-PCP32 was also devoid of methionine.

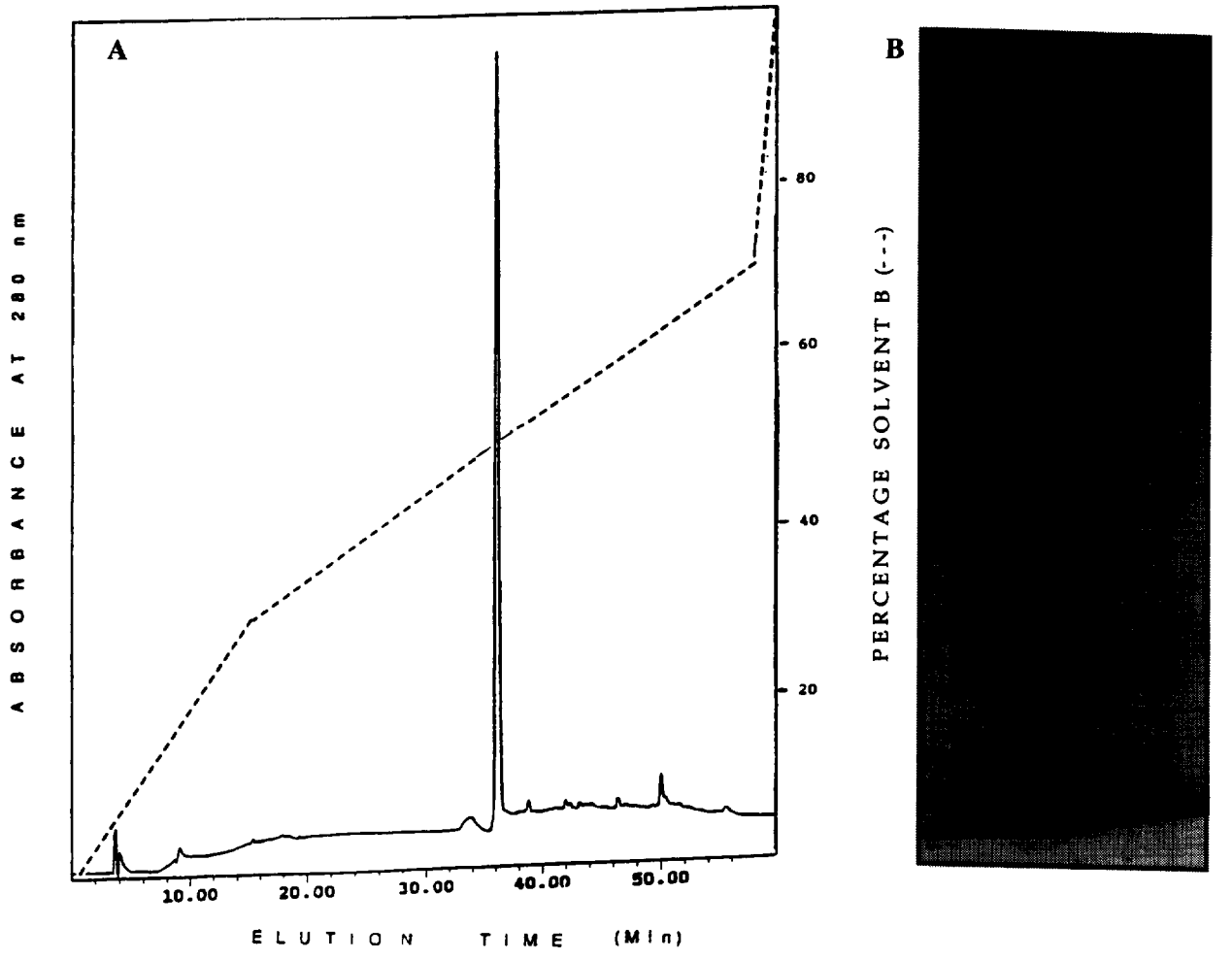

FIGURE 2. (A) Further purification of peak "a" fraction shown in Fig. 1 by semipreparative LC C8 reversed phase chromatography as described in Materials and Methods. (B) SDS–PAGE electropherogram of the peak "a" purified fraction from (A). Lane 3 shows a single Coomassie brilliant blue stained band of a 32 kDa protein (MS-PCP32). CP, cuticle protein extract; MW, molecular mass protein markers as in Fig. 3(B).

MS-PCP41 had a very high content of glycine (31%) and a moderate amount of serine (9%). Glutamate and aspartate with their amides amounted to 15%, and the basic amino acid content was relatively low (7%). Hydrophobic amino acids, such as proline (10%), alanine (8%) and valine (6%), accounted for 36% of the total amino acids. The aromatic amino acids and arginine were not detected. A relatively high methionine level (4%) for cuticle proteins was detected.

N-Terminal amino acid sequences

MS-PCP32, MS-PCP41 and MS-PCP48 were subjected to automated Edman degradation N-terminal amino acid sequencing (Table 3). The first 29 amino acids from the N-terminus of MS-PCP32 were sequenced and 23 of those were identified unequivocally. This terminus is fairly hydrophobic and characterized by an AAPV motif which also occurs in a number of other cuticular proteins and may be important for optimal folding (Andersen *et al.*, 1995). The procedure unambiguously identified 27 of the 29 residues for MS-PCP41 and 40 of 41 residues for MS-PCP48. The N-termini of both proteins are rich in glycine and contain appreciable amounts of histidine. Repeats of a triplet sequence GGX, in which X is either phenylalanine, tyrosine, or histidine, are common features in both proteins.

A cuticle protein sequence database search was performed for similarities between these sequences and those of other cuticle proteins, employing the Pearson search program. No N-terminal sequence homologies with the proteins occurred in the database or in comparisons against one another. Similarities were found only by aligning amino acid overlaps. MS-PCP32 is a relatively unique cuticular protein, with the closest similarity being a 28% identity in a 29 amino acid overlap with the locust cuticle protein LM-ACP38 (Andersen *et al.*, 1995).

MS-PCP41 and MS-PCP48 can be aligned with 46% identity for 28 of their amino acids. Yet this similarity is not significant when tested by the RDF program of Lipman and Pearson (1985). Both MS-PCP41 and MS-PCP48 can be aligned with cuticular proteins from a diversity of species. For example, MS-PCP41 had 59% identity with *D. melanogaster* cuticular proteins DM-CP56 and DM-EDGCP91, 53% with *Tenebrio molitor* TM-ACP20 in a 17 amino acid overlap, and 37% with *H. cecropia* HC-LCP49 in a 19 amino acid overlap. The

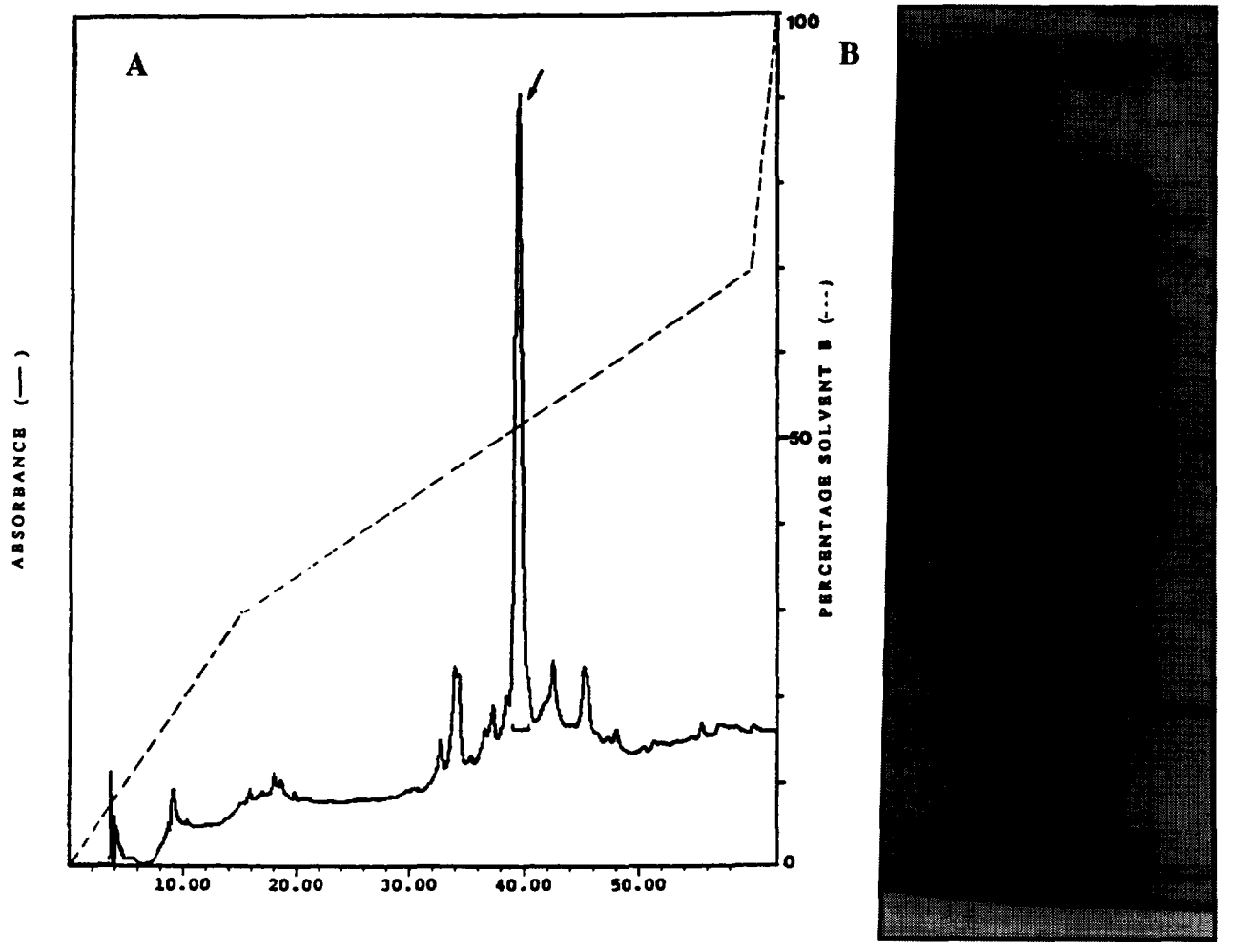

FIGURE 3. (A) Further purification of the peak "b" fraction as shown in Fig. 1 (arrow) by semipreparative LC C8 reversed phase chromatography. The fraction was subjected to chromatography as described in Materials and Methods. Bracket denotes the area collected. (B) SDS–PAGE electropherogram of the peak "b" purified fraction from (A). Lane 1 shows a single band of Coomassie brilliant blue stained 48 kDa protein (MS-PCP48) band. MW, molecular mass protein markers (kDa); CP, cuticle protein extract.

N-terminus of the *Manduca* sequences were aligned to internal regions of the *Drosophila* sequences. Likewise, MS-PCP48 was similar to DM-CP56 with a 36% identity in a 33 amino acid overlap, a 54% identity with locust LM-ACP64 in a 13 amino acid overlap, and a 37% identity with *T. molitor* TM-ACP20. There was also a 38% identity in a 42 amino acid overlap with a cockroach oothecal protein (oothecin), from *Periplaneta americana* PA-OOC. Given the high glycine content of the N-termini of MS-PCP41 and MS-PCP48, it would be premature to speculate on whether the similarities identified are indicative of homology or are merely a reflection of the high glycine regions found in many cuticular proteins.

DISCUSSION

Three cuticle proteins were purified to apparent homogeneity from the partially sclerotized cuticle of pharate pupae of *M. sexta*. Semipreparative C8 reversed phase LC was effective for the purification of these proteins when used in both the initial and the final steps. We reported earlier the purification by SEC of two high molecular weight cuticle proteins from the same source (Okot-Kotber *et al.*, 1994). SEC also proved useful as the first step in the purification of the relatively low molecular size proteins reported here and compared favorably with the reversed phase procedure. Each of the two procedures had advantages and disadvantages. Whereas the SEC column had a higher protein recovery rate, the resolution was inferior to that obtained using the C8 reversed phase column. SEC resolved fewer than 13 peaks, whereas the C8 column separated up to 40 peaks. The latter number is close to the nearly 50 proteins resolved by SDS–PAGE (Okot-Kotber *et al.*, 1994). The main disadvantage of starting the purification using the reversed phase column is the lower recovery of the cuticle proteins. Notwithstanding this obstacle, the reversed phase LC procedure was effective for the purification, and recoveries could be improved by use of isopropanol in the mobile phase and the addition of urea to the sample solution before injecting onto the column. The difficulties encountered in the purification of cuticle proteins have

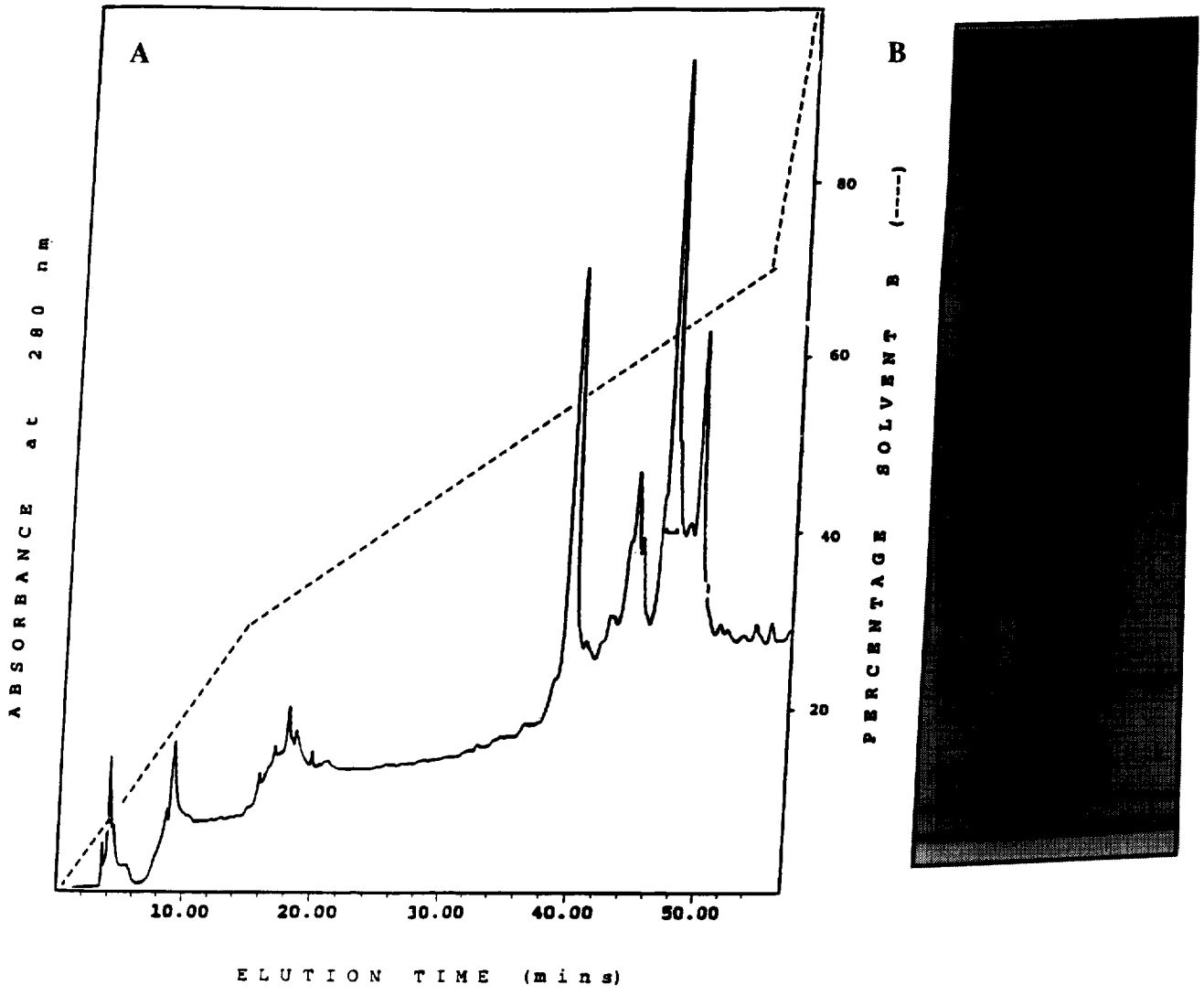

FIGURE 4. (A) Further purification of the peak "c" shown in Fig. 1 by semipreparative LC C8 reversed phase chromatography as described in Materials and Methods. (B) SDS–PAGE electropherogram of the peak "c" purified fraction shown in (A). Lane 2 shows a single Coomassie brilliant blue stained band of a 41 kDa protein (MS-PCP41). MW, molecular mass protein markers as in Fig. 3(B); CP, cuticle protein extract.

TABLE 1. Concentrations of *N*-β-alanylnorepinephrine (NBANE) released by mild acid hydrolysis from a crude cuticle protein extract, and purified 32 kDa (MS-PCP32), 41 kDa (MS-PCP41) and 48 kDa (MS-PCP48) proteins from tanning pharate pupal cuticle of *M. sexta*

Sample[1]	μmol/g protein[2]	Molar ratio[3]
Crude extract	2.33 ± 0.31	–
MS-PCP32	+	–
MS-PCP41	3.91 ± 0.03	0.16
MS-PCP48	2.75 ± 0.09	0.13

[1] Samples were heated for 10 min in 1 M acetic acid at 110°C under nitrogen.

[2] Mean value ± 1 2 range, $n = 2$, +, detected but not quantified because of interference.

[3] Molar ratio of NBANE to protein.

usually included poor recoveries and low resolution because of their lack of solubility in aqueous buffers. Poor solubility may be related to the hydrophobic nature of these proteins in cuticle (Hillerton and Vincent, 1983; Andersen *et al.*, 1995).

Other resaons also could exist for the poor solubility of these cuticular proteins. Previously, and in the present study, we showed that these proteins contain covalently bonded catecholamine adducts (Okot-Kotber *et al.*, 1994). Catechols can form hydrogen bonds with hydrophilic polymers such as chitin and protein (Hagerman and Butler, 1981). Therefore, the catecholamine adducts of cuticle proteins might interact with the hydrophilic regions of other cuticle proteins and/or chitin with a stronger affinity than that of water and, consequently, displace water from the protein matrix. A similar situation appears to exist in the case of adhesive proteins from aquatic invertebrates, such as the DOPA-containing proteins of *Fasciola hepatica* (Waite and Rice-Ficht,

TABLE 2. Amino acid composition of purified MS-PCP32 (32 kDa) and MS-PCP41 (41 kDa) proteins from *M. sexta* tanning pharate pupal cuticle

Amino acid	MS-PCP32	MS-PCP41
Aspartate[1]	4.2	4.5
Threonine	0.7	2.6
Serine	5.5	9.2
Glutamate[1]	9.2	10.6
Proline	10.8	10.2
Glycine	9.6	31.2
Alanine	25.7	7.8
Valine	13.1	5.8
Methionine	ND	3.6
Isoleucine	2.6	2.9
Leucine	3.5	4.7
Tyrosine	ND	ND
Phenylalanine	0.8	ND
Histidine	6.6	1.9
Lysine	4.5	4.9
Arginine	3.2	ND
Total	100	99.9

Proteins were hydrolyzed in 6 M HCl with 4% phenol under argon at 110°C for 24 h. Amino acid composition is expressed as mol %. ND, not detected.

[1] Aspartate and glutamate values include asparagine and glutamine, respectively.

TABLE 3. N-Terminal amino acid sequences of *M. sexta* pharate pupal cuticle proteins MS-PCP32, MS-PCP41 and MS-PCP48

Protein	N-Terminal Sequence[1]
MS-PCP32	G??AAPVHYSPAESV??Q?IVRHDQP?AA . . . 10 20
MS-PCP41	HHGLIDVG? HGGFDGGYGGGH? GYYGGHH . . . 10 20
MS-PCP48	GIHDFGSYGGHGGFGGADEGHSFGGHEGI 10 20 SLGGHEGGH?FG . . . 30 40

[1] Proteins are denoted by MS-PCP- *M. sexta* cuticle protein followed by their molecular mass in kDa. The triplet sequences GGX in MS-PCP41 and MS-PCP48 and the sequence AAPV in MS-PCP32 are underlined.

1987) and *Phragmatopoma californica* (Waite *et al.*, 1992), which are relatively insoluble in aqueous systems. A DOPA-containing peptide that is a potent inhibitor of phenol oxidase was detected recently in the hemolymph of the housefly, *Musca domestica* (Daquinag *et al.*, 1995).

As we found previously with higher molecular weight cuticular proteins (Okot-Kotber *et al.*, 1994), the three medium-sized proteins, MS-PCP32, MS-PCP41 and MS-PCP48, all contained covalently bound NBAD bonded via the β-carbon that releases NBANE under mild hydrolytic conditions. The molar ratios of NBANE released from these low molecular weight proteins were four- and 12-fold lower than the ratios for the 120 kDa and 246 kDa proteins, respectively (Okot-Kotber *et al.*, 1994). However, on a protein weight basis, the relative amount of NBANE was not more than two-fold lower. The concentration of NBANE in the crude extract of the proteins from cuticle was also about two-fold lower in this study than in the earlier study. Nevertheless, the overall trend indicates that the concentration of NBANE released from individual proteins is similar to that of the crude protein extract. This conclusion is also supported by a similarity in the staining intensity obtained after fluorography of SDS–PAGE-resolved cuticular protein from *M. sexta* pupae that were injected with ^{14}C-labeled β-alanine (Okot-Kotber *et al.*, 1994). Therefore, all the cuticular proteins from *M. sexta* pupae analyzed to date appear to acquire comparable amounts of NBAD. Although the number of individual cuticular proteins that has been analyzed for catecholamines is small, SDS–PAGE of the cuticular proteins, extracted from *M. sexta* pupae injected with ^{14}C-labeled β-alanine shortly before the start of sclerotization, showed a large number of proteins with low to high molecular masses labeled presumably with NBAD (Okot-Kotber *et al.*, 1994).

Previous studies have shown that high quantities of NBANE can be obtained from the pupal cuticle of *M. sexta* only by acid extraction and not by extraction in aqueous buffers or methanol (Hopkins *et al.*, 1984; Morgan *et al.*, 1987). The former authors suggested that NBANE was a hydrolytic product of NBAD covalently linked by its β-carbon to cuticular components. In the present study, unbound NBAD and NBANE were eliminated from the extract by repeated ultrafiltration. The NBANE released from the ultrafiltered preparations, therefore, represents NBAD covalently linked at the side-chain β-carbon probably to amino acid nucleophilic groups in the cuticular protein. Such a linkage between the ^{13}C-labeled β-carbon of the dopamine side-chain and the ^{15}N-ring-labeled histidinyl residue in protein was detected in the pupal cuticle of *M. sexta* by solid state NMR spectroscopy (Christensen *et al.*, 1991). A similar bond between NBAD and kynurenine in a butterfly wing pigment was shown to be extremely labile to weak acid and to release NBANE as a hydrolysis product (Rembold *et al.*, 1978; Rembold and Umebachi, 1984).

Protein-bound catecholamines have been detected in rat neostriatal tissue slices incubated with [^{3}H]dopamine (Hastings and Zigmond, 1994). Acid hydrolysis of the protein extract revealed the presence of cysteinyldopamine and cysteinyldihydroxyphenylacetic acid residues, suggesting that dopamine oxidizes to form reactive metabolites, presumably quinones, which then form covalent bonds with nucleophilic sulfhydryl groups on protein cysteinyl residues. Tyrosinase-catalyzed binding of DOPA with several proteins, including bovine serum albumin through the sulfhydryl group and perhaps other nucleophilic side-chains *in vitro*, has been demonstrated (Kato *et al.*, 1986). A similar mechanism probably occurs in insect cuticle during sclerotization, but the residues in

the cuticular protein that act as nucleophiles for the quinones have not been identified directly. Histidine and lysine residues have been implicated to be nucleophiles by solid-state NMR analyses (Schaefer *et al.*, 1987; Christensen *et al.*, 1991).

Catechol adducts and cross-links contribute to the hydrophobicity of the cuticular protein matrix as does the abundance of certain hydrophobic amino acids (Andersen *et al.*, 1995). MS-PCP32 and MS-PCP41 have a relatively high content of hydrophobic amino acids (57% and 36%, respectively), which is often a characteristic of cuticular proteins. The amino acid compositions of MS-PCP32 and MS-PCP41 show a relatively high abundance of alanine in one and of glycine in the other, whereas the contents of tyrosine and pheylalanine are either very low or undetectable. This is similar to the composition of cuticular proteins from *L. migratoria*, although moderate amounts of tyrosine do occur in locust proteins (Hojrup *et al.*, 1986; Andersen, 1988; Andersen *et al.*, 1993).

The N-terminal amino acid sequences of the three cuticle proteins of *M. sexta* exhibited similarities with several other insect cuticular proteins. The typical AAPV motif which occurs repeatedly in the exocuticular proteins of locusts (Andersen *et al.*, 1993) was found in the N-terminus of MS-PCP32. Because MS-PCP32 has a high content of alanine, valine and proline, the occurrence of additional AAPV motifs is possible.

The other *Manduca* cuticle proteins sequenced, MS-PCP41 and MS-PCP48, had stretches of GGX triplet repeats in common with a number of cuticle proteins from *D. melanogaster*, *T. molitor*, *L. migratoria* and *H. cecropia*. The GGX triplet may be common, especially in MS-PCP41, whose amino acid composition shows a high glycine content. The role of these repeats might be related to facilitating turns and bends in polypeptide chains, which are possibly important features for the secondary and tertiary structures of cuticular proteins (Andersen *et al.*, 1995). Proline and glycine residues have been reported as being sites in a protein chain with a high tendency to undergo turns and bends (Richardson and Richardson, 1989). Andersen *et al.* (1993) concluded that this type of protein is characteristic of the exocuticle. The pharate pupal cuticle of *M. sexta* largely becomes sclerotized into exocuticle, and the endocuticular proteins are secreted after ecdysis (Hopkins and Kramer, 1992). Therefore, certain exocuticular proteins apparently have unique differences in amino acid composition and sequence, and these differences undoubtedly influence the physical properties of sclerotized cuticle not only by their intrinsic structures, but also by how they interact with quinone tanning agents to form supramolecular structures.

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