



Characterization of Two High Molecular Weight Catechol-containing Glycoproteins from Pharate Pupal Cuticle of the Tobacco Hornworm, *Manduca sexta**

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Two high molecular weight cuticular proteins (MSCP120 and MSCP246) were extracted in acidic guanidine hydrochloride solution from tanning abdominal cuticle of *Manduca sexta* pharate pupae and purified by size exclusion high performance liquid chromatography. The apparent molecular weights were ca. 120 and 246 kDa as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Both proteins contained high levels of glutamate/glutamine, glycine, serine, alanine and aspartate/asparagine. MSCP120 was enriched in histidine relative to MSCP246, but the reverse was true for valine and proline. Small quantities of β -alanine and 3,4-dihydroxyphenylalanine (DOPA), as well as other catechols and carbohydrates, also were detected in the hydrolysates. The proteins became radiolabeled when [1-¹⁴C]- β -alanine was injected into pharate pupae, presumably by the formation of adducts with N- β -alanyldopamine metabolites during sclerotization. Mild acid hydrolysis released N- β -alanyl norepinephrine and 3,4-dihydroxyphenylketoethanol from both proteins. Strong acid hydrolysis yielded predominantly 3,4-dihydroxyphenylketoethylamine (arterenone), but also DOPA and dopamine. The N-terminal amino acid sequences of the two cuticular proteins were dissimilar, and that of MSCP246 was more hydrophobic than MSCP120. Both of these proteins were glycosylated with glucose, N-acetylglucosamine and traces of N-acetylgalactosamine, and MSCP246 also contained galactose. These structural glycoproteins, which occur in cuticle undergoing sclerotization, apparently react post-translationally with quinonoid tanning agents to yield catecholamine-protein adducts. Small amounts of peptidyl DOPA probably are formed by hydroxylation of tyrosyl residues. Results from this study are consistent with the hypothesis that these catechol-containing glycoproteins participate in cross-linking reactions in *M. sexta* pupal cuticle during sclerotization.

Sclerotization Cuticle Glycoproteins Catechols Catecholamines Carbohydrate Tanning
Tobacco hornworm *Manduca sexta*

INTRODUCTION

Although proteins constitute a substantial portion of the dry mass of an insect's cuticle (Neville, 1975; Hepburn, 1985), their structures and the mechanisms by which they are modified or sclerotized into rigid exocuticle

remain poorly understood. Several studies have focused on alterations in electrophoretic properties of cuticular proteins as indications of the hormonal basis of metamorphic changes in the integument (Willis *et al.*, 1981; Chihara *et al.*, 1982; Riddiford, 1981; Willis and Cox, 1984; Kiely and Riddiford, 1985; Wolfgang and Riddiford, 1986). Individual proteins have been used as markers of developmental programs and differential expression of genes by epidermal cells in specific anatomical areas of a number of insect species (Cox and Willis, 1985, 1987; Willis, 1987; Lemoine and Delachambre, 1986; Skelly and Howells, 1987, 1988; Andersen and Hojrup, 1987; Souliotis *et al.*, 1988; Stiles and Leopold, 1990; Rabossi *et al.*, 1991). However, little is known about the functional roles of individual cuticular pro-

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teins or about their post-translational modifications that occur during sclerotization. A few proteins have been purified and characterized, notably several in *Locusta migratoria* (Hojrup *et al.*, 1986; Andersen and Hojrup, 1987; Andersen, 1988; Talbo *et al.*, 1991; Andersen *et al.*, 1993) and *Hyalophora cecropia* cuticles (Willis, 1989). Most of these proteins were in the lower molecular weight range and, therefore, more amenable for structural analysis than higher molecular weight proteins. The post-translational modification of cuticular proteins during sclerotization by quinonoid cross-linking agents has not been well investigated. Results from analyses of catechol from tanning cuticle of *Manduca sexta* (tobacco hornworm) pupae (Hopkins *et al.*, 1982; Morgan *et al.*, 1987) and solid-state NMR analyses of bonding between catecholamine aromatic or β -carbons and imidazole nitrogens of histidyl residues in proteins of sclerotized cuticle (Schaefer *et al.*, 1987; Christensen *et al.*, 1991) encouraged us to attempt the isolation of quinonoid-modified proteins from the cuticle of *M. sexta*. In the present study, we describe the purification and characterization of two high molecular weight cuticular proteins from abdominal cuticle of pharate pupae of *M. sexta* and demonstrate that they possess covalently bound catechols and carbohydrates.

MATERIALS AND METHODS

Chemicals

α -Methyl dopa (AMD), 3,4-dihydroxyphenylalanine (DOPA), *N*-acetyldopamine (NADA), norepinephrine (NE), dopamine (DA) and pyrocatechol (CAT) were purchased from Sigma (St Louis, Mo.). 3,4-Dihydroxyphenylketoethanol (DOPKET) and 3,4-dihydroxyphenylketoethylamine or arterenone (ART) were gifts from Dr S. O. Andersen (Copenhagen, Denmark). *N*- γ -Alanyl dopamine (NBAD) and *N*- β -alanyl norepinephrine (NBANE) were synthesized according to Hopkins *et al.* (1982) and Rembold *et al.* (1978), respectively. The tripeptide, Gly-Tyr-Gly, was purchased from Research Plus (Denville, NJ) and [1- 14 C]- β -alanine from DuPont (Wilmington, DE). Arylphorin was purified from *M. sexta* hemolymph in our laboratory by Dr Billy R. Thomas. Unless noted otherwise, other chemicals, proteins and solvents were purchased from Fisher Scientific (Springfield, NJ), Pierce (Rockford, IL) or Sigma.

Insect rearing

Eggs of *M. sexta* were supplied by the Biosciences Research Laboratory, USDA, ARS (Fargo, ND). The insects were reared at $27 \pm 1^\circ\text{C}$, 16:8 light:dark photoperiod on an artificial diet essentially as described by Baumhover *et al.* (1977). Pharate pupae were collected at the late brown bar stage (a few hours before pupal ecdysis) and either frozen in dry ice or in a freezer at -20°C before dissections were performed. Freezing facilitated the cleaning of tissues adhering to the cuticle.

Cuticle dissection

Frozen pharate pupae were washed quickly in distilled water to remove loosely attached remnants of larval cuticle, immersed in ice-cold dissection buffer composed of Ringer's solution fortified with a protease inhibitor [1 mM phenylmethylsulphonyl fluoride (PMSF)], phenoloxidase inhibitors [1 mM phenylthiourea (PTU), 1 mM sodium cyanide], and a reducing agent (6 mM ascorbic acid). Abdominal cuticle was collected by carefully cutting the cuticle all around the edge between the first abdominal and metathoracic segments, and along the lateral sides without penetrating deeply into frozen underlying tissues. This technique facilitated pulling off posteriorly both the dorsal and the ventral halves of the abdominal cuticle without having to detach much of the underlying tissues. The cuticle then was placed in a dissecting dish containing ice-cold dissection buffer and the remaining pieces of muscle, fat body and epidermis were scraped off using a small spatula under a dissecting microscope. Cleaned pieces of the cuticle then were rinsed several times in cold dissection buffer, blotted and weighed.

Protein extraction

Several solvents were tested for the extraction of cuticular proteins: (1) 25 or 50 mM potassium or sodium tetraborate pH 9 containing 1 mM PMSF, 6 mM ascorbic acid and either 0.1% sodium dodecyl sulfate (SDS), 0.1% Triton X-100 or 10 mM thiourea; (2) 100 mM sodium phosphate, pH 7.2 containing 0.1% SDS and the protease inhibitor and anti-oxidant mentioned above; and (3) 8 M guanidine hydrochloride buffered with 100 mM ammonium acetate pH 3.5, two protease inhibitors [1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride and 1 mM ethylenediaminetetraacetic acid], the phenoloxidase inhibitors mentioned above together with 1 mM dithiothreitol and 6 mM ascorbic acid as antioxidants. The latter buffer was the most effective in extracting cuticular proteins, so it was used routinely. Homogenization of cuticle was performed in a glass tissue grinder (Kontes Dual type), driven by an electric motor, with intermittent cooling in ice. The homogenate was stirred at 4°C overnight to maximize extraction of proteins and subsequently centrifuged 5000 g for 10 min at 8°C to remove debris. The supernatant was pipetted out, and the residue reextracted several times. All of the supernatants were pooled and finally centrifuged at 27,000 g for 60 min at 8°C . The supernatant was subsequently diluted with 0.8 M acetic acid (pH 2.4), such that the final concentration of guanidine hydrochloride was ca. 6 M. This concentration of guanidine hydrochloride was compatible with use in a 3 kDa molecular weight cut-off Centriprep centrifugal ultrafilter (Amicon), which was employed for filtering the supernatant according to the manufacturer's instructions. The retentate obtained was resuspended in 0.8 M acetic acid, which maintained cuticular protein solubility and also minimized oxidation of diphenols.

The retentate then was ultrafiltered four more times to remove traces of guanidine hydrochloride, noncovalently bonded catechols and other low molecular weight components. The final retentate was divided into two portions, one diluted in 0.05% trifluoroacetic acid (TFA) pH 2.4 for use in purification procedures and the other in 0.8 M acetic acid for use in acid hydrolysis experiments. The yield of proteins extracted was estimated by determining the amount of protein (Bradford, 1976) as a percentage of the wet weight of cuticle.

HPLC protein purification

Several purification strategies were attempted in order to purify the acid-soluble cuticular proteins. First, we tried semipreparative size exclusion HPLC followed by cation-exchange chromatography and reversed-phase HPLC. Preliminary experiments showed that good recoveries of all proteins were achieved when size exclusion chromatography was used, although resolution of lower molecular weight proteins were relatively poor. On the other hand, low recoveries of proteins were experienced when the other two chromatographic methods were employed. Higher molecular weight proteins were resolved well by size exclusion chromatography, and, therefore, they were purified by this method. The size exclusion column, BIOSEP SEC S3000 (300 × 7.8 mm, 7.8 μ, Phenomenex), was equilibrated with 0.05% TFA, loaded with the crude cuticular protein extract in the same mobile phase, and eluted at a flow rate of 0.25 ml/min. This relatively slow flow rate was critical for good separation. The eluent was monitored at 280 nm. Peak fractions were concentrated under vacuum, and aliquots subjected to SDS-PAGE to determine purity.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially following the method of Laemmli (1970) as modified by Ames (1974) using 1 mm-thick discontinuous slab gels with a 4–15% linear gradient of polyacrylamide for the separating gel and a 3% stacking gel. Sample mobilities were compared with those of broad-range molecular weight marker proteins (6.5–200 kDa, Bio-Rad). Electrophoresis was carried out using the Bio-Rad Protean II apparatus at 8°C and 70 V constant voltage for 17 h. After electrophoresis, gels were first rinsed in a destaining solution consisting of methanol:water:acetic acid (50:40:10) for a few minutes and then stained for at least 2 h in 0.2% Coomassie Brilliant Blue R-250 dissolved in the destaining solution. Finally, the gels were destained until the background was clear.

Protein size and concentration

Molecular weights of proteins were estimated from SDS-PAGE (4–15% gradient gel) by plotting log molecular weight of marker proteins (broad-range molecular weight marker proteins, Bio-Rad) versus their relative mobilities. The molecular weight 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).

Protein concentrations were estimated using the Bio-Rad protein assay dye reagent (Bio-Rad) based on the method of Bradford (1976) using bovine serum albumin (Pierce) as the standard.

Detection of catechol-containing proteins

Protein extracts were prepared from abdominal cuticle and muscle of pharate pupae. Electrophoresis of samples was performed as described above, and electroblotting to a poly(vinylidenedifluoride) (PVDF) membrane carried out as described below. Catechol-containing proteins were indicated by the redox-cycling glycine-nitroblue tetrazolium staining technique performed according to Paz *et al.* (1991).

Catechol analysis

Catechols present in the cuticular proteins were quantified by HPLC with electrochemical detection (LCEC) as described by Morgan *et al.* (1987) on the basis of their peak areas relative to standard compounds and relative to recovery of an internal standard catechol, α-methyl dopa (AMD). Unknown compounds were quantified by assuming the same recovery and electrochemical response as AMD. Crude or purified protein samples were hydrolyzed by two methods: (1) strong acid hydrolysis in 6 M constant-boiling HCl (Pierce) containing 5% redistilled phenol at 110°C for 24 h *in vacuo* and (2) weak acid hydrolysis in 1 M acetic acid for 20 min at 110°C under nitrogen. Strong acid hydrolysates were subjected to alumina adsorption as previously described (Murdock and Omar, 1981; Hopkins *et al.*, 1984) to recover catechols and eliminate undesirable byproducts. Acetic acid-hydrolyzed samples were cooled and spun briefly in a bench-top minicentrifuge to sediment any precipitated proteins. The supernatant from the mild acid hydrolysis or alumina-recovered catechols from strong acid hydrolysis were then subjected to LCEC analysis using a C18 reversed-phase column and an amperometric, dual electrode EC detector. Two mobile phases, one using 26% acetonitrile plus SDS and the other 13% methanol plus sodium octyl sulfate (SOS), were used for isocratic elution of the catechols as previously described (Morgan *et al.*, 1987) with minor modifications. Two proteins, bovine serum albumin and *M. sexta* arylphorin, and a tripeptide, Gly-Tyr-Gly, were used as control proteins/peptide to assess whether any spontaneous hydroxylation of protein/peptidyl tyrosyl residues occurred when utilizing the strong acid hydrolytic conditions used to process the cuticular proteins.

¹⁴C-β-Alanine labeling

Fifth instar larvae near the end of the wandering stage (about 5 days into the instar) or early pharate pupae

were anesthetized with carbon dioxide, and each was injected through the base of an abdominal proleg with 4 μ Ci of [1- 14 C]- β -alanine in 0.1 M HCl. The larvae were held until they developed into brown metathoracic bar stage pupae close to ecdysis or newly ecdysed pupae, after which they were frozen at -20° C. Then, the abdominal cuticles were dissected and proteins extracted as described above. Radioactivity in these extracts was determined by scintillation counting and an aliquot containing about 500 cpm was subjected to SDS-PAGE as described above. The gel subsequently was processed and fluorographed as described by Skinner and Griswold (1983) and modified by Mohamed *et al.* (1989).

Amino acid analysis

The amino acid compositions of purified proteins were determined at the Experiment Station Chemical Laboratories, University of Missouri at Columbia. Proteins were hydrolyzed in 6 M constant boiling HCl containing 4% phenol at 110 $^{\circ}$ C for 24 h under argon. The amino acids were analyzed on a Beckman 6300 amino acid analyzer using cation-exchange chromatography and postcolumn derivatization with ninhydrin as the detection procedure.

N-Terminal amino acid sequencing

Purified proteins were subjected to SDS-PAGE (4–15% gradient gel) and electroblotted onto PVDF membranes as described above. Proteins stained by Coomassie Brilliant Blue were cut out, and N-terminal amino acid sequences determined by automated Edman degradation on an Applied Biosystem sequencer at the Biotechnology Support Facility, University of Kansas at Kansas City. HPLC purified proteins were sequenced at the Biotechnology Microchemical Core Facility, Kansas State University at Manhattan. The N-terminal sequences were aligned for comparison with other cuticular proteins using Pearson's FASTP search program by Dr Judith H. Willis, University of Georgia at Athens (Lipman and Pearson, 1985).

Carbohydrate determination

Glycoprotein detection was done with the GlycoTrack carbohydrate detection kit from Oxford GlycoSystems, which utilized carbohydrate oxidation by periodate, biotinylation and visualization with streptavidin-alkaline phosphatase. Samples pre-labeled with biotin to minimize background staining were subjected to SDS-PAGE, with one of the lanes containing biotinylated standard marker proteins provided with the kit. After electrophoresis, samples were electroblotted onto a PVDF membrane using a Bio-Rad Trans-Blot Cell at 400 mA constant current for 2 h at 8° C, essentially as described by Towbin *et al.* (1979) and modified by Burnette (1981). However, we added 10% methanol and 0.1% SDS to the transfer buffer to facilitate a more efficient transfer of the cuticular proteins.

Carbohydrate compositions were determined at the Experiment Station Chemical Laboratories, University

of Missouri at Columbia. The samples were hydrolyzed, and the liberated sugars were reduced and derivatized to alditol or hexosaminitol acetates, which were separated by gas-liquid chromatography and quantified by mass spectrometry (Mawhinney *et al.*, 1980; Mawhinney, 1986; Tilley *et al.*, 1993).

RESULTS

Protein extraction

Several solvents used for the extraction of *M. sexta* pharate pupal cuticle gave rather low yields of proteins. Tetraborate buffers with either 1% Triton X-100 or SDS extracted $\leq 1\%$ of the wet weight of the cuticle as protein, but when 10 mM thiourea was added with or without the detergents, the yield increased to about 2.5%. Phosphate buffer with 1% SDS extracted the least amount of protein ($< 1\%$). However, acetate-buffered 8 M guanidine hydrochloride extracted *ca.* 10% of the fresh weight as proteins. To further process the extracted proteins, centrifugal ultrafiltration was used to remove the guanidine hydrochloride. Unbound catechols also were eliminated as was verified by analysis of the last filtrate by LCEC. The retentate had a yellowish coloration and was diluted with 0.8 M acetic acid or 0.05% trifluoroacetic acid for storage and subsequent processing.

Protein purification

Gradient SDS-PAGE was used to resolve the mixture of cuticular proteins extracted by guanidine hydrochloride. Approximately 50 proteins were discernible with molecular weights ranging from about 6 to 250 kDa (Fig. 1), and among these, 13 were considered to be major proteins. The bulk of the proteins ranged in size from 6 to 50 kDa, but two relatively large proteins were also present, one being a major protein with an apparent molecular weight of *ca.* 120 kDa (MSCP120) and the other a relatively minor protein of *ca.* 246 kDa (MSCP246).

The two high molecular weight cuticular proteins were purified to apparent homogeneity by two steps of size exclusion HPLC (SEC). In the first step, nine fractions were collected (Fig. 2) and analyzed by SDS-PAGE (Fig. 1). It was evident from the electropherogram that fractions 1 and 2 were enriched in MSCP246. These fractions were then pooled for a second step of SEC. The second SEC profile revealed a single peak of protein (Fig. 3). The early fractions of this peak were homogeneous in MSCP246, as revealed by SDS-PAGE (Fig. 4). However, it was necessary to rechromatograph some of the later fractions to eliminate several smaller proteins.

Fractions 3–5 from the first SEC step of the crude cuticular extract contained predominantly MSCP120 (Fig. 1). These fractions were pooled and subjected to a second step of SEC (Fig. 5). Homogeneity of fractions collected also was assessed by SDS-PAGE (Fig. 6).

Several fractions showed a high degree of purity for MSCP120 with only a minor protein having an apparent molecular weight of 107 kDa also present. This protein appeared to be related to MSCP120, as will be discussed later.

Catechol-containing proteins

Results of preliminary experiments involving redox-cycling glycine-tetrazolium staining of proteins extracted from abdominal cuticle and muscle of *M. sexta* pharate pupae suggested that several cuticular proteins, including MSCP120 and MSCP246, reacted positively for the presence of catechols, as judged by intense bands on protein blots from SDS-PAGE separations. No protein in the muscle extract stained positive for catechols.

^{14}C - β -Alanine labeling

β -Alanine is a precursor of *N*- β -alanyldopamine (NBAD), which supplies most of the quinonoid cross-linking agents in *M. sexta* pupal cuticle (Hopkins *et al.*, 1982; 1984). Several cuticular proteins were radiolabeled after [^{14}C]- β -alanine was injected into the larvae, as seen in the fluorogram of Fig. 7, suggesting that *N*- β -

alanylated catecholamines were bound to these proteins. The strongest signal was exhibited by a protein with the same apparent molecular weight as MSCP120. MSCP246 also was labeled, but it displayed a weaker signal. Other proteins, predominantly of lower molecular weight, had relatively strong radiolabeling as well.

Catechol determination

Because catecholamines serve as precursors to cuticular protein cross-linking agents and because redox-cycling staining and radiolabeling with ^{14}C - β -alanine had indicated the presence of catecholic components in these proteins, we wished to determine whether catechols were bonded covalently to any of the cuticular proteins extracted from pharate pupal cuticle of *M. sexta*. Catechols bound to the crude extract and to purified proteins were identified and quantified by LCEC following either mild or strong acid hydrolysis and alumina recovery. Mild acid hydrolysis released one major catecholamine, NBANE, and a minor product identified as DOPKET (Fig. 8). Because DOPKET was resolved poorly in the acetonitrile-SDS mobile phase, a second mobile phase containing methanol-SOS was used to quantify this

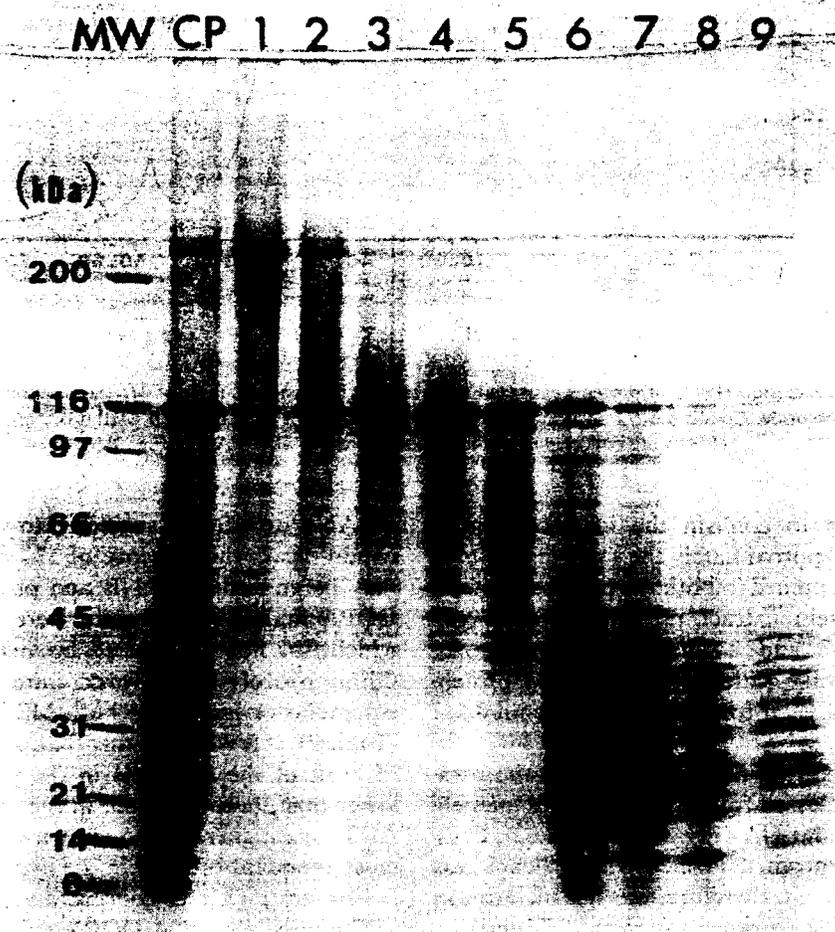


FIGURE 1. Electropherogram (4–15% SDS-PAGE) of *M. sexta* pharate cuticle protein fractions separated by size exclusion HPLC (SEC). MW—protein molecular weight markers; CP—whole cuticular extract; 1–9 fractions collected from HPLC (Fig. 2). Fractions 1–2 and 3–5 were pooled and used in subsequent steps of purification of MSCP120 and MSCP246, respectively.

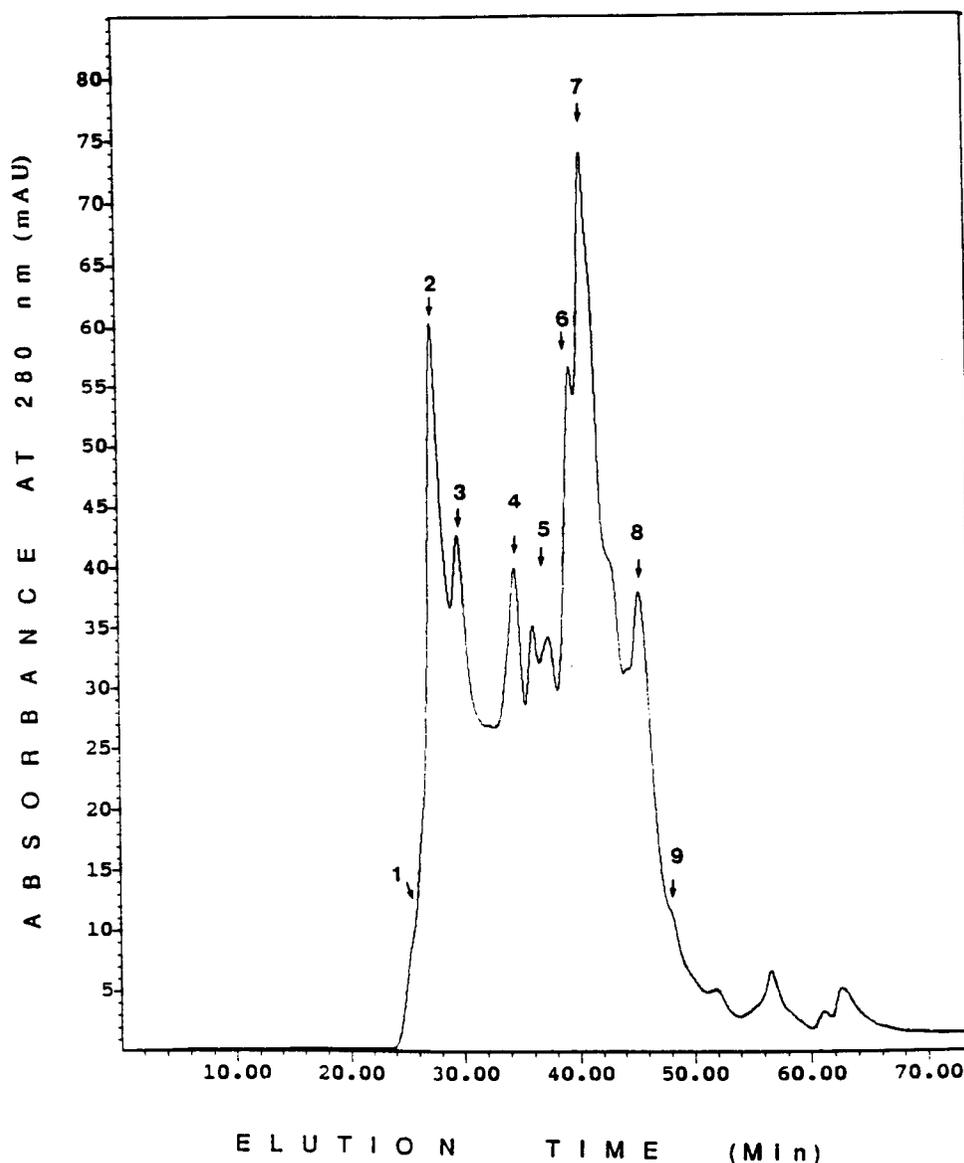


FIGURE 2. Size exclusion HPLC of proteins extracted from tanning abdominal cuticle of *M. sexta* pharate pupae with 8 M guanidine hydrochloride. Conditions for separation were as described under Materials and Methods. Nine protein fractions (1-9) were collected and aliquots subjected to SDS-PAGE (Fig. 1).

compound and also to confirm the identification of NBANE (Table 1). Approximately the same amounts of each catechol were obtained in both the purified proteins and the crude protein extract (about $5-7 \mu\text{mol g}^{-1}$ protein for NBANE and $2-3 \mu\text{mol g}^{-1}$ protein for DOPKET), with molar ratios of catechol to protein estimated to be about 1.2:1 for NBANE and about 0.5:1 for DOPKET.

Hydrolysis under strong acid conditions in the presence of 5% phenol released four other catechols, namely DOPA, CAT, ART and DA (Fig. 9 and Table 2). CAT was apparently a byproduct formed when phenol was heated in strong acid and, therefore, was not included in Table 2. Two major unknown components, X_1 and X_2 , also were resolved in the chromatography (Fig. 9). The structure of X_1 remains obscure. X_2 displayed chromatographic properties identical to a product formed when

NBANE or NE was subjected to these same hydrolytic conditions in the presence of 5% phenol. Therefore, X_2 may be an adduct of NE and phenol. Molar ratios of DOPA and DA to protein were less than equimolar, which was not unexpected because of oxidative losses during hydrolysis. However, those of ART were nearly equimolar or higher for both MSCP120 and MSCP246 (Table 2). It is worth noting that, although the quantities of most of the catechols in the purified proteins were lower than those observed in the crude extract, levels of ART were higher. The unknown compound, X_2 , was the most abundant product in the hydrolysates, being about 5-10 times higher in concentration than DOPA.

The possibility that DOPA was produced as an artifact from hydroxylation of tyrosine during acid hydrolysis was tested by subjecting non-DOPA-containing proteins and a tyrosine-containing tripeptide to strong

acid hydrolysis and amino acid analysis. Hydrolysis of the two proteins, arylphorin and bovine serum albumin, and also Gly-Tyr-Gly under strong acidic conditions yielded only traces of DOPA (Table 2). At least 10-fold less DOPA was generated than detected in either MSCP120 or MSCP246. Molar ratios of DOPA to protein or the tripeptide were <0.01 . No other catechols were detected in the hydrolysates of the control proteins or tripeptide.

Amino acid compositions

The amino acid compositions of MSCP120 and MSCP246 are presented in Table 3. Cysteine and tryptophan were not determined, and aspartate and glutamate were not distinguished from their corresponding amides. Both proteins contained high levels of glutamate/

glutamine, glycine, serine, alanine and aspartate/asparagine. Aromatic amino acid content did not differ substantially in the two proteins, except for histidine, which was very high (11.4%) in MSCP120. Tyrosine was a prominent amino acid in both proteins (6–7%), but phenylalanine was relatively low (1–2.5%). Valine and proline were higher in MSCP246. Trace quantities ($\leq 0.1\%$) of β -alanine, methionine and hydroxyproline also were detected.

N-Terminal amino acid sequences

The N-terminal amino acid sequences of MSCP120 and MSCP246 were determined by automated Edman degradation. The sequences of the first 20 amino acid residues for MSCP120 and of the first 12 for MSCP246 were established (Fig. 10). No similarity was found

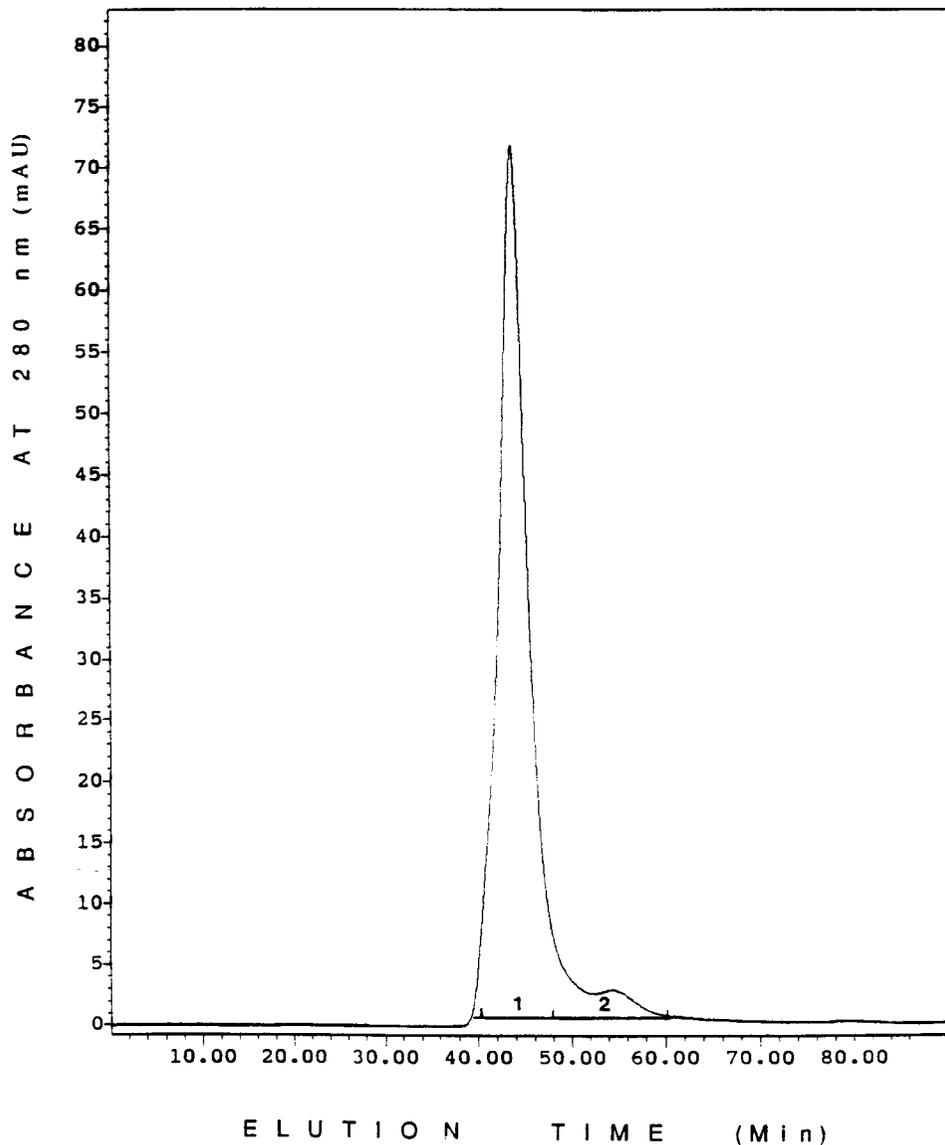


FIGURE 3. HPLC of a purified *M. sexta* high molecular weight cuticular protein (246 kDa, MSCP246) obtained by size exclusion chromatography of pooled fractions 1 & 2 from the first chromatographic step (Fig. 2) as described in Materials and Methods. Two fractions were collected as designated in the chromatogram with numbers 1 and 2 and subjected to SDS-PAGE (Fig. 4) to check for purity.

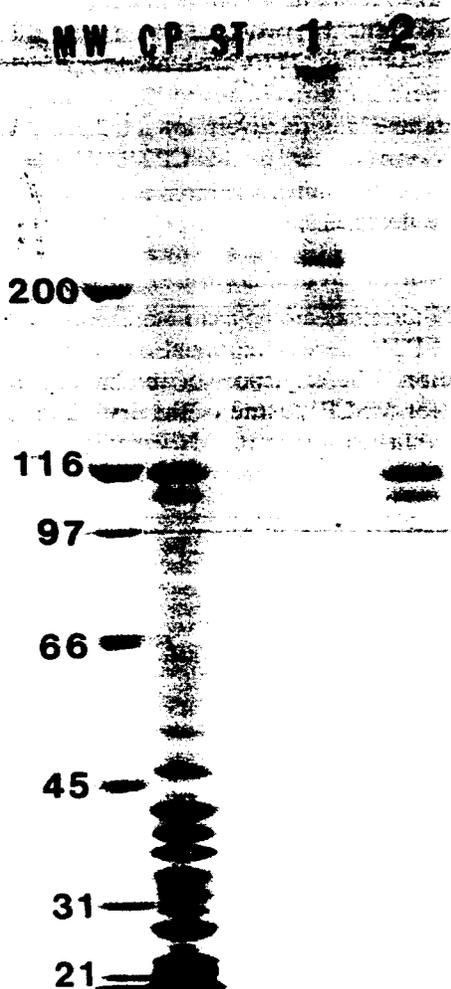


FIGURE 4. Electropherogram of protein fractions collected from SEC of partially purified MSCP246 (Fig. 3). The homogeneity of the two fractions (1 and 2) collected was assessed on a gradient (4–12%) SDS-PAGE. The samples were loaded as follows: MW, protein molecular weight markers; CP, whole extract; ST, pooled fractions 1 and 2 from SEC of whole extract; 1, first fraction from the column containing purified MSCP246; 2, second fraction containing primarily MSCP120.

between the two proteins, and the N-terminus of MSCP246 was slightly more hydrophobic than that of MSCP120. A search was made for similarities between these sequences with those of other cuticular proteins using Pearson's search program (Willis J., personal communication). MSCP120 and MSCP246 showed no homology with cuticular proteins from other species, but the N-terminal 17 amino acid sequence of the former protein was similar to the signal peptide region of a protein encoded by the *yellow* locus of *Drosophila melanogaster*, which is associated with the pigmentation pattern of adult cuticle and larval mouth parts (Chai *et al.*, 1986; Walter *et al.*, 1991). The N-terminal of the 107 kDa protein, which was present in the purified fractions of MSCP120, also was sequenced and found to be identical to the N-terminal

sequence of MSCP120, indicating that the two proteins were related.

Carbohydrate composition

Results obtained from analysis of protein blots using the GlycoTrack carbohydrate detection kit indicated that MSCP120 and MSCP246, as well as several other smaller proteins ranging in size from 6 to 50 kDa, were glycosylated. The carbohydrate residues of MSCP120 and MSCP246 were determined by gas-liquid chromatography and mass spectrometry (Table 4). Glucose was the major sugar found in both proteins with relatively high molar ratios (4–9 mol of glucose:mole of protein), followed by *N*-acetylglucosamine and only trace amounts of *N*-acetylgalactosamine. Galactose also was found in MSCP246, but not in MSCP120.

DISCUSSION

The composition and structure of sclerotized insect cuticle, which is formed primarily from chitin microfibrils embedded in a protein matrix and stabilized by quinonoid derivatives of catechols, confer a diversity of mechanical properties to the exoskeleton that are required for locomotion and protection from environmental stresses (Neville, 1975; Andersen, 1979, 1980, 1990; Sugumaran, 1988; Hopkins and Kramer, 1992). However, because of the stability of sclerotized cuticle, studying its constituents, without altering the biopolymers involved, has been difficult. We tested several detergents and chaotropic agents for extraction of proteins from tanning pharate pupal cuticle of *M. sexta*, and the results showed that SDS and Triton X-100 solutions with or without thiourea in tetraborate or phosphate buffers were not as efficient as acidic-buffered guanidine hydrochloride. Approximately 5 to 10 times more protein was extracted with the latter than with any of the other extraction solvents. Guanidine hydrochloride solutions have been used previously to solubilize cuticular proteins with good recoveries from *H. cecropia* (Cox and Willis, 1985) and *Anthonomus grandis* (Stiles and Leopold, 1990). Therefore, we routinely used guanidine hydrochloride buffered with acetate (pH 3.5) containing ascorbic acid and dithiothreitol as antioxidants, and phenoloxidase and protease inhibitors for extraction of cuticular proteins. The low pH, antioxidants and phenoloxidase inhibitors minimized the possibility of catechol oxidation, a condition important for successful handling of catechol-containing proteins.

Solid state ^{13}C -NMR analysis of the cuticle from newly emerged pupae of *M. sexta* revealed that proteins constituted about 14% of the wet weight (Kramer *et al.*, 1988). In the present study, acidic guanidine hydrochloride solvent extracted about 10% of the wet weight of cuticle as protein, which accounted for *ca.* 75% of the total. This result suggested that the remaining unextractable protein may have been cross-linked in the exocuticle at the pharate pupal stage.

Purification of cuticular proteins was hindered by their poor solubility in aqueous buffers. Conventional chromatographic purification procedures, such as ion-exchange, gel permeation, hydrophobic interaction or affinity chromatography, usually did not afford high recoveries because the proteins tended to adsorb irreversibly to the stationary phase, leading to unacceptable losses as has been observed for DOPA-containing proteins from other invertebrates including *Fasciola hepatica* (Waite and Rice-Ficht, 1987) and *Phragmatopoma californica* (Waite *et al.*, 1992). However, by using an elution buffer containing 6 M guanidine hydrochloride, we found semipreparative size exclusion HPLC to be very effective for the purification of two high molecular weight proteins, MSCP120 and MSCP246, with rela-

tively high recoveries (about 90%). Trifluoroacetic acid (0.05%) also worked fairly well as a solvent and was convenient to use because ultrafiltration to remove non-volatile components was not required before subsequent analyses. Both of the high molecular weight proteins were purified to apparent homogeneity by SEC. The persistent satellite protein of 107 kDa, which copurified with MSCP120, appeared to be related to it. This conclusion is based on two observations. First, these two proteins were practically inseparable by chromatographic methods based on charge or hydrophobicity. Second, they had the same amino acid N-termini for the first five residues. The size difference measured by SDS-PAGE may reflect a difference in post-translational modification between MSCP120 and the 107 kDa

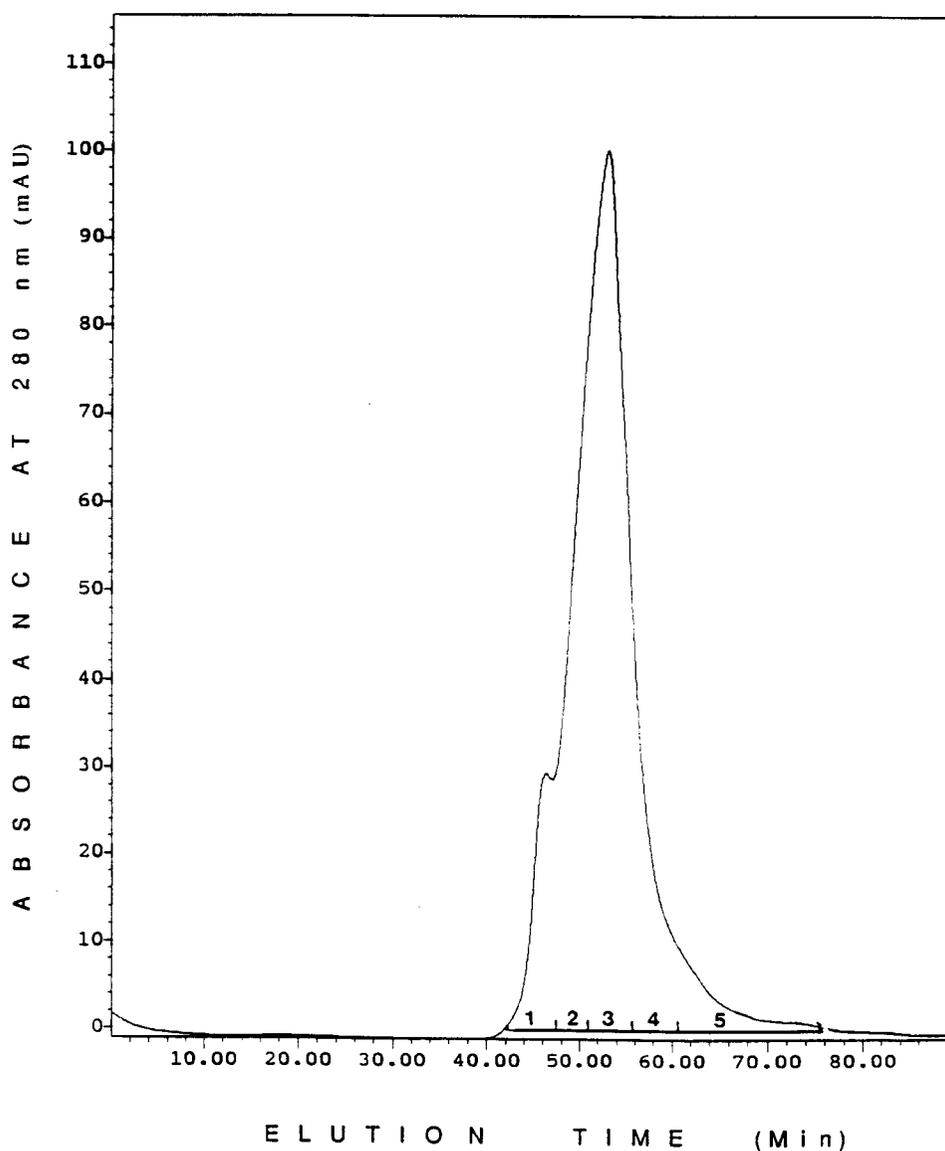


FIGURE 5. Size exclusion HPLC (SEC) of a high molecular weight *M. sexta* cuticular protein (MSCP120). This protein was isolated following SEC chromatography of the crude extract (Fig. 2). Pooled fractions 3–5 from that SEC were rechromatographed by SEC following equilibration with 0.05% trifluoroacetic acid and elution with the same solution at a flow rate of 0.2 ml/min. Protein was monitored at 280 nm. Numbers 1–5 denote fractions collected and subjected to SDS-PAGE (gradient gel) as shown in Fig. 6.

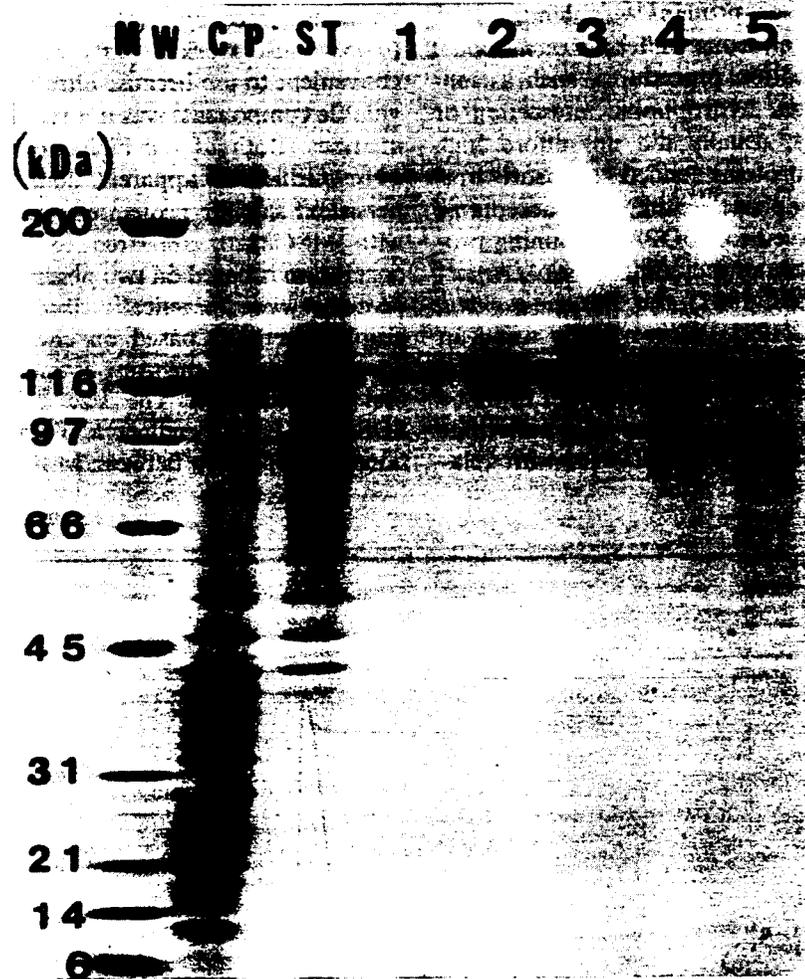


FIGURE 6. SDS-PAGE (4–15% gradient gel) electropherogram of fractions from size exclusion HPLC (Fig. 5) of *M. sexta* pharate pupal cuticle 120 kDa protein (MSCP120). Fractions 1–5 were loaded on the gel and stained with Coomassie Brilliant Blue R-250 after electrophoresis. Lanes: MW, molecular weight markers (Bio-Rad); CP, crude extract of cuticle proteins; ST, a fraction rich in MSCP120 obtained from the first SEC and 1–5, fractions from a second SEC (designated as 1–5 in Fig. 5). Note that lanes marked 2 and 3 contain the protein of highest purity.

protein, the latter perhaps being derived from the former.

The preliminary results obtained from redox-cycling glycine-tetrazolium staining suggested that a number of proteins from extracts of pharate pupal cuticle possessed covalently bonded catechols. These data were consistent with previous results from solid-state NMR analyses, which demonstrated that catecholamines were linked covalently to histidyl residues in proteins of sclerotized cuticle of *M. sexta* (Schaefer *et al.*, 1987; Christensen *et al.*, 1991). Furthermore, fluorography of cuticular proteins extracted from tanning abdominal cuticle of *M. sexta* pharate pupae injected with ^{14}C - β -alanine revealed radiolabeling of these proteins. Because NBAD synthesis in *M. sexta* requires not only DA but also β -alanine (Krueger *et al.*, 1989), it is reasonable to assume that the labeling of cuticular proteins observed here was due to incorporation of ^{14}C -N- β -alanyldopamine. Indeed, Grun and Peter (1984) reported that radiolabeled NBAD injected into *M. sexta* pharate pupae was incorporated into the cuticle.

Analysis of the purified proteins as well as the crude protein extract showed that several catechols were covalently bonded to the proteins. This conclusion was based on the following observations: (1) chaotropic agents, which disrupt noncovalent bonds, did not eliminate the catechols bound to the proteins, even after repeated ultrafiltration steps; (2) catechols persisted in the purified proteins, even after incubation in 1M acetic acid overnight at ambient temperature; and (3) catechols were released only after heating the proteins in mild or strong acid. Therefore, the catechols released by mild acid hydrolysis, NBANE and DOPKET, were derived from catechol-protein adducts joined by relatively weak covalent linkages. Acid extractable NBANE was detected previously in the pupal cuticle of *M. sexta* during sclerotization (Hopkins *et al.*, 1984; Morgan *et al.*, 1987). NBANE was not extracted readily from the cuticle with aqueous buffers or alcohol as was its precursor NBAD, but instead required harsher acidic solvents (Morgan *et al.*, 1987). Therefore, NBAD is probably bound covalently to cuticular macromolecules at the

β -carbon and the acidic extraction hydrolyzed the catecholamine-protein bond releasing NBANE. The present data are consistent with that hypothesis because crude protein extracts and the purified proteins required heating in mild acid to release NBANE. NBANE is apparently a hydrolytic product of NBAD bonded through the β -carbon of the side chain to histidyl or other amino acid nucleophilic groups of cuticular protein. In some cases, a small peak of NE was observed as a secondary product from NBANE hydrolysis in mild acid, as was observed previously (Morgan *et al.*, 1987).

The origin of DOPKET released by mild acid hydrolysis of cuticular proteins in our studies remains unknown. Andersen and Roepstorff (1981) showed that equimolar amounts of DOPKET and *N*-acetyldopamine (NADA) were formed readily as a result of mild acid hydrolysis of benzodioxin-type NADA dimers obtained from *Schistocerca gregaria* cuticle. Andersen *et al.* (1992) also showed that DOPKET was released from NADA trimers isolated from extracts of adult *L. migratoria* sclerotized cuticle. These data suggested that oligomer-

ization of NADA was important in generating the precursor of DOPKET. We obtained substantial amounts of DOPKET after mild acid hydrolysis of *M. sexta* pupal wing cuticle and puparial cuticle of *Musca domestica*, structures in which only small amounts of NADA relative to NBAD occur (Morgan *et al.*, 1987). Furthermore, we also demonstrated that DOPKET was produced by acid hydrolysis following incubation of either NBAD or NADA with an insoluble fraction from

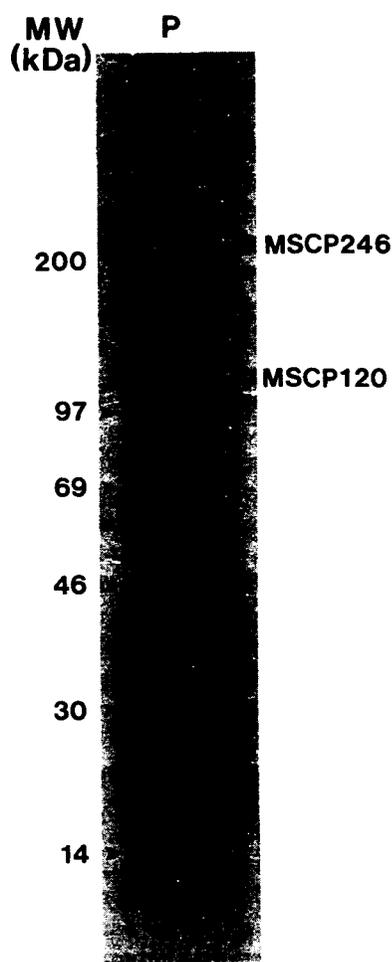


FIGURE 7. A fluorogram obtained after SDS-PAGE of cuticular proteins from abdominal cuticle of *M. sexta* newly ecdysed pupae radiolabeled with [^{14}C]- β -alanine injection. Note that the two high molecular weight proteins, MSCP120 and MSCP246 (arrows), were labeled as were several smaller proteins. MW—Molecular weight markers. P—abdominal pupal cuticular protein extract.

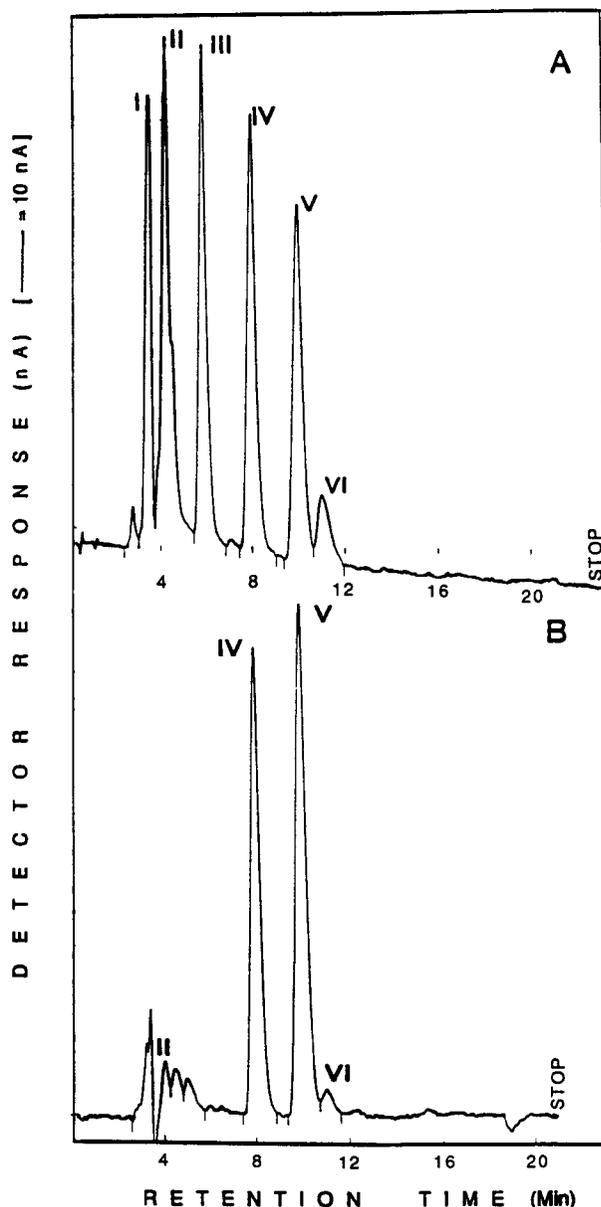


FIGURE 8. HPLC of catechols in: A. standard mixture and B. 20 min (110°C) 1M acetic acid hydrolysate of *M. sexta* pharate pupal cuticular protein extract. The catechols were resolved on a RP C18 column using an isocratic mobile phase of 26% acetonitrile, 1 mM EDTA, 60 mM H_3PO_4 and 0.1% SDS, pH 2.85 at a flow rate of 1 ml/min. Catechols were monitored using a dual electrochemical detector with the downstream electrode set at -0.1 V after oxidation at $+0.8$ V with an upstream electrode. Full scale response was 100 nA. Standard mixture: NANE (I), DOPKET (II), DOPA (III), AMD (IV), NBANE (V) and NE (VI).

M. sexta wing cuticle homogenates (Morgan *et al.*, 1987). This result indicated that derivatives of *N*-acyldopamines were capable of generating DOPKET upon hydrolysis. We were unable to detect NADA or NANE in mild acid hydrolysates of *M. sexta* pharate pupal cuticle protein, but we did recover substantial amounts of NBANE in addition to DOPKET, indicating that NBAD metabolites may be the primary DOPKET precursors in this species. Therefore, DOPKET apparently is an oxidative/hydrolytic metabolite of *N*-acyldopamines bonded to protein.

Strong acid hydrolysis of the crude protein extract and purified proteins yielded several catecholic products including DOPA, ART and DA. The identification of DOPA in the strong acid hydrolysates was of particular interest, because we know of no other reports of DOPA found in hydrolysates of insect cuticular proteins. Hydrolysis of two noncuticular proteins, bovine serum albumin and arylphorin, as well as the tripeptide, Gly-Tyr-Gly, yielded less than one-tenth the amount of DOPA found in the cuticular proteins, indicating that DOPA recovered from cuticular protein hydrolysates was not an artifact generated by the hydrolytic conditions (Gieseg *et al.*, 1993). Although the possibility that DOPA resulted from the hydroxylation of tyrosine by integumental tyrosinase (Aso *et al.*, 1984) while the cuticle was being processed cannot be ruled out despite the presence of antioxidants and phenoloxidase inhibitors, we propose that DOPA occurs *in vivo* as a residue of the primary structures of MSCP120, MSCP246 and perhaps other cuticular proteins. Several DOPA-containing proteins were characterized from a variety of marine invertebrates (Waite, 1992), and amino acid analysis plus sequencing data revealed that DOPA was part of the peptide backbone (Waite and Rice-Ficht, 1987, 1989). The phylogeny of quinone-tanned DOPA proteins in invertebrates was described by Waite (1990) and indirect evidence for the participation of peptidyl DOPA in the tanning process was presented.

The origin of peptidyl DOPA in invertebrates remains unknown. However, in some organisms, for example, *F. hepatica*, DOPA residues in a major eggshell protein were coded for by tyrosine codons (Rice-Ficht *et al.*, 1989), suggesting that the protein became post-translationally modified by hydroxylation of tyrosyl residues in

a reaction catalyzed by a putative tyrosyl-3-hydroxylase (Waite, 1990). In *M. sexta*, this role may be played by an integumental tyrosinase, which has been shown to hydroxylate tyrosine to DOPA (Morgan *et al.*, 1990). Relatively low levels of DOPA were found in *M. sexta* cuticular proteins in comparison to levels found in DOPA-containing proteins from marine invertebrates, e.g., in cement precursor proteins from the reef-building polychaete, *P. californica* (Jensen and Morse, 1988; Waite *et al.*, 1992). The catecholamines, NADA and NBAD, are the major precursors for quinonoid tanning agents in insect cuticle (Hopkins *et al.*, 1982; Hopkins and Kramer, 1992), whereas peptidyl DOPA appears to be the primary sclerotization precursor in marine invertebrates (Waite, 1992). Thus, some invertebrates rely on high molecular weight peptidyl DOPA for tanning of proteinaceous structures, whereas insects appear to utilize primarily *N*-acyldopamines for sclerotizing precursors for different regions of the cuticle. However, the low level of peptidyl DOPA in the pupal cuticular proteins may indicate a minor role for DOPA as a tanning precursor in *M. sexta*. Another invertebrate, black coral, *Antipathes fiordensis*, has both 3,4-dihydroxybenzaldehyde and peptidyl DOPA in its skeletal material, suggesting that both of these catechols could be involved in the stabilization of coral skeleton (Holl *et al.*, 1992).

3,4-Dihydroxyphenylketoethylamine or ART was the major identified catechol recovered from strong acid hydrolysates of tanning pharate pupal cuticle from *M. sexta*. The levels were comparable with those of NBANE obtained by mild acid hydrolysis and about twice those of DOPKET, indicating that the source of ART may be derived from NBANE. Preliminary experiments have shown that hydrolysis of NBANE under strong acid conditions yielded minor amounts of ART, in addition to major amounts of the unknown compound X₂ (unpublished observations). Benzodioxin-type NADA dimers do not produce ART during hydrolysis in 1 M HCl, but do when heated in 6 M HCl (Andersen *et al.*, 1980). ART may be a derivative of NADA bound to protein (Andersen, 1970). Because NBAD was found to be a major tanning agent in stiff brown cuticle (Hopkins *et al.*, 1982, 1984; Kramer and Hopkins, 1987; Morgan *et al.*, 1987), ART in the hydrolysates of these proteins is probably derived from NBAD rather than NADA bound to cuticular protein.

Dopamine also was found in the cuticular protein hydrolysates and may be a hydrolytic product of protein-bound NBAD. Two unidentified catechols, X₁ and X₂, were also detected in the strong acid hydrolysates. The former compound has not been characterized, but the latter appeared to be a phenol adduct of NE. Heating NBANE or NE in strong acid containing phenol yielded a single major peak having the same HPLC retention time as X₂. Heating these compounds or the cuticular proteins in concentrated HCl in the absence of phenol did not produce X₂. More studies are needed to elucidate the structure of X₂. Finally, a product with the same

TABLE 1. Catechols released by mild acid hydrolysis from a crude extract and purified high molecular weight proteins MSCP120 and MSCP246 from *Manduca sexta* tanning pharate pupal cuticle*

Sample	NBANE†	DOPKET†
Crude extract	5.49 ± 0.61	2.21 ± 0.44
MSCP120	4.55 ± 0.01 (0.6)	2.67 ± 0.05 (0.3)
MSCP246	7.17 ± 0.39 (1.8)	2.48 ± 0.16 (0.6)

*Hydrolysis for 20 min in 1 M acetic acid at 110 °C. Mean values ($\mu\text{mol g}^{-1}$ protein) \pm 1/2 range, $n = 2$. Molar ratios of catechol to protein are shown in parentheses.

†NBANE = *N*- β -alanyl-norepinephrine; DOPKET = 3,4-dihydroxyphenylketoethanol.

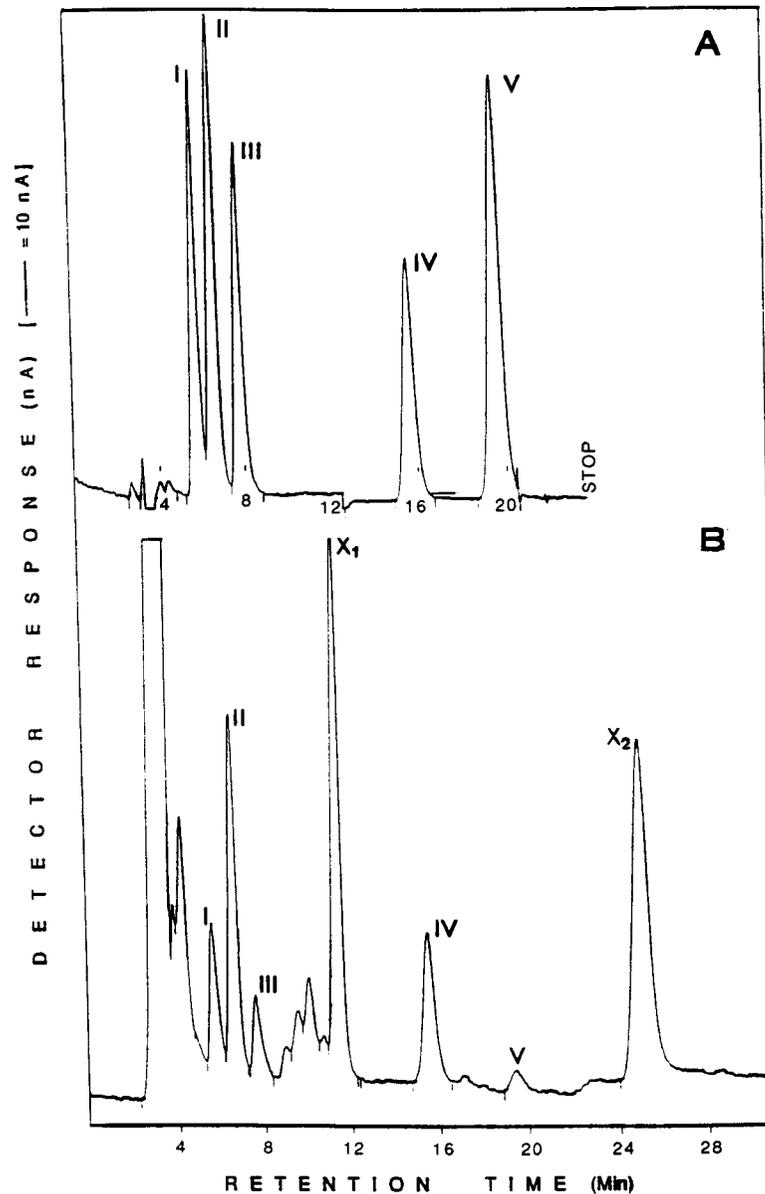


FIGURE 9. HPLC of catechols in A, standard mixture; B, products of *M. sexta* pharate pupal cuticle proteins after 24-h hydrolysis in 6 M HCl plus 5% phenol. The peaks were resolved and monitored as in Fig. 8. Full-scale response was 100 nA. Catechols detected were DOPA (I), CATECHOL (II), AMD (III), ART (IV), DA (V). Major unknowns in protein hydrolysates were X_1 and X_2 . X_2 had the same retention time as that of a major product produced by heating NBANE or NE with phenol under similar conditions.

TABLE 2. Catechols released by strong acid hydrolysis of *Manduca sexta* tanning pharate pupal cuticle crude extract, high molecular weight proteins MSCP120 and MSCP246, arylphorin, bovine serum albumin and a tripeptide, Gly-Tyr-Gly*

Sample	DOPA	Arterenone	Dopamine	X_2 †
Crude extract	3.30 ± 0.21	4.11 ± 0.03	1.82 ± 0.02	16.08 ± 1.69
MSCP120	0.87 ± 0.04 (0.10)	5.02 ± 0.02 (0.60)	0.57 ± 0.11 (0.07)	8.39 ± 0.56 (1.01)
MSCP246	0.96 ± 0.10 (0.24)	6.99 ± 0.96 (1.75)	1.72 ± 0.73 (0.42)	11.92 ± 2.0 (2.93)
Arylphorin	0.09 ± 0.01 (0.01)	—	—	—
Bovine serum albumin	0.07 ± 0.01 (0.01)	—	—	—
Gly-Tyr-Gly	0.18 ± 0.04 (<0.01)	—	—	—

*24-h hydrolysis in 6 M HCl containing 5% phenol at 110 C. X_2 —unidentified major catechol, presumably a norepinephrine-phenol adduct. Mean values ($\mu\text{mol g}^{-1}$ protein or peptide) ± 1 2 range, $n = 2$. Molar ratios of catechol to protein or peptide are given in parentheses.

† X_2 was quantified assuming it had the same electrochemical response as α -methyl DOPA.

TABLE 3. Amino acid composition of purified high molecular weight proteins MSCP120 and MSCP246 from *Manduca sexta* tanning pharate pupal cuticle*

Amino acid†	MSCP120	MSCP246
Aspartate	8.9	7.6
Threonine	2.5	5.7
Serine	2.3	9.3
Glutamate	18.5	11.0
Proline	3.7	7.8
Glycine	11.7	10.7
Alanine	8.9	8.5
Valine	6.6	9.0
Isoleucine	4.7	3.8
Leucine	1.9	3.9
Tyrosine	6.4	7.0
Phenylalanine	1.2	2.4
Histidine	11.4	5.8
Lysine	3.1	4.8
Arginine	2.4	2.5

*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 %.

†Aspartate and glutamate values include asparagine and glutamine. Traces ($\leq 0.1\%$) of β -alanine, methionine and hydroxyproline also were detected.

retention time as CAT was detected after strong acid hydrolysis of either MSCP120, MSCP246, NBANE or NBAD in the presence of phenol, or of just phenol alone. Apparently, CAT was an artifact produced by oxidation of phenol in hot acid.

MSCP120 and MSCP246 were found to be glycoproteins. Preliminary evidence for this came from the staining of proteins electroblotted into PVDF membranes. Because catechols can be oxidized by the periodate reagent, there was the possibility that the catechols could have mimicked the carbohydrate staining. Nonetheless, we were able to show by GC-MS analysis that both proteins were glycosylated with glucose, *N*-acetylglucosamine and *N*-acetylgalactosamine, and that galactose was also present in MSCP246. Sugar residues have been identified previously in partially purified *D. melanogaster* pupal cuticular proteins (Silvert *et al.*, 1984), cuticular proteins of *H. cecropia* (Cox and Willis, 1987) and abdominal cuticular proteins extracted from *L. migratoria* (Talbot *et al.*, 1991). The significance of glycosylation in the function of insect cuticular proteins is still not understood.

TABLE 4. Carbohydrate composition of high molecular weight proteins MSCP120 and MSCP246 from tanning pharate pupal abdominal cuticle of *M. sexta**

Protein	Glucose	Galactose	<i>N</i> -Acetylglucosamine	<i>N</i> -Acetylgalactosamine
MSCP120	0.56 (3.8)	0	0.35 (1.9)	Trace
MSCP246	0.63 (8.6)	0.14 (1.9)	0.12 (1.4)	0.02 (0.3)

*Values were determined by GC-mass spectrometry and are expressed as percentage of protein weight. Molar ratios of sugar to protein are shown in parentheses.

PROTEIN*	N-TERMINAL SEQUENCE
MSCP120	EEHQGFTLQTLIKHSNPINI..
MSCP246	GLVLRGLYHEIL..

*Proteins are denoted by their apparent molecular mass in kDa. MSCP = *Manduca sexta* cuticle protein.

FIGURE 10. N-Terminal amino acid sequences of *M. sexta* pharate pupal abdominal cuticle proteins MSCP120 and MSCP246.

Hojrup *et al.* (1986) purified a number of cuticular proteins from pharate migratory locusts and found that soft cuticular and endocuticular proteins were glycine-rich and that hard and stiff cuticular proteins were alanine-rich. The proteins described in this study, which were extracted from a cuticle with a relatively high degree of stiffness, had about 30% more glycine than alanine, and both of those amino acids were major constituents of the proteins.

Trace levels of β -alanine and hydroxyproline were found in the high molecular weight cuticular proteins from *M. sexta* pupal cuticle. The former likely was released from *N*- β -alanylated catecholamines covalently bonded to the proteins as previously discussed. The amount of β -alanine (0.1 mol %) recovered from the cuticular proteins was easily accounted for by the level of NBANE and the other catechols that may be hydrolytic products of NBAD or its derivatives. These data were consistent with the hypothesis that β -alanine is not present in the peptide backbone, but instead is incorporated post-translationally as covalently bound *N*- β -alanylated catecholamines (Andersen, 1979). Hydroxyproline has so far not been detected in cuticular proteins, and its significance is unknown.

This study clearly shows that some proteins from tanning pupal cuticle of *M. sexta* have covalently bound catechols and sugars. Such catechol-protein adducts may participate in cross-linking reactions during cuticle sclerotization. Post-translation modification of protein with quinonoid derivatives of catechols would increase the hydrophobicity of these proteins as well. Future studies will elucidate more fully the functional roles played by these catecholic glycoproteins in insect cuticular sclerotization.

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