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Pupal cuticle proteins of *Manduca sexta*: characterization and profiles during sclerotization

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Abstract

Proteins in pupal abdominal cuticle of the tobacco hornworm, *Manduca sexta*, were characterized during the pre-ecdysial and post-ecdysial periods of sclerotization and endocuticle formation. Protein extractability decreased dramatically as the cuticle became sclerotized through 6 h post-ecdysis, but increased rapidly from 9 to 48 h as endocuticular layers were secreted. Nearly 100 proteins that were extracted from pre-ecdysial cuticle became largely insoluble during sclerotization. Three major proteins in this group destined to become exocuticle had apparent molecular masses (Mapp) of 20, 27 and 36 kDa, and were designated MS-PCP20, MS-PCP27, and MS-PCP36. Amino acid analysis revealed glycine to predominate in all three proteins, and alanine, aspartate, glutamate, proline and serine were also relatively abundant. Histidine residues, which provide sites for adduct and cross-link formation with quinone metabolites of *N*- β -alanyldopamine during sclerotization of pupal cuticle, ranged from 2 to 3 mol %. *N*-Terminal amino acid analysis of MSPC-20 and MSPC-36 also revealed some sequence similarities indicating they may be related. An almost entirely new group of proteins appeared by 9 h as endocuticle secretion began, and these increased in abundance through 48 h post-ecdysis. Two of these were major proteins with Mapps of 33 and 34 kDa, and they also had close similarities in their *N*-terminal amino acid sequences. This study showed that the large number of proteins secreted into the presumptive exocuticle of the pupa before ecdysis are involved in sclerotization reactions and as a consequence become largely insoluble. The epidermis then switches to the secretion of an entirely new group of proteins that are involved in formation of the endocuticle. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Sclerotization of insect cuticle partly involves the reactions of functional groups of certain amino acid residues of proteins in the presumptive exocuticle with quinone metabolites of *N*- β -alanyldopamine (NBAD) and *N*-acetyldopamine (NADA) to form adducts and cross-links (Hopkins and Kramer, 1992; Andersen et al., 1996; Sugumaran, 1998). We have previously isolated and characterized several proteins from the tanning pupal cuticle of the tobacco hornworm, *Manduca sexta*, which contained covalently bonded NBAD. This was evidenced by the release of *N*- β -alanyl norepinephrine

(NBANE) and other catechols during acid hydrolysis of the purified proteins, and also by labeling of cuticular proteins in situ by ¹⁴C- β -alanine (Okot-Kotber et al., 1994, 1996). These proteins had different *N*-terminal amino acid sequences and ranged in apparent molecular weight from 32 to 256 kD. Although they appeared to be unrelated in structure, they were similar in containing relatively large amounts of glycine, glutamic and aspartic acids, alanine and serine, which are typical of some other insect cuticular proteins. Small amounts of peptidyl DOPA were detected in some of the proteins, indicating the possible involvement of the catechol moiety in cross-linking of proteins (Okot-Kotber et al., 1994). The proteins also contained low to moderate amounts of histidine, an important amino acid in the peptide chain for covalent bonding to the *N*-acetyldopamine quinones of NBAD and NADA (Schaefer et al., 1987; Christensen et al., 1991). Recently, we isolated and

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identified both histidyl–dopamine and histidyl–DOPET (3,4-dihydroxyphenylethanol) adducts from the sclerotized pupal exuviae of *M. sexta*, confirming the existence of protein–catechol covalent linkages in the sclerotization process (Xu et al., 1997; Kerwin et al., 1999).

Our objectives in this research were to map the proteins secreted into the presumptive exocuticle prior to pupal ecdysis of *M. sexta*; to determine the time course of their disappearance during sclerotization, as well as the appearance of new proteins in the exoskeleton as endocuticle is secreted; and to isolate the major proteins involved in the sclerotization process as identified by this procedure, for further characterization.

2. Materials and methods

2.1. Insect rearing and cuticle preparation

Manduca sexta was reared as described by Bell and Joachim (1976) at 27°C with a photoperiod of 16L:8D. Insects were selected at pre-ecdysial and post-ecdysial intervals for extraction of cuticular proteins: pharate pupae within a few hours of ecdysis (brown metathoracic bars); at ecdysis (0 h); 3, 6, 9, 24 and 48 h (fully tanned) after ecdysis and were frozen at –20°C. For dissection, the frozen insects were placed in an ice-cold solution of 0.1 M acetic acid and 10 mM boric acid, and the exoskeleton was cut between the metathoracic and first abdominal segments. The abdominal cuticle then was cut laterally along each side through the spiracles, and the dorsal and ventral halves of the exoskeleton were peeled away from the underlying tissue. In pharate pupae, the remnants of larval cuticle were separated from the underlying pupal cuticle. The inner surface of the abdominal cuticles was scraped with a small spatula to remove large pieces of muscle and other adhering tissue and given a final cleaning by brushing with a small, stiff-bristle, fabric brush. The cleaned cuticles were rinsed, air dried for 30 min at room temperature, weighed, and stored at –20°C until extraction of proteins.

2.2. Scanning electron microscopy of cuticle cross-sections

Abdominal cuticle dissected from pupae was fixed in Karnosky's solution for 2 h, dehydrated in an ascending ethanol series from 70 to 100%, and then critical point dried. The dried cuticle was fractured to expose cross-sections, fixed to aluminum stubs with silver paste, sputter coated with gold-palladium, and viewed in an ETEC Autoscan scanning electron microscope (SEM).

2.3. Protein extraction

Cuticle samples were vacuum dried, weighed, and placed in 1.5 ml plastic microcentrifuge tubes containing

0.05 ml of an extraction fluid consisting of 5% sodium dodecyl sulfate (SDS), 50 mM acetic acid, 10 mM boric acid, 4 M urea, 10% glycerol, and 0.00125% bromophenol blue for each milligram of dried cuticle. The tubes were capped and placed on a reciprocating shaker for 24 h at room temperature. Following extraction, the tubes were centrifuged to remove solids, and the supernatants used for gel electrophoresis. Protein concentration was measured using a DC protein assay kit (Bio-Rad Labs., Hercules, CA) as modified from Lowry et al. (1951).

2.4. Gel electrophoresis

Proteins in the cuticle extracts were separated initially by one dimensional discontinuous SDS–PAGE in Mini-PROTEAN II Cells (Bio-Rad). The acrylamide was 4.5% in the stacking section and 12% in the separating section of the gel, and 150 V at approximately 15°C was used for separation until the tracking dye reached the bottom edge. The gels then were stained with 0.1% Coomassie brilliant blue R-250 in 40% methanol and 10% acetic acid for about 6 h, followed by destaining in a 40% methanol and 10% acetic acid solution.

Two-dimensional isoelectric focusing (IEF), SDS–PAGE of the cuticle extracts and blotting of protein onto PVDF membranes was done by Kendrick Labs, Madison, WI. Isoelectric focusing was done in glass tubes of 2.0 mm inner diameter using 2% pH 4 to 8 ampholines for 9600 volt-hours. After equilibration in 10% glycerol, 50 mM dithiothreitol, 2.3% SDS, and 0.0625 M Tris (pH 6.8), the tube gel was sealed to the top of a stacking gel on a 10% slab gel. Electrophoresis was carried out at 12.5 mA/gel for about 4 h. The gels were stained with Coomassie brilliant blue, destained, and dried between cellophane sheets. To quantify the proteins, known amounts of bovine serum albumin also were stained and dried with the gel. Protein quantities were determined by scanning the gel with an LGS-50 laser densitometer, and the stain optical density measured. The densitometer was calibrated with the Coomassie brilliant blue-stained BSA standard strip. Duplicate gels were placed in transfer buffer and transblotted onto PVDF membranes overnight at 200 mA and approximately 100 V/2 gels. The membranes then were stained with Coomassie brilliant blue to locate the proteins of interest. The apparent molecular masses (Mapp) of proteins were estimated by comparison with standard proteins run on the SDS–PAGE gels.

2.5. Amino acid composition of proteins

Proteins were separated on preparative one-dimensional gels and then negative stained with 0.3 M CuCl₂ and 5 mM boric acid (Lee et al., 1987). Individual protein bands were cut from the gels, the copper was

removed by chelation with EDTA, and the protein was electroeluted from the gel with a Bio-Rad Electro-Eluter. The electroelution buffer was 50 mM ammonium bicarbonate, 0.1% SDS, 5 mM boric acid, and 5 mM EDTA. The proteins then were absorbed onto PVDF membrane filters by centrifugation, and the buffer was discarded. Samples were hydrolyzed in 6 M HCl containing 4% phenol at 110°C for 24 h, and the amino acids analyzed by HPLC with ninhydrin detection by the Experiment Station Chemical Laboratories, University of Missouri, Columbia, MO.

2.6. N-Terminal amino acid sequencing of proteins

The proteins were obtained in pure form by either electroelution from bands cut from copper-stained one-dimensional preparative polyacrylamide gels as described above or by cutting the individual Coomassie brilliant blue stained protein spots from PVDF membranes after blotting from two dimensional (2-D) gels. Sequencing was achieved by automated Edman degradation on an Applied Biosystem Sequencer at the Biotechnology Core Facility, Kansas State University, Manhattan, KS.

3. Results

3.1. Cuticular protein solubility during sclerotization

Total extractable proteins in pupal abdominal cuticle significantly decreased from nearly 250 $\mu\text{g}/\text{mg}$ dry weight in pharate pupae that recently had initiated sclerotization to 190 $\mu\text{g}/\text{mg}$ in the newly ecdysed pupae ($P=0.05$, $n=3$, Fig. 1). Protein solubility continued to decrease through 6 h post-ecdysis to a low of about 90 $\mu\text{g}/\text{mg}$ or about 60% less than that in pharate pupal cuticle. However, by 9 h, total proteins extracted had

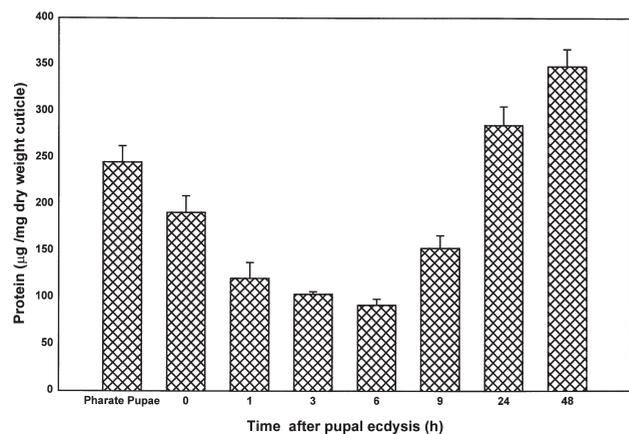


Fig. 1. Solubility of protein in the pupal cuticle of *Manduca sexta* ($\mu\text{g}/\text{mg}$) from the pharate pupal stage to 48 h post-ecdysis. Bars represent S.E.M.

increased significantly by about 70%. By 24 and 48 h, total extractable protein increased sharply and was greater than that of pharate pupae (Fig. 1).

3.2. Scanning electron microscopy of cuticle cross-sections

Cross-sections of pupal cuticle were examined by SEM at intervals during sclerotization to correlate the protein solubility experiments with the physical appearance of the cuticle (Fig. 2). Newly ecdysed pupal abdominal cuticle (Fig. 2A) is partially sclerotized and is similar to the more fully sclerotized cuticle collected at 3–6 h after ecdysis (Fig. 2B). Sclerotization is apparent in both examples with the outer layers showing a fused indistinct appearance compared to the thicker inner layers. However, no endocuticular layers are visible during the main period of sclerotization from zero to 6 h. By 24 h post-ecdysis, a large number of thick endocuticular layers are visible beneath the sclerotized exocuticle that were not present at 6 h (Fig. 2C). Endocuticular layers continued to be secreted through 48 to 72 h (data not shown).

3.3. One dimensional SDS-PAGE analysis of cuticular proteins

Extracts of total proteins adjusted to the same dry weight of cuticle were analyzed by SDS-PAGE during the main period of sclerotization (Fig. 3). Nine major bands and several minor bands of proteins occurred in the extracts of pharate pupal cuticle and had Mapps ranging from below 10 to over 100 kDa. These generally showed a progressive decrease and disappearance through the first 6 h post-ecdysis as the cuticle sclerotized. By 9 h post-ecdysis, several new bands of proteins began to appear in the extracts and these generally increased through 48 h, correlating with the secretion of the endocuticle (Fig. 2). Because the bands observed in one-dimensional gel electrophoresis may have contained more than a single protein, two-dimensional separation of the extracts also was done.

3.4. Two-dimensional IEF-PAGE electrophoresis analysis of proteins

The proteins in pharate pupal cuticular extracts were resolved more completely by two-dimensional IEF-SDS-PAGE (Fig. 4). Nearly 100 pre-ecdysial proteins were observed in these extracts, but two of the spots, #3 and #19, predominated in concentration (Table 1). Spot #11 and spots #16, 17, 18, 20 and 21 were of intermediate concentrations (Fig. 4, Table 1). The latter group of more basic proteins had Mapps of 20–21 kDa. Spots #3, 11, and 19 with Mapps of 20, 27 and 36, respectively, were selected for further characterization, and therefore

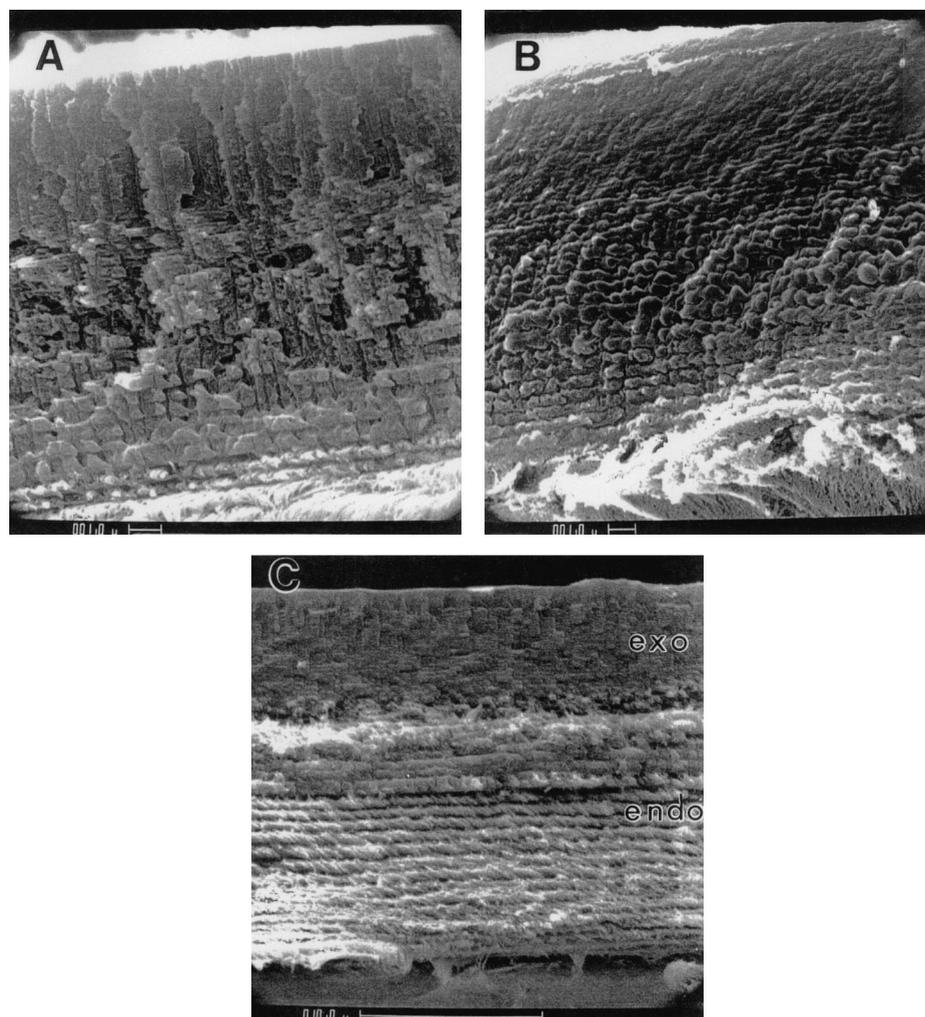


Fig. 2. Scanning electron micrographs of cross-sections of pupal abdominal cuticle of *Manduca sexta*. Outer surface of the cuticle at top of micrograph; epidermal surface at bottom of micrograph. (A) Newly ecdysed partially sclerotized cuticle (6000 \times). (B) Cuticle at 3–6 h post-ecdysis (5000 \times). (C) Cuticle at 24 h post-ecdysis (3500 \times) showing the sclerotized exocuticle and the more recently secreted endocuticle.

were designated MS-PCP 20, MS-PCP 27 and MS-PCP 36. All of these proteins essentially disappeared from extracts of the pupal cuticle as sclerotization proceeded.

Extracts of pupal cuticle 48 h after ecdysis showed an almost entirely new set of proteins, but fewer in total number compared to the pharate pupal cuticle extracts (Fig. 5). The two major post-ecdysial proteins had almost identical Mapps of 32.9 and 33.6 kDa and pI values of 5.6 and 5.7. Four proteins with Mapps of 9–10 kDa and pI values of 5.2–5.7 appeared for the first time, as well as a number of more basic proteins in the 20–30 kDa range with pI values of 7.1–7.8.

3.5. Amino acid composition of proteins

The amino acid compositions of MS-PCP 20, MS-PCP 27, and MS-PCP 36 are shown in Table 2. Glycine was the predominant amino acid in all three proteins, ranging from 17 to 32%. Relatively high levels of glutamate, aspartate, serine, and alanine also occurred in these

proteins. The basic amino acid residues of histidine and lysine, whose side chains are potential nucleophilic sites for adduct formation with quinone sclerotizing agents, accounted for approximately 3–6% of the total amino acids. The aromatic amino acids tyrosine and phenylalanine ranged from 3 to 5 mol % each.

3.6. N-terminal amino acid sequences of proteins

The N-terminal amino acid sequences of the exocuticular and endocuticular proteins are shown in Table 3. The predominant exocuticular or pre-ecdysial proteins MS-PCP 20 and MS-PCP 36 showed similarities in amino acid alignments and composition in the first 30 residues. A tyrosine, leucine, proline, proline, arginine sequence occurred in both proteins starting at residue 7, as well as arginine, leucine, and either aspartic acid or glutamic acid at residues 1, 2, and 3. Glycine, proline, and alanine content were relatively high in both proteins. The N-terminal sequence of MS-PCP 27 showed little

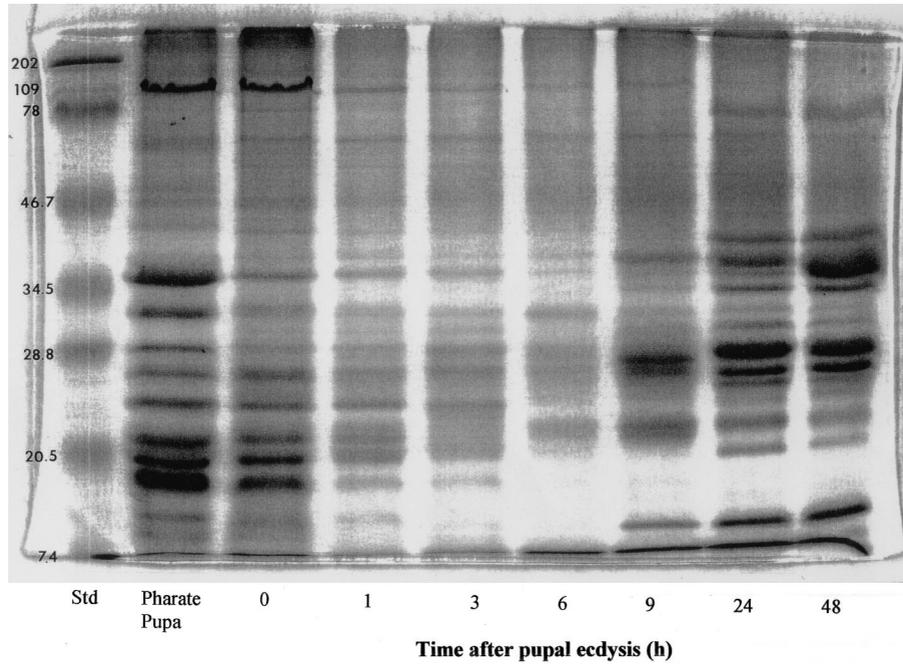


Fig. 3. 1-D SDS-PAGE of protein extracts of *Manduca sexta* cuticle from pharate pupae to pupae at 48 h post-ecdysis. Protein MW standards are in left lane.

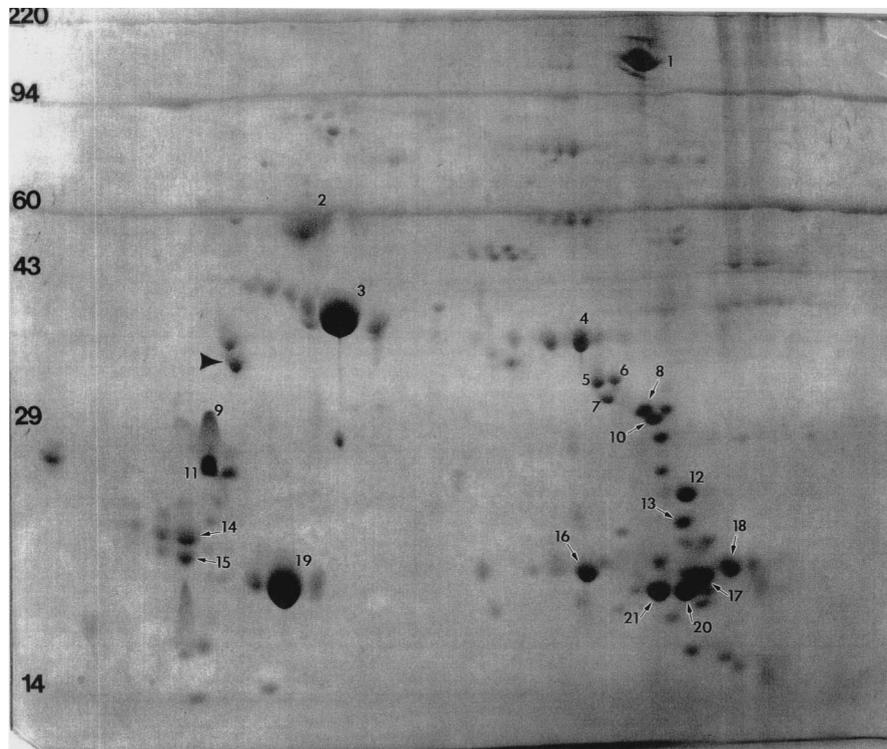


Fig. 4. 2D IEF SDS-PAGE of proteins extracted from pharate pupal cuticle of *Manduca sexta*. Arrow indicates tropomyosin standard MW 33 kDa. Horizontal lines are protein MW standards: myosin (220 kDa), phosphorylase A (64 kDa), catalase (60 kDa), actin (43 kDa), carbonic anhydrase (29 kDa), lysozyme (14 kDa). Protein numbers correspond to those in Table 1.

Table 1

Two-dimensional IEF–SDS–PAGE analysis of proteins from pharate pupal cuticle of *Manduca sexta*

Protein # (see Fig. 4)	App. mol. mass (kDa)	pI	μg/spot	pmol/spot	Mole % of total protein/spot
1	143.1	7.1	2.3	15.9	1.7
2	50.7	5.5	1.1	21.3	2.3
3	MS-PCP 36 36.7	5.7	5.1	139.0	15.1
4	34.5	6.8	0.6	17.4	1.9
5	31.7	6.9	0.3	8.1	0.9
6	31.7	7.0	0.2	6.9	0.7
7	30.7	7.0	0.2	6.4	0.7
8	29.9	7.1	0.5	16.8	1.8
9	29.7	5.1	0.4	14.6	1.6
10	29.4	7.2	0.6	19.7	2.1
11	MS-PCP 27 27.2	5.1	1.0	37.5	4.1
12	25.2	7.4	0.6	24.6	2.7
13	23.6	7.3	0.4	15.6	1.7
14	23.3	5.0	0.5	20.8	2.3
15	22.2	4.9	0.3	14.7	1.6
16	21.0	6.9	0.9	45.1	4.9
17	21.0	7.5	1.4	68.1	7.4
18	21.0	7.6	1.4	65.9	7.1
19	MS-PCP 20 20.4	5.4	4.4	214.7	23.3
20	20.4	7.4	1.3	62.6	6.8
21	19.9	7.2	1.2	62.5	6.8

Table 2

Amino acid compositions of three major proteins involved in the sclerotization of pupal cuticle of *Manduca sexta*

Amino acid	Mol %		
	MS-PCP 20	MS-PCP 27	MS-PCP 36
Aspartic acid/asparagine	8.4	10.3	7.1
Threonine	4.8	4.9	3.1
Serine	11.5	8.6	7.6
Glutamic acid / glutamine	10.8	15.2	7.6
Proline	7.1	5.9	4.7
Glycine	21.1	16.8	31.5
Alanine	8.4	7.7	14.4
Valine	3.4	3.5	2.6
Methionine	0.4	0.7	0.2
Isoleucine	3.4	3.5	2.3
Leucine	4.4	4.3	3.3
Tyrosine	3.4	3.2	3.2
Phenylalanine	3.3	2.8	4.9
Histidine	3.1	2.7	1.4
Lysine	1.4	2.8	1.8
Arginine	5.1	7.1	4.3

similarity to the major proteins, although it had a relatively high glycine and proline content.

The *N*-terminal amino acid sequences of the two major endocuticular or post-ecdysial proteins were quite different from the exocuticular proteins, but showed

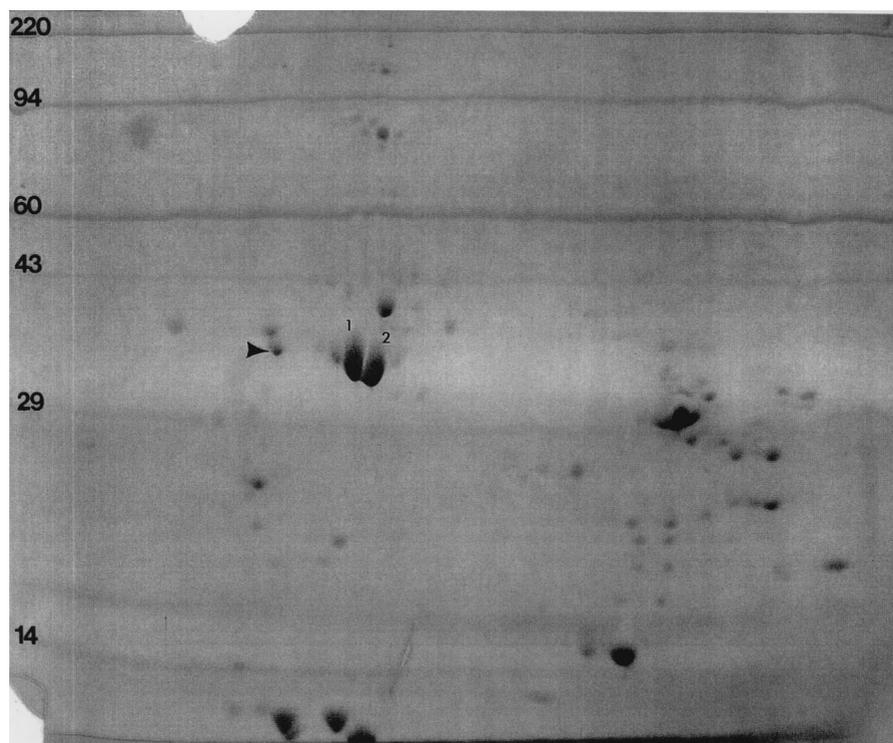


Fig. 5. 2D IEF SDS–PAGE of proteins extracted from pupal cuticle of *Manduca sexta* at 48 h post-ecdysis. Protein standards are as indicated in Fig. 4.

Table 3

N-Terminal amino acid sequences of cuticular proteins from the pharate pupa of *Manduca sexta*

*Protein	Amino acid sequence	
MS-PCP20	10	20
1	G R L E P Q Y L P P R P G N G F G G G A ? S	
2	G R L E P Q Y L P P R R G N N F F A G A A A V S N P S L P	30 S G E
MS-PCP27	10	20
1	A E L P S R N Y I P Q D Q G S G P Y P G	
2	A E L P S R N Y I P Q D Q G P G P Y P G P H G S	
MS-PCP36	10	20
1	D R L D N K Y L P ? R G N A G A G F G P G F G G A G P K K G G	30
2	D R L D N K Y L P P R G D A G G A F F P P F G G A ? P P S G ? S	
MS-PCP33	10	20
2	D G Y I S R A A ? ? ? ? P A ? P I G A K P Q V	
	H L	A
MS-PCP34	10	20
2	A H L L P Y A A Y S Y I P A I P I G A L A L V A P K D I Q A Q A	30 Y

*Pre-ecdysial proteins: MS-PCP 20, MS-PCP 27, MS-PCP 36; post-ecdysial proteins: MS-PCP 33, MS-PCP 34. 1 = Protein electroeluted from 1-D polyacrylamide gels. 2 = Protein from PVDF blots of 2-D polyacrylamide gels.

some similarity to each other. A proline, alanine, isoleucine, proline, isoleucine, glycine, alanine stretch starting at residue 13 was the longest common sequence observed, although two of residues in MS-PCP 33 were not unequivocally identified. Alignments of five of amino acids also occurred, further indicating their relatedness.

4. Discussion

Sclerotization or the interactions of quinone metabolites of NADA and/or NBAD with cuticular proteins greatly reduces the solubility of the resulting exocuticle (see reviews by Hopkins and Kramer, 1992; Andersen et al., 1996). Andersen and Hojrup (1987), Andersen et al. (1986) and Hojrup et al. (1986) have previously shown that over 100 proteins can be extracted from the unsclerotized cuticle of newly ecdysed adult *Locusta migratoria*, but not after sclerotization has occurred. Also, proteins from pharate adult cuticle of *L. migratoria* could not be extracted 1 d after ecdysis, demonstrating the dramatic effect of sclerotization on reduction of protein solubility. Further, the proteins from unsclerotized pre-ecdysial cuticle of nymphs and adults of *L. migratoria*, were reported to be completely different

from those extracted from the fully formed post-ecdysial cuticle (Andersen et al., 1995).

In the present study, a temporal analysis of the solubility of cuticular proteins showed a progressive decrease from a few hours before ecdysis when the abdominal cuticle begins to tan, through 6 h post-ecdysis. Pre-ecdysial tanning or sclerotization of pharate pupal cuticle is visible in the thorax and abdomen approximately 12–24 h before ecdysis, as evidenced by the appearance of dark brown pigmentation in localized areas of the sclerites. This tanning continues to spread until expansive areas of abdominal and thoracic cuticle are brown pigmented in the newly ecdysed pupa, and the exoskeleton is totally darkened a few hours later. Examination of cross-sections of abdominal cuticle by SEM showed that the presumptive exocuticle was secreted before ecdysis, and endocuticle secretion began only after tanning of the exocuticle was essentially completed during the first 6 h post-ecdysis. The decrease in the amounts of total protein extracted during this time was correlated with the progressive sclerotization of the exocuticle and no further secretion of protein matrix. By 9 h post-ecdysis, however, total extractable protein increased significantly, and this trend continued through 48 h. Cross-sections of 24 h pupal abdominal cuticle showed a well differentiated endocuticle of many layers

that exceeded the thickness of the exocuticle. Deposition of additional endocuticular layers continued through 48 h post-ecdysis, which correlated with the rapid increase in extractable protein. Gel electrophoresis showed that the pre-ecdysial proteins decreased in cuticle extracts through 6 h post-ecdysis and new proteins began to appear by 9 h. Therefore, the exocuticular proteins appear to be totally secreted prior to ecdysis and become largely sclerotized and insoluble before secretion of endocuticle begins. A comparison of 2-D gels of *M. sexta* pharate pupal cuticle extracts with 48 h extracts showed an almost complete difference between exocuticular or pre-ecdysial proteins and endocuticular or post-ecdysial proteins.

Previous studies of pharate pupal cuticle of *M. sexta* revealed a large number of proteins with Mapps ranging from less than 14 kDa to more than 200 kDa (Okot-Kotber et al., 1994). Most of these proteins appeared to be involved in sclerotization reactions and formation of the exocuticle, because they become labeled by injected ^{14}C - β -alanine, presumably by the formation of adducts with NBAD via quinonoid intermediates (Xu et al., 1997). Heating purified proteins in acetic acid also released *N*- β -alanyl norepinephrine and other catechols, by hydrolyzing weakly bonded NBAD (Okot-Kotber et al. 1994, 1996). We extracted almost 100 proteins from the pre-ecdysial or exocuticular pupal abdominal cuticle ranging from less than 14 kDa to nearly 200 kDa, confirming the results of Okot-Kotber et al. (1994), although the extraction procedures in the two studies differed. The major group observed on 2-D gels was slightly acidic, and contained the two predominant proteins MS-PCP 20 and MS-PCP 36, as well as MS-PCP 27, whereas the smaller group of proteins was neutral or slightly basic. Sridhara (1994) had previously observed two groups of about 30 distinct proteins extracted from recently ecdysed pupal cuticle of *M. sexta*. The larger group of proteins had pI values in the neutral range, whereas the second smaller group contained more alkaline proteins. The patterns of proteins extracted from pupal abdominal and wing cuticles were almost identical, as were those extracted from intersegmental and sclerite regions of the abdomen (Sridhara, 1994). Therefore, the protein composition of the pupal cuticle of *M. sexta* appears to be similar in all regions of the exoskeleton. The larger number of proteins that we detected may have been due to more efficient extraction procedures and less sclerotization in the pharate pupal cuticle than the recently ecdysed cuticle used by Sridhara (1994).

Three proteins in the extracts selected for further characterization were MS-PCP 20, 27 and 36. Amino acid analysis of protein hydrolysates showed that glycine predominated in all three of the proteins. Relatively high levels of alanine, glutamic acid, aspartic acid and serine also occurred in these proteins. The aromatic amino acids phenylalanine and tyrosine ranged from 3 to over

4 mol% each and could serve as precursors for peptidyl DOPA. Small but significant amounts of peptidyl DOPA were previously detected in pharate pupal cuticular proteins of *M. sexta* and may play a role in sclerotization (Okot-Kotber et al., 1994). Therefore, post-translational hydroxylation of tyrosyl residues to DOPA by cuticular phenoloxidases may be involved in the cross-linking of cuticular proteins (Thomas et al., 1989; Morgan et al., 1990). The basic amino acids histidine and lysine, which can form adducts and cross-links with quinone sclerotizing agents, were also present in the pre-ecdysial proteins. Histidyl-dopamine and histidyl-DOPET adducts have previously been isolated from hydrolysates of sclerotized pupal cuticle of *M. sexta* (Xu et al., 1997; Kerwin et al., 1999).

N-Terminal amino acid analysis of the two major proteins extracted from pharate pupal cuticle, MS-PCP 20 and MS-PCP 36, showed sequence similarities in a YLPPR region starting at residue 7 and an RLD/E stretch starting at residue 2. MS-PCP 27 showed little similarity to MS-PCP 20 and 36. A search of the BLAST program for protein sequence homology revealed no other proteins with close similarities to the *N*-terminal sequences we obtained for MS-PCP 20, 27 and 36. These proteins are apparently stage specific to pupal cuticle and are distinct from the deduced amino acid sequences of *M. sexta* larval cuticle proteins so far obtained (Rebers and Riddiford, 1988; Horodyski and Riddiford, 1989; Rebers et al., 1997). The two major endocuticular proteins partially characterized in this study appeared to be closely related to each other, but not to the exocuticular proteins or to cuticle proteins from other insect species.

Results of this study showed that a large number of proteins, varying widely in concentration, are secreted into the pre-ecdysial pupal cuticle of *M. sexta* and become involved in sclerotization. Only after formation of the exocuticle do the epidermal cells switch to the secretion of an entirely new group of endocuticular proteins. Full structural elucidation of these proteins will be necessary to further determine their relatedness to other cuticular proteins, as well as the sites on the pre-ecdysial proteins involved in interactions with quinone sclerotizing agents to produce the functional exoskeleton.

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