Available online at www.sciencedirect.com
SCIENCE DIRECT
Insect Biochemistry and Molecular Biology 36 (2006) 353–365
Insect Biochemistry and Molecular Biology
www.elsevier.com/locate/ibmb

Model reactions for insect cuticle sclerotization: Cross-linking of recombinant cuticular proteins upon their laccase-catalyzed oxidative conjugation with catechols

Richard J. Suderman[a], Neal T. Dittmer[a], Michael R. Kanost[a,*], Karl J. Kramer[a,b]

[a]*Department of Biochemistry, 103 Willard Hall, Kansas State University, Manhattan, KS 66506-3706, USA*
[b]*Grain Marketing and Production Research Center, Agricultural Research Service, US Department of Agriculture, Manhattan, KS 66502-2736, USA*

Abstract

The quinone-tanning hypothesis for insect cuticle sclerotization proposes that *N*-acylcatecholamines are oxidized by a phenoloxidase to quinones and quinone methides, which serve as electrophilic cross-linking agents to form covalent cross-links between cuticular proteins. We investigated model reactions for protein cross-linking that occurs during insect cuticle sclerotization using recombinant pupal cuticular proteins from the tobacco hornworm, *Manduca sexta*, fungal or recombinant hornworm laccase-type phenoloxidase, and the cross-linking agent precursor *N*-acylcatecholamines, *N*-*β*-alanydopamine (NBAD) or *N*-acetyldopamine (NADA). Recombinant *M. sexta* pupal cuticular proteins MsCP36, MsCP20, and MsCP27 were expressed and purified to near homogeneity. Polyclonal antisera to these recombinant proteins recognized the native proteins in crude pharate brown-colored pupal cuticle homogenates. Furthermore, antisera to MsCP36, which contains a type-1 Rebers and Riddiford (RR-1) consensus sequence, also recognized an immunoreactive protein in homogenates of larval head capsule exuviae, indicating the presence of an RR-1 cuticular protein in a very hard, sclerotized and nonpigmented cuticle. All three of the proteins formed small and large oligomers stable to boiling SDS treatment under reducing conditions after reaction with laccase and the *N*-acylcatecholamines. The optimal reaction conditions for MsCP36 polymerization were 0.3 mM MsCP36, 7.4 mM NBAD and 1.0 U/μl fungal laccase. Approximately 5–10% of the monomer reacted to yield insoluble oligomers and polymers during the reaction, and the monomer also became increasingly insoluble in SDS solution after reaction with the oxidized NBAD. When NADA was used instead of NBAD, less oligomer formation occurred, and most of the protein remained soluble. Radiolabeled NADA became covalently bound to the MsCP36 monomer and oligomers during cross-linking. Recombinant *Manduca* laccase (MsLac2) also catalyzed the polymerization of MsCP36. These results support the hypothesis that during sclerotization, insect cuticular proteins are oxidatively conjugated with catechols, a posttranslational process termed catecholation, and then become cross-linked, forming oligomers and subsequently polymers.

Keywords: Tyrosinase; Catechol; Exoskeleton; Phenoloxidase; Laccase; Cross-linking; Cuticle; Sclerotization; Tanning; Catechol; Quinone; Tobacco hornworm; *Manduca sexta*; Catecholation

1. Introduction

Sclerotized insect cuticles are so stable that they do not easily give up information when they are examined to determine how they were assembled (Andersen, 2005). As the *Manduca sexta* pupal cuticle becomes sclerotized, the extractability of most pre-ecdysial cuticular proteins decreases until they are no longer extractable (Hopkins et al., 2000; Willis et al., 2005). It has long been held that the proteins become inextractable due to the formation of covalent cross-links to cuticular polymers, such as protein and chitin. One hypothesis for the mechanism of protein cross-linking is the quinone-tanning hypothesis, first put forward by Pryor (1940), and modified by many others including Kramer et al. (2001). In this hypothesis, *N*-acylcatecholamines in the cuticle are oxidized to *o*-quinones by laccase and/or tyrosinase or to *p*-quinone

*Corresponding author. Tel.: +1 785 532 6121; fax: +1 785 532 7278.
E-mail address: kanost@ksu.edu (M.R. Kanost).

0965-1748/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ibmb.2006.01.012

methides by laccase. The electrophilic quinones and quinone methides are then attacked by nucleophilic side chain functional groups of amino acid residues of cuticular proteins, whereby the nucleophile forms a covalent bond to either a ring carbon or the β-carbon of the *N*-acylcatecholamine by a non-enzymatic spontaneous Michael addition reaction (Xu et al., 1997), which is a posttranslational process denoted as catecholation. The protein bound *N*-acylcatecholamine may then be reoxidized in a redox-coupled reaction by diffusible quinones and quinone methides formed by oxidation of *N*-β-alanyldopamine (NBAD) and/or *N*-acetyldopamine (NADA) or perhaps directly by laccase and/or tyrosinase. Whereas evidence exists for cuticular proteins being bound to *N*-acylcatecholamines (Okot-Kotber et al., 1994, 1996), the linkages that have been characterized were mono histidine and lysine residues of cuticular proteins being bound to *N*-acylcatecholamines (Schaefer et al., 1987), and no evidence has been reported so far for two or more such residues bound to a single *N*-acylcatecholamine.

A second hypothesis for the mechanism of sclerotization is that incorporation of large quanitities of oxidized *N*-acylcatechols displaces water and dehydrates the cuticle to such an extent that cuticular proteins become trapped in a hydrophobic matrix of polymerized *N*-acylcatechols (Vincent and Hillerton, 1979). In this hypothesis *N*-acylcatechols are oxidized to quinones that then react with histidine and lysine residues of cuticular proteins, just as in the quinone-tanning hypothesis. However, this is proposed to decrease the number of hydration sites, increasing the hydrophobicity of the proteins, thus serving to drive water out of the cuticle. Both hypotheses assert that *N*-acylcatechols that are polymerized via benzodioxin-type linkages may serve as space fillers in the exoskeleton (Andersen, 1985, 2005). Active resorption of water from the cuticle by the epidermal cells may also occur.

Because the inextractability of cuticular proteins once they become cross-linked precludes most methods of protein analysis, development of model systems for cuticle sclerotization may provide insight concerning the fate of particular cuticular proteins after sclerotization. Several such models have been constructed, although none have consisted entirely of biologically relevant components found in the cuticle (Sugumaran et al., 1987; Hasson and Sugumaran, 1987; Grün and Peter, 1983; Peter et al., 1988). The purpose of this study was to develop simple in vitro model sclerotization reactions that specifically tested insect cuticular proteins and *N*-acylcatecholamines known to be present in insect cuticle. The model that we have developed consisted of recombinant proteins from the pupal cuticle of *M. sexta*, the phenoloxidases laccase or tyrosinase from either insect or fungal sources, and the *N*-acylcatecholamines, NBAD or NADA, which are the most abundant catechols in *M. sexta* and serve as precursors for protein cross-linking agents in the exoskeleton (Hopkins and Kramer, 1992; Andersen, 2005).

2. Materials and methods

2.1. Expression of recombinant M. sexta cuticular proteins

Primers used for reverse transcription-polymerase chain reactions (RT-PCR) and PCR in constructing plasmids for expressing recombinant cuticular proteins are listed in Table 1. cDNA clones containing the complete open reading frames of cuticular proteins MsCP20, MsCP27 and MsCP36 were constructed by two-step RT-PCR of RNA from pharate pupal integument. In the first step, single stranded cDNA was reverse transcribed using an oligo-dT primer. This cDNA pool served as a template in the second step of PCR using gene-specific primers from the 5′ and 3′ untranslated sequences of each cDNA (primers 584 and 585 for MsCP20, 582 and 583 for MsCP27, and 580 and GR3P for MsCP36). These products were cloned into pGEM-T (Promega). To create the expression constructs, the region of the open reading frame encoding each mature cuticular protein was amplified using the pGEM-T clones as templates with primers designed to contain restriction sites (Table 1), and the resulting PCR products were cloned into the expression vector pProEXH-Ta (Life Technologies). Platinum Taq High Fidelity DNA Polymerase (Invitrogen) was used per the manufacturer's recommendations under the following reaction conditions for MsCP20 and 27: 94 °C, 2 min; 25 cycles of 94 °C, 30 s; 68 °C, 4 min; followed by 68 °C, 10 min. For MsCP36, the following reaction conditions were used: 94 °C, 2 min; 30 cycles of 94 °C, 30 s; 50 °C, 30 s; 72 °C, 3 min; followed by 72 °C, 10 min. Reaction products were gel purified, digested with the appropriate restriction enzymes, gel purified again and ligated to the pProEXHTa vector. The expression vectors containing the cDNAs for the cuticular proteins were then used to transform *Escherichia coli* strains XL1-blue or DH5α (Life Technologies). Transformed *E. coli* were grown in 2 L LB containing 100 μg/ml ampicillin at 37 °C with shaking at 275 rpm until the A_{600} reached 0.8–1.0, at which time expression was induced by adding IPTG to 1 mM. Expression proceeded for 4–5 h, after which the cells were pelleted by centrifugation, frozen at −20 °C for at least 10 h, thawed and resuspended in 4 volumes of lysis buffer (50 mM Tris–HCl, pH 8.5 at 4 °C, 5 mM 2-mercaptoethanol and protease inhibitor cocktail (Sigma P8849) at 50 μl/g cell pellet) on ice, and lysed by adding 1 mg/ml lysozyme for 45 min and then sonicating. The cell debris was removed by centrifugation at 15,000*g* for 1 h at 4 °C. The soluble recombinant cuticular proteins, which contained N-terminal six-His tags, were purified from the clarified supernatant (approximately 50 ml) by incubating the supernatant with 2.5 ml Ni-NTA (Qiagen) beads for 1 h and mixing by tumbling end over end at 4 °C. The beads were then pelleted by centrifugation at 100*g* for 5 min, and the supernatant was removed. The beads were then resuspended with an equal volume of Buffer A (20 mM Tris–HCl, pH 8.5, 0.5 M KCl, 5 mM β-mercaptoethanol, 10% glycerol, 20 mM imidazole) at room

Table 1
Oligonucleotide primers used for polymerase chain reactions for constructing plasmids to express cuticular protein cDNAs as recombinant proteins

Primer name	Cuticular protein	Primer sequence
584(+)	MsCP20	5′ TGAACAAGGAGCGCCTGTCCTA 3′
585(−)	MsCP20	5′ GTCGCATATAAAACCCGACGTTAC 3′
590(+)NcoI	MsCP20	5′ GTAACCATGGTAGGTCGTTTGGAGCCGCAGTACCTCCCC 3′
591(−)NotI	MsCP20	5′ TGTAGCGGCCGGGTATTGAATGAGATCAGTAGTGGTAGCCG 3′
582(+)	MsCP27	5′ TTTAAGTCCACCACTTAAAGGAAC 3′
583(−)	MsCP27	5′ TTCTATAAGTTTATTGTACAGTCCATT 3′
588(+)NcoI	MsCP27	5′ GTAACCATGGTAGCTGAGCTTCCATCAAGGAATTATATC 3′
589(−)NotI	MsCP27	5′ TGTAGCGGCCGGTACAGTCCATTGGTAATCTTCAGTAG 3′
580(+)	MsCP36	5′ GCACACAGCATCCGAACCACTA 3′
GR3P	MsCP36	5′ GCTGTCAACGATACGCTACGTAACG 3′
597(+)EcoRI	MsCP36	5′ GTAAGAATTCGATCGCCTCGACAACAAGTACCTG 3′
599(−)SpeI	MsCP36	5′ TGTAACTAGTGGTCGCGAGTGGTCGTCTAGTACT 3′

temperature and quantitatively transferred to a 15 ml column at room temperature. The column was then packed at 1.5 ml/min with buffer A until the bed volume was constant, then washed with 10 volumes of buffer A at 1 ml/min, followed by two volumes of buffer B (buffer A, containing 1 M KCl and no imidazole). The column was then washed with two more volumes of buffer A, and the protein was eluted with buffer C (buffer A with 100 mM imidazole) until the $A_{280} < 0.1$. Fractions (1.5 ml) were analyzed by SDS–PAGE and immunoblotting using α-pentaHis monoclonal antibody (Qiagen) and stored at 4 or −80 °C.

2.2. Production of rabbit polyclonal antisera to full-length cuticular proteins

Full-length recombinant cuticular proteins were expressed as described above. Approximately, 0.7 mg of each protein excised from polyacrylamide gels was injected into rabbits for polyclonal antisera production (Cocalico Biologicals). Antisera to all cuticular proteins were shown by immunoblot analysis to recognize their respective recombinant proteins as well as proteins of the expected size from *M. sexta* pharate pupal cuticle homogenates.

2.3. Removal of N-terminal His-tag from recombinant proteins

The recombinant proteins expressed from vector pProEXHTa contained an amino-terminal six His tag with a tobacco etch virus (TEV) protease cleavage site between the His tag and the recombinant protein. The His tag and the cleavage site add approximately 2400 Da to the recombinant protein. After affinity purification using Ni-NTA, the His tag was removed using recombinant TEV protease (Invitrogen). Briefly, the Ni-NTA elution buffer (buffer C, above) was replaced with TEV protease reaction buffer (50 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 1 mM dithiothreitol) by filtration using Amicon Microcon or Centriplus devices or by gel filtration using PD-10 columns (Amersham Biosciences). The protein was then concentrated by ultrafiltration from 0.1 to 2 mg/ml, dithiothreitol was added to 1 mM, and TEV protease was added to a final concentration of 500 U/mg cuticular protein. The reaction was incubated at room temperature for 1–2 h, then 4 °C overnight with rocking. The cleaved protein was purified by applying the entire reaction mixture to a Ni-NTA column equilibrated with TEV protease reaction buffer. The flow-through contained the cleaved protein, while the TEV protease, uncleaved protein, and cleaved His tags all bound to the column. Yield and purity were examined by SDS–PAGE and immunoblot analyses using cuticular protein-specific polyclonal antisera and α-pentaHis monoclonal antibodies.

2.4. M. sexta integument protein homogenate preparation

To prepare larval head capsule homogenates, the head capsules of 8 fourth instar larvae at head capsule slippage were removed and the underlying pharate fifth instar head capsules were carefully dissected. These samples of fourth and fifth instar head capsules were frozen in liquid nitrogen and crushed with a mortar and pestle. Approximately, 50 mg of each powder was immediately suspended in 0.4 ml 1X SDS sample loading buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS w/v, 1.55% DTT w/v, 0.01% bromophenol blue w/v), vortexed, and boiled for 10 min. To prepare pharate pupal integument homogenates, fifth instar larval cuticle from at least two late bar stage prepupae was removed, and the underlying pupal cuticle was carefully dissected. The cuticle was then treated as described above to prepare the homogenate.

2.5. Synthesis of [^{14}C]-labeled N-acetyldopamine

The synthesis of radiolabeled NADA was carried out as described by Andersen (1985). The products were analyzed by thin layer chromatography (3 M cellulose TLC plates without fluorescent indicator) using dopamine and NADA standards and a mobile phase of butanol: acetic acid: water

at 3:1:1 (v:v:v). The chromatographic plates were developed by spraying with 2% $FeCl_3$, and spots were scraped into scintillation fluid for measurement of radioactivity.

2.6. Immunoblot analysis

Protein samples were separated by SDS–PAGE on either 1 mm thick 12% NuPage Novex Bis-Tris Gels (Invitrogen), 1 mm thick 8–16% Express Gels (ISC Bioexpress), or 1 mm thick 3–8% NuPage Novex Tris-Acetate gels according to manufacturer's recommendations. Protein was then transferred to nitrocellulose by semidry transfer and blocked in 3% dried milk or 5% BSA for 1 h in 1X TTBS (25 mM Tris–HCl, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween-20, pH 7.4). Primary polyclonal rabbit antiserum against recombinant cuticular protein was then added (1/3000 dilution) and incubated for 1–2 h. The blot was then washed 3 times for 5 min each with TTBS, after which the blot was incubated in TTBS containing 1% dried milk or 1% BSA with a 1/3000 dilution of the secondary antibody, goat anti-rabbit IgG (H + L)-AP conjugate (Bio-Rad), for 1–4 h. The blot was then washed three times for 5 min with TTBS, and the protein was visualized by reaction of the enzyme bound to the secondary antibody with a chromophore using the AP Conjugate Substrate Kit (Bio-Rad). For immunoblots of proteins using primary antibody to the His tag, the same procedure was followed, except the blots were blocked and incubated with antisera in TTBS with BSA instead of dried milk protein. The primary antibody was α-pentaHis monoclonal antibody (Qiagen), and the blots were incubated with primary antibody (1/2000 dilution) overnight. The secondary antibody was goat anti-mouse IgG (H + L)-AP conjugate (Bio-Rad).

2.7. Circular dichroism spectroscopy of cuticular proteins

Samples of recombinant proteins with N-terminal histidine tags removed were prepared at 2 mg/ml except for MsCP36, which was 1 mg/ml, in 50 mM sodium phosphate buffer, pH 7, and loaded into a 0.02 cm path length cylindrical cell. The cell was analyzed in a Jasco J-720 spectropolarimeter at 10 nm/min and averaged over three runs. The CD signal was recorded as the mean molar elipticity per residue.

2.8. Cross-linking reactions

NADA, fungal laccase (*Pyricularia oryzae*), and mushroom tyrosinase were purchased from Sigma. NBAD was synthesized according to Yamasaki et al. (1990). NADA and NBAD concentrations were determined by measuring absorbance at 280 nm using an extinction coefficient for both of 2600/cm M. *M. sexta* recombinant laccase (MsLac2) was produced in a baculovirus expression system (N. Dittmer and M.R. Kanost, unpublished results). The parameters of the cross-linking reactions varied considerably between experiments and are described in the Results section and in the figures. Generally, a standard cross-linking reaction consisted of 4.5 μl of 12 mg/ml recombinant cuticular protein in 50 mM sodium phosphate, pH 7, 0.5 μl of 85 mM NADA or NBAD in 1 mM acetic acid, 0.5 μl of 12U/μl fungal laccase (1 unit produces ΔA_{530} of 0.001/min at pH 6.5, 30 °C with syringaldazine) in 50 mM sodium phosphate buffer, pH 7, and 0.5 μl protease inhibitor cocktail mixed in a 0.6 ml microcentrifuge tube. In controls in which one component was left out, the buffer for that component was substituted. After the reactants were added, the tubes were vortexed and then centrifuged briefly to collect liquid at the bottom of tubes. Reactions were carried out at 30 °C. In some experiments, immediately following the cross-linking reaction, the tubes were centrifuged at 16,000*g* for 10 min to separate soluble (S) and pellet (P) fractions. The appropriate concentration of SDS sample buffer was then added to equal volumes of each fraction and boiled for 4 min before centrifuging for 1 min. Equivalent fractions of S and P samples were then analyzed by SDS–PAGE.

3. Results

3.1. Expression of recombinant cuticular proteins

Full length cDNAs of MsCP36, MsCP27, and MsCP20 (Suderman et al., 2003) obtained by RT-PCR of RNA isolated from *M. sexta* pharate pupal integument using primers flanking the open reading frame were cloned and sequenced to check for errors. The coding regions for the mature proteins were then subcloned into the expression vector pProEXHTa. Recombinant proteins expressed in *E. coli* were recovered from the soluble fraction obtained after sonication of the bacteria and removal of cellular debris by centrifugation. The recombinant proteins contained an N-terminal 6-His tag, which was used to help purify the proteins to near homogeneity by affinity chromatography using Ni-NTA resin. After the His tag and adjoining linker sequence (Fig. 1) were removed by incubation with recombinant tobacco etch virus (TEV) protease, both the His tag and the TEV protease (which contains its own His tag) were separated from the cuticular protein by binding to Ni-NTA resin. Cleavage of the His tag and homogeneity of the cleaved protein preparations were confirmed by SDS–PAGE analysis (Fig. 2). No detectable uncleaved protein remained in the MsCP36, MsCP20, or MsCP27 preparations, which was confirmed by using anti-His tag monoclonal antibody (data not shown) and by amino-terminal sequencing of TEV-cleaved proteins. Although the theoretical masses of cleaved MsCP27, 20, and 36 are 17785, 18085, and 30386 Da, respectively, the mobilities of these proteins during SDS–PAGE were lower than expected when compared to the mobilities of molecular weight standard proteins (Fig. 2). To ensure that the recombinant proteins were indeed the correct size, the masses of cleaved MsCP20 and MsCP36 were verified by MALDI–TOF mass

```
MsCP36
                    MSYYHHH HHHDYDIPTT ENLYFQGAMDPEF
  1 DRLDNKYLPP RGNAGAGFGP GFGGAGPKGG GSGFGGAGSG
 41 FGGAGSGFGG AGSGFGGGAG GAFGHGGAGS GFGGQAGGYS
 81 GAGGAGGYSG GASGQYSGRG GAASADANAQ ILRLNNEVTA
121 EGFAYDFETS NGIRADAQGV ATNGVQSQGS FAYKGDDGQD
161 YSITYTADEN GFVPQGAHLP TPPPIPEEIL KSLEQNARDE
201 AAGIVDDGTY RGEGAGAGVA GGYSGAGGYS GGAGGAGGFG
241 AGGAGRAGGA GGFGTGGAGR AGGAGGFGAG GAGGAGGAGG
281 FGAGAGGFGG RGSGGFGGAA SRQYLAPNAG GRGSGSGNFN
321 AQTGYQY

MsCP20
                    MSYYHHH HHHDYDIPTT ENLYFQGAMD
  1 GRLEPQYLPP RPGNGFGGGA GSGGNAGLGG GAGGSGAGAG
 41 GFGGSGAGAY GGSGGSHGGG AQIPITSFEN VNNGDGSYHF
 81 SYETGNGISA QESGAPRAPG PEGLAVTAEG AFSYRTPDGQ
121 QIALTYTADE NGFHPLGPHL PTPPPIPEEI LRSIEFNRQN
161 PSSEGSYNGG NGGSGGSNGY HY

MsCP27
                    MSYYHHH HHHDYDIPTT ENLYFQGAMD
  1 AELPSRNYIP QDQGSGPYPG PHGGIGGDTG NRRPQQDAEK
 41 NSNVVKQEQE ISDSGNYHFG FETSNGIRAE EAGGPEQAQG
 81 GYSYKGDDGQ TYTLIYTSGE GGFKPQGEHL PVAPPTPEAI
121 LIALQQNERD EAAGIFDDGQ YHPENNGQSR PGASAGAGTG
161 SGYHY
```

Fig. 1. Amino acid sequences of recombinant MsCP36, MsCP20, and MsCP27. Histidine and lysine residues, which are the strongest nucleophiles in these proteins for cross-linking reactions at neutral pH, are shown highlighted in blue and green, respectively. Tyrosine residues are highlighted in yellow. Bold residues denote conserved residues of the R&R consensus sequence. Numbering begins with the first residue of the mature cuticular protein. The unnumbered vector-encoded sequence at the amino-terminus is cleaved by TEV protease at the position shown with arrowheads, leaving a few vector-encoded residues (underlined) in the purified recombinant proteins.

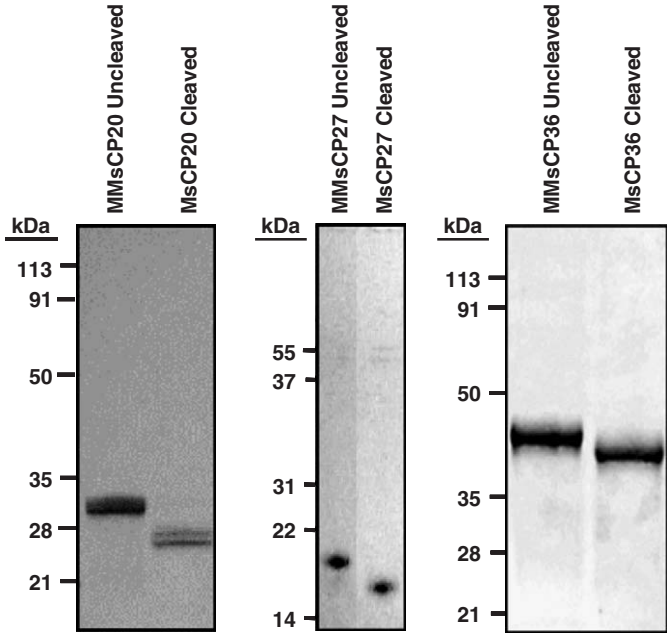

Fig. 2. Verification of TEV cleavage of the amino-terminal six-His tag from recombinant cuticular proteins. SDS–PAGE analysis of uncleaved and cleaved proteins (MsCP20, 1.3 μg; MsCP27, 1 μg; MsCP36 3 μg).

spectrometry (data not shown). Other cuticular proteins have been observed to exhibit abnormal electrophoretic behavior (Cox and Willis, 1987; Andersen et al., 1995).

3.2. Immunoblot analysis of native proteins in cuticle homogenates

The specificity of antisera raised against the recombinant cuticular proteins was analyzed by immunoblotting. Each antiserum recognized its respective recombinant protein antigen (Fig. 3A). MsCP20 and MsCP36 antisera were slightly cross-reactive. MsCP27 antiserum did not cross-react with MsCP20 or MsCP36. Antisera to MsCP20, 27, and 36 recognized proteins in pharate pupal integument homogenates, which were slightly larger than their theoretical sizes (Fig. 3B), but the masses were consistent with the apparent sizes observed when they were first

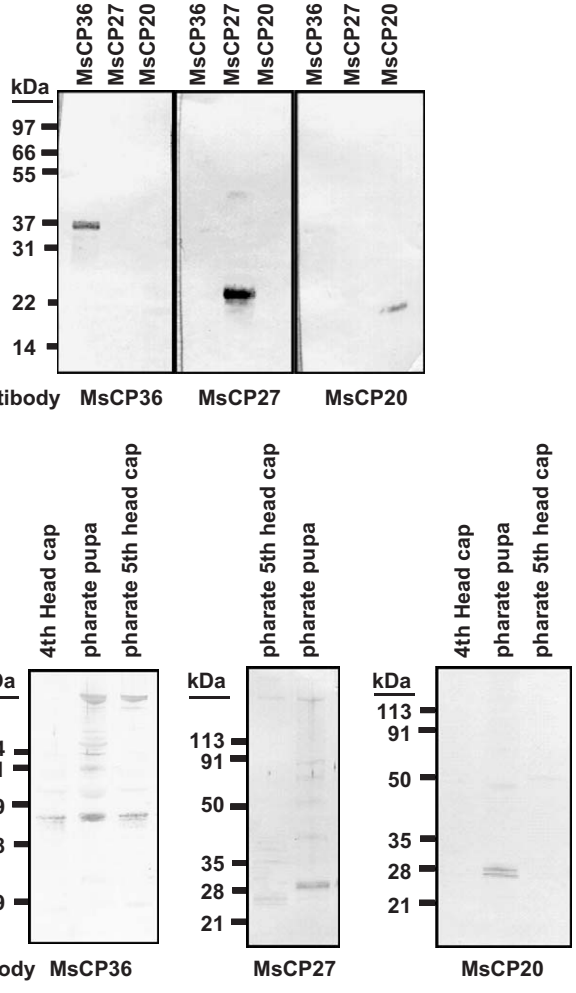

Fig. 3. Immunoblot analysis of cuticular proteins. (A) Specificity of antisera to recombinant cuticular proteins. Aliquots of 50 ng of each recombinant cuticular protein were separated on a 12% acrylamide gel and transferred to nitrocellulose. The blots were incubated in 1:3000 dilutions of the indicated primary antisera. (B) Detection of cuticular proteins in homogenates from *M. sexta* abdominal integument and head capsules. Proteins were detected using antisera to recombinant cuticular protein (1:3000 dilution).

purified from cuticle (Hopkins et al., 2000). MsCP36 was also detected in fourth and fifth instar head capsule homogenates. MsCP27 and MsCP36 antisera also labeled rather large proteins. These may represent cross-linked forms of these cuticular proteins or perhaps related proteins that cross-react with the antisera. In control experiments the preimmune sera were not immunoreactive with proteins in integumental homogenates (data not shown).

3.3. Secondary structure analysis of recombinant cuticular proteins

Recombinant MsCP36, 20, and 27 were analyzed by circular dichroism to obtain information about the nature of their secondary structures (Fig. 4). None of the proteins exhibited spectra characteristic of proteins with a high content of either α-helical or β-sheet secondary structure. Instead, their spectra indicated that the proteins are composed mostly of a random-coil conformation with some β-sheet. Spectra from random coil proteins are normally positive in absorbance between 215 and 250 nm, but the spectra of the cuticular proteins are negative from 215 to 250 nm, perhaps because the presence of β-sheet has decreased absorbance in this region. A minimum at just below 200 nm, which is usual for a protein with a random coil conformation, is shifted positively to 206 nm for MsCP20 and MsCP27, with both spectra increasing nearly to zero at approximately 200 nm. This spectral pattern may reflect the influence of some β-sheet sequences, which typically exhibit a positive peak at this wavelength. MsCP36, which contains 36% glycine, has a spectrum indicative of a larger percentage of random coil than either MsCP20 or MsCP27. The glycine-rich C-terminal region of MsCP36, which is substantially longer than those of MsCP20 and MsCP27 (Fig. 1), may lack any typical secondary structure.

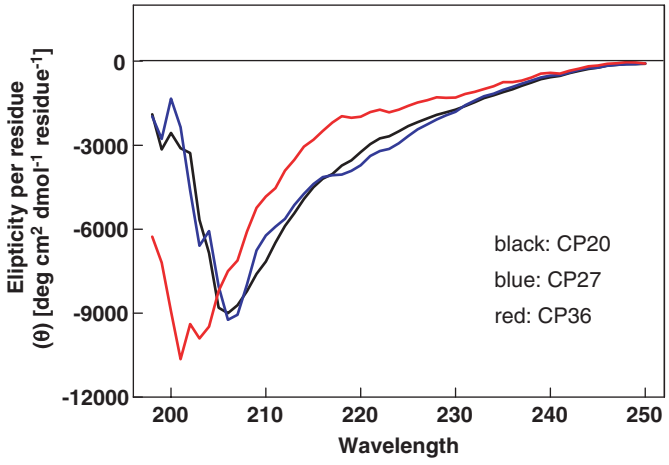

Fig. 4. Circular dichroism spectra of recombinant cuticular proteins. MsCP20 and 27 were analyzed at 2 mg/ml in 50 mM sodium phosphate buffer, pH 7, while MsCP36 was analyzed at 1 mg/ml in the same buffer. The spectra were corrected by subtracting the spectrum of the buffer alone and are presented as mean molar elipticity per residue.

3.4. Oxidative conjugation with catechols and cross-linking of recombinant cuticular protein

All three of the recombinant cuticular proteins were employed in reactions to investigate their cross-linking in the presence of catechols and laccase. MsCP36, which was observed in integument samples in a high molecular weight form speculated to represent cross-linked material (Fig. 3), was selected for more detailed study. Upon long-term storage of MsCP36, oligomers slowly accumulated spontaneously (Fig. 5A). Reaction of MsCP36 with NBAD and laccase resulted in rapid cross-linking of the protein and the formation of oligomers that appeared as a ladder of bands extending to very high molecular weight aggregates or polymers that did not migrate into the SDS–PAGE gel (Fig. 5A). The oligomers had apparent molecular masses consistent with the formation of the dimer, trimer, tetramer, etc. of the CP. Such cross-linking was not observed in the absence of either NBAD or laccase. These products were stable in boiling SDS (Fig. 5A) and in 8 M urea (data not shown) under reducing conditions, which

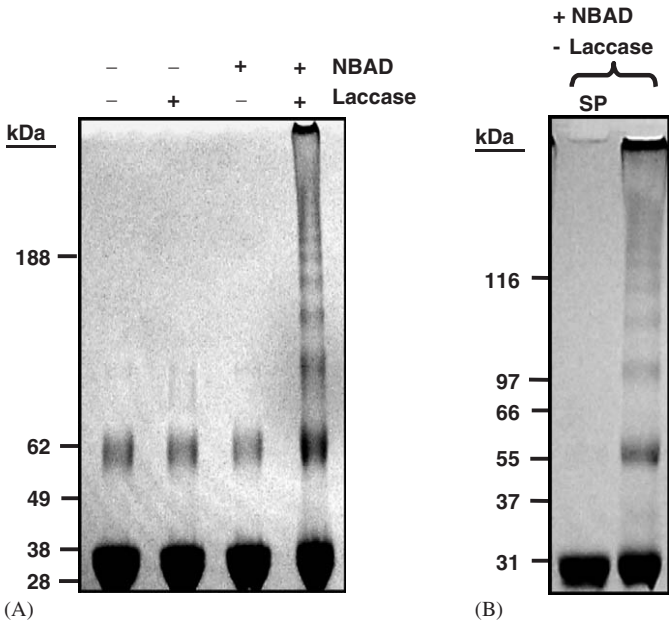

Fig. 5. Analysis of MsCP36 cross-linking reaction products. MsCP36 was incubated for 1 h at 30 °C in the presence or absence of NBAD and fungal laccase, indicated by (+) and (−), and the products were then analyzed by SDS–PAGE. (A) Requirement for catechol and laccase at pH 7. When a component was omitted, an equal volume of the component's buffer was added to maintain a constant reaction volume. Reaction conditions: [MsCP36] = 3.9 μg/μl, [NBAD]: [MsCP36] = 56:1 (molar ratio), [fungal laccase] = 1 U/μl, reaction volume = 6 μl, reaction buffer: 50 mM sodium phosphate, pH 7. An MsCP36 dimer, which formed slowly and spontaneously during storage, is visible at ~65 kDa in all lanes. (B) Cross-linking in the absence of laccase at alkaline pH. After the reaction, soluble (S) and pellet (P) fractions were separated by centrifugation. Both fractions were diluted with SDS sample buffer to the same volume and boiled for 5 min. Equal aliquots were then applied to the gel. Reaction conditions: 1 h at 30 °C, [MsCP36] = 9 μg/μl, [NBAD]: [MsCP36] = 27:1 (molar ratio), reaction volume = 6 μl, reaction buffer: 50 mM sodium bicarbonate, pH 8.3.

suggests that the cross-links formed were covalent. When the reaction was carried out at a pH greater than 8, cross-linking occurred to about the same extent even without the addition of laccase, which is most likely due to the spontaneous auto-oxidation of the *N*-acylcatecholamine (Fig. 5B). When the reaction products were separated by centrifugation into soluble (S) and insoluble (P) fractions prior to analysis by SDS–PAGE, we observed that both monomeric and oligomeric proteins became insoluble as the reaction proceeded until there were no oligomers remaining in the soluble fraction. Apparently, when MsCP36 is oxidatively conjugated with catechols, a process denoted as catecholation, and before any oligomers have formed, the catecholated monomeric protein has already become very hydrophobic and insoluble.

A comparison of the three recombinant cuticular proteins in the cross-linking reaction demonstrated that MsCP36 became cross-linked to a much greater degree than either MsCP20 or MsCP27 (Fig. 6). In addition, in contrast to MsCP36 and MsCP20, products from the oxidative conjugation with NBAD and cross-linking of MsCP27 were completely soluble. This result indicated that the type of protein used for catecholation and cross-linking influences the solubility of the products and perhaps the type of cross-linking reaction that occurs.

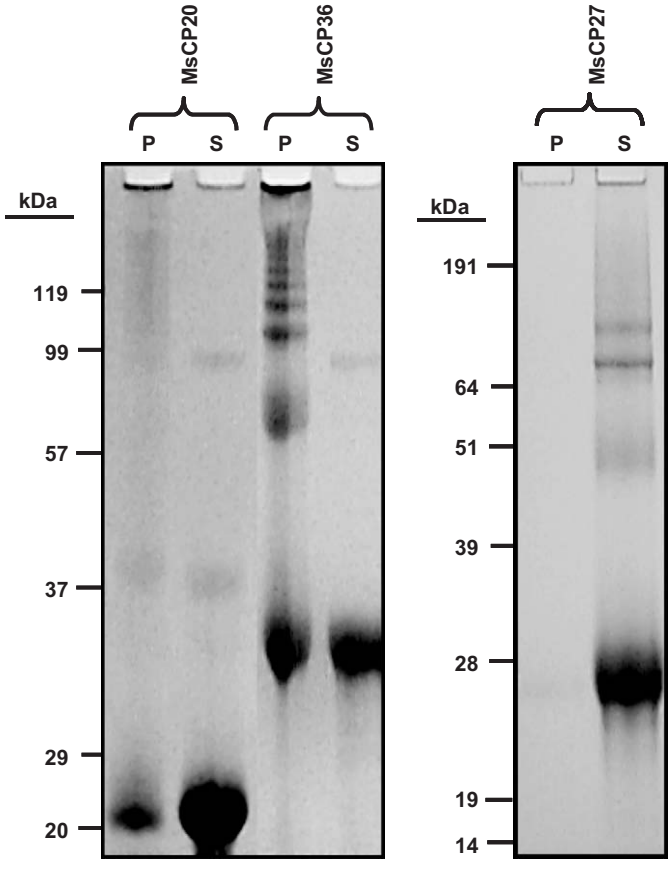

Fig. 6. Comparison of oligomer/polymer formation by MsCP36, MsCP27, and MsCP20. After reaction of MsCP36, MsCP27, or MsCP20 (each at 3.9 μg/μl) with a 56-fold molar excess of NBAD and fungal laccase (1 U/μl) for 1 h at 30 °C in 50 mM sodium phosphate, pH 7, the soluble (S) and pellet (P) fractions were recovered after centrifugation. After separation both fractions were diluted with SDS sample buffer to the same volume and boiled for 5 min before analysis of by SDS–PAGE.

A series of experiments was carried out to identify optimal conditions for cross-linking reaction of MsCP36 (Fig. 7). When the concentration of MsCP36 was varied, formation of oligomers visible by SDS–PAGE was apparent at MsCP36 concentrations of 5–43 mg/ml, but was optimal when the concentration was 9 mg/ml (0.3 mM) (Fig. 7A). At every protein concentration tested, the oligomers were present only in the pellet obtained after centrifugation, indicating that oligomer formation was accompanied by a loss of solubility. When the NBAD concentration was increased ten-fold in the reaction containing the highest MsCP36 concentration (43 mg/ml), there was a marked decrease in the amount of monomer that remained in the supernatant, and the pellet formed a stringy mass. Analysis of this pellet fraction by SDS–PAGE indicated that it contained mostly monomer with only a small amount of oligomers. It appears that the high concentrations of protein and NBAD led primarily to formation of catecholated monomer, which was insoluble and did not cross-link efficiently. The possibility remains that some very large protein polymers also formed, which were undetected because they are too large to enter into the stacking gel.

To test the effect of the molar ratio of NBAD to MsCP36 on cross-linking, the reactions were carried out at the optimal protein concentration of 9 mg/ml in the presence of varying concentrations of NBAD (Fig. 7B). The formation of oligomers detectable by SDS–PAGE analysis was optimal when the NBAD concentration was 7.4 mM, a molar ratio of NBAD to MsCP36 of approximately 25:1. A precipitate formed at the higher concentrations of NBAD that could possibly be an uncharacterized NBAD polymer rather than protein, as most of the protein remained as the monomeric form.

Formation of insoluble oligomers of MsCP36 increased with reaction time (Fig. 7C). The amount of catecholated monomer in the pellet fraction also increased from 0.5 to 2 h but then decreased as the amount of protein present as oligomers and very high molecular weight polymers increased steadily up to 8 h. By 24 h, hardly any monomer remained, and the products were too large to analyze by SDS–PAGE. The 24 h pellet fraction was a highly viscous gel that could not be pipetted onto the SDS–PAGE gel even after boiling in SDS sample buffer. These results are consistent with a two-step, biphasic reaction, in which the protein first becomes insoluble by reaction with oxidized *N*-acylcatecholamines (catecholation), which then undergoes cross-linking to produce oligomers and finally polymers. Reaction 8C was carried out for 8 h with no protein present to test whether the oligomerized material was indeed composed of MsCP36 and not just polymerized catecholamine. In this reaction, laccase catalyzed conversion of NBAD to an orange product, which precipitated. This material was soluble in boiling SDS, but it did not stain with Coomassie blue after SDS–PAGE.

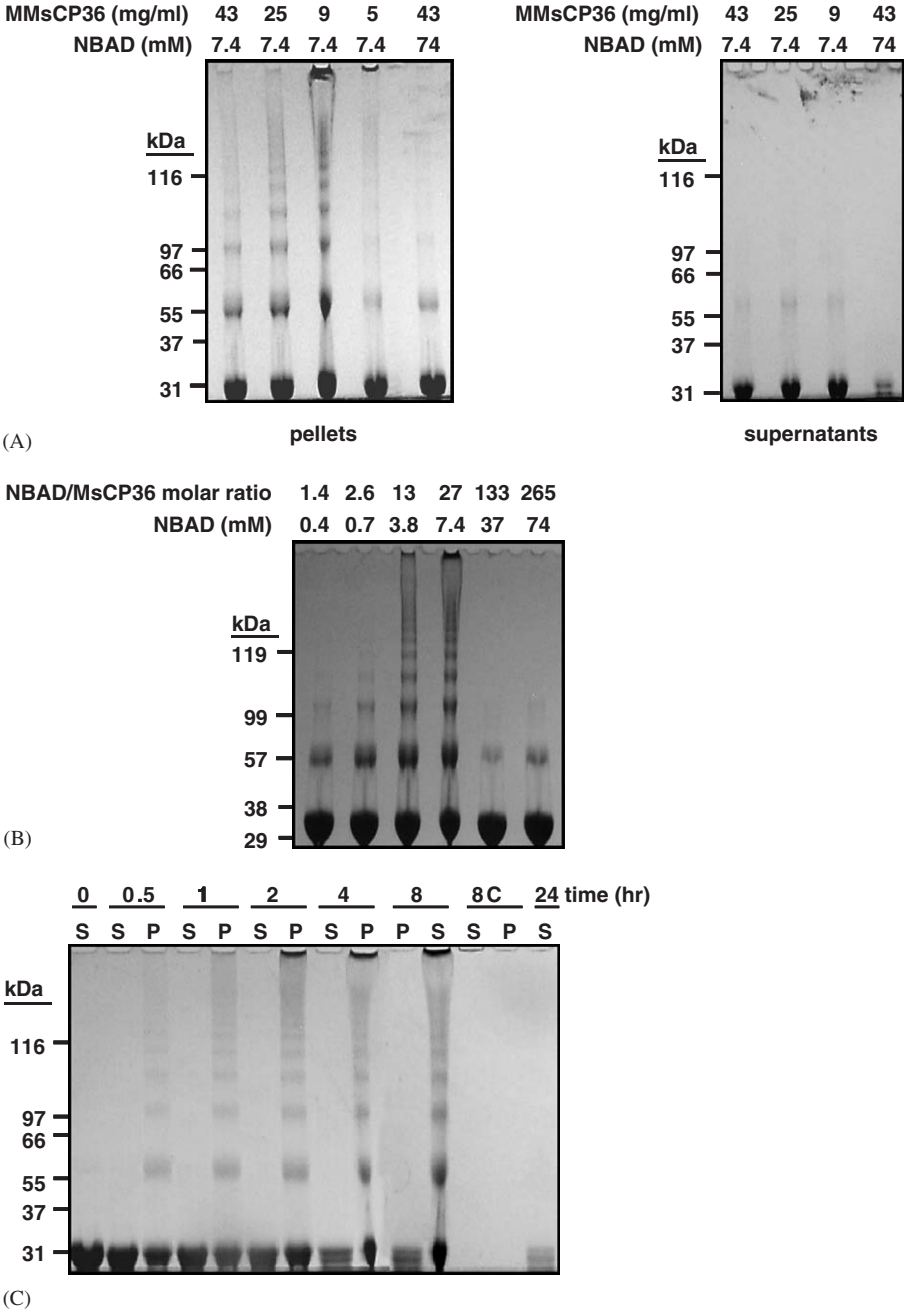

Fig. 7. Effect of reactant concentrations and reaction time on MsCP36 cross-linking. (A) Effect of MsCP36 concentration on cross-linking. After reaction for 2 h at 30 °C, the samples were centrifuged, and the supernatants were removed from any insoluble material. For each sample, the volume of supernatant that contained 25 μg MsCP36 protein in the original reaction mixture was analyzed by SDS–PAGE (gel marked "supernatants"), which represents the soluble protein remaining after the reaction. The pellets were resuspended to the original reaction volume with SDS sample loading buffer, vortexed, boiled, and centrifuged again, and from the supernatant the volume equivalent to 25 μg protein in the initial reaction mixture was loaded on the gel labeled "pellets." The reactions were carried out with the concentrations of MsCP36 indicated for each lane and fungal laccase at 1 U/μl in 50 mM sodium phosphate, pH 7. Positions of molecular weight markers are indicated at the left of each gel. (B) Effect of NBAD concentration on MsCP36 cross-linking. Reactions were carried out for 2 h at 30 °C in 50 mM sodium phosphate, pH 7 containing 9 μg/μl MsCP36, 1 U/μl fungal laccase, and varying concentrations of NBAD as indicated above each lane. Reaction products (without separation by centrifugation) were analyzed by SDS–PAGE. (C) Time dependence of the cross-linking reaction. After different reaction times, the soluble (S) and pellet (P) fractions were separated by centrifugation, and both fractions were diluted with SDS sample buffer to the same volume and boiled for 5 min. Half of each sample was then analyzed by SDS–PAGE. Reaction 8C is a control that contained no MsCP36. The pellet of the 24 h reaction could not be resuspended or pipetted due to high viscosity. Reactions were carried out at 30 °C with 9 μg/μl MsCP36, 7 mM NBAD, 1 U/μl fungal laccase in 50 mM sodium phosphate, pH 7.

NBAD and NADA differed in their properties as conjugating and cross-linking agent precursors. During incubation for 1–2 h, reactions involving NBAD resulted in a higher degree of MsCP36 cross-linking to form oligomers and polymers than did reactions utilizing NADA (Fig. 8). Furthermore, MsCP36 that was oligomerized in the

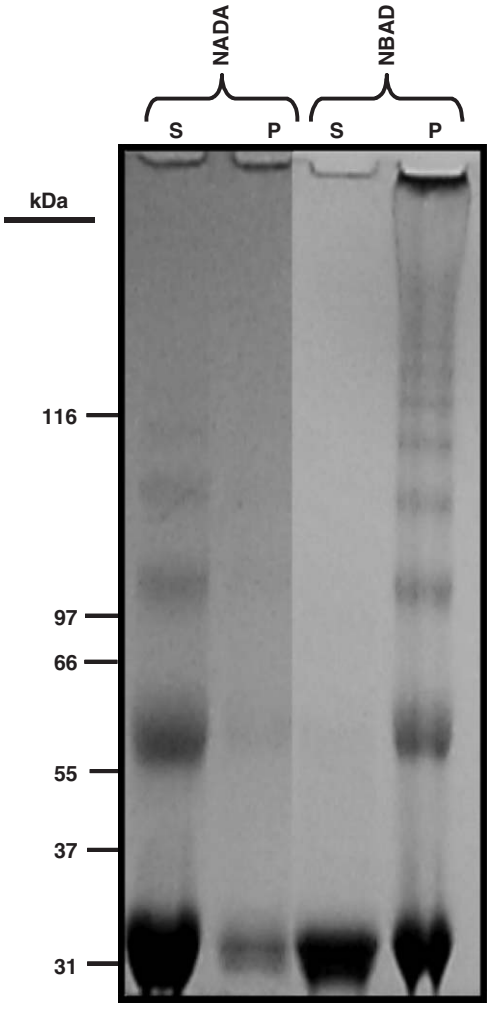

Fig. 8. Comparison of MsCP36 cross-linking by laccase oxidation products of NBAD and NADA. Reactions were carried out in 50 mM sodium phosphate, pH 7 for 2 h at 30 °C with 9 μg/μl MsCP36, 1 U/μl fungal laccase and either 7.4 mM NBAD or 7.4 mM NADA. The soluble (S) and pellet (P) fractions were separated by centrifugation, and both fractions were diluted with SDS sample buffer to the same volume and boiled for 5 min. Half of each sample was then analyzed by SDS–PAGE.

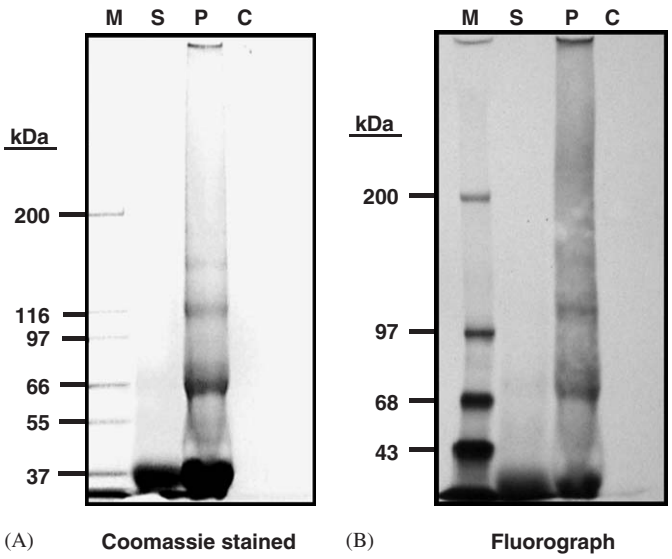

Fig. 9. Reaction of [^{14}C]-NADA oxidation products with MsCP36. The cross-linking reaction was carried out using NADA labeled with [^{14}C] at the carbonyl carbon. Reactions were carried out for 7 h at 30 °C with 5.4 μg/μl MsCP36, 5 mM [^{14}C]-NADA, 1 U/μl fungal laccase in 50 mM sodium phosphate, pH 7. The soluble (S) and pellet (P) fractions were separated by centrifugation, and both fractions were diluted to the same volume with SDS sample buffer and boiled for 5 min. The samples were then analyzed by SDS–PAGE in duplicate gels followed by either staining with Coomassie blue (A) or detection of ^{14}C by fluorography (B). A control reaction included laccase and [^{14}C]-NADA but no MsCP36 (lane C).

reaction containing NADA remained soluble in the supernatant, whereas all of the oligomerized MsCP36 and some of the monomer became insoluble during reaction with NBAD and laccase. However, with longer reaction times with laccase and NADA, MsCP36 also formed very high molecular weight polymers and became insoluble (Fig. 9A). Apparently, the type of catechol used for conjugation and cross-linking affects the solubility of the products and perhaps also the type of conjugating and cross-linking reactions that occur.

To test whether the *N*-acylcatecholamine was covalently bound to the protein during conjugation and cross-linking, [^{14}C]-labeled NADA (labeled in the carbonyl carbon) was incorporated into the cuticle sclerotization model reaction. Analysis of the products by SDS–PAGE and fluorography indicated that radioactivity was incorporated into protein bands representing the MsCP36 monomer and a ladder of bands that reached the sample well (Fig. 9). This result indicated that during the reaction, NADA became covalently bound to the cuticular protein monomer and was also present in the oligomers and polymers.

To investigate whether the catecholation, oligomerization and polymerization of MsCP36 were due to an oxidative mechanism, the reducing agent dithiothreitol (DTT) was included in the cross-linking reactions. The formation of oligomers visible by SDS–PAGE was decreased to control levels in the presence of 100 mM DTT and nearly to control levels with 10 mM DTT (Fig. 10). Therefore, DTT effectively prevented MsCP36 from cross-linking in the presence of NBAD and laccase, presumably by its ability to reduce the quinones and quinone methides back to catechols before the former could react with nucleophiles in the protein. DTT might also serve as a trapping agent and compete with the nucleophilic side chains of MsCP36 for forming covalent adducts to the quinones and quinone methides (Huang et al., 1998).

Tyrosinase was substituted for laccase as the phenoloxidase in the cross-linking reaction to determine the effect of a different oxidative enzyme on MsCP36 catecholation and cross-linking (Fig. 11A). Prior to adding the tyrosinase to the reaction mixture, its NBAD-oxidizing activity was adjusted to be the same as the fungal laccase used to catalyze the cross-linking reactions. The reaction utilizing tyrosinase did cross-link MsCP36, although not to the same extent as the reaction using fungal laccase (compare lane 3 with lane 2, Fig. 11A). The laccase reaction also

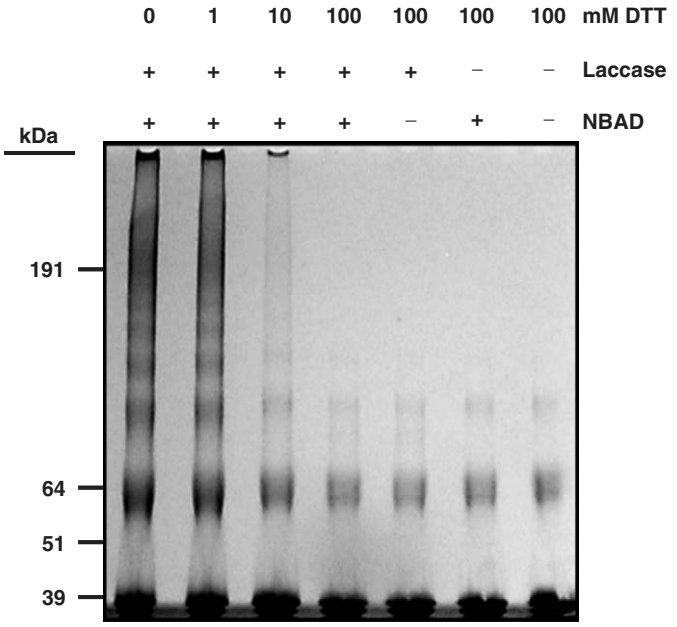

Fig. 10. Inhibition of cross-linking reaction of MsCP36 by dithiothreitol (DTT). Reactions were carried out at indicated concentrations of DTT and in the presence (+) or absence (−) of fungal laccase and NBAD. Reaction mixtures were analyzed by SDS–PAGE. Reactions were carried out for 1.5 h at 30 °C with 8.3 μg/μl MsCP36, 7 mM NBAD, 1 U/μl fungal laccase in 0.1 M sodium bicarbonate, pH 8.3 and DTT at the indicated concentrations. An MsCP36 dimer, which formed slowly and spontaneously during storage, is visible at ~65 kDa in all lanes.

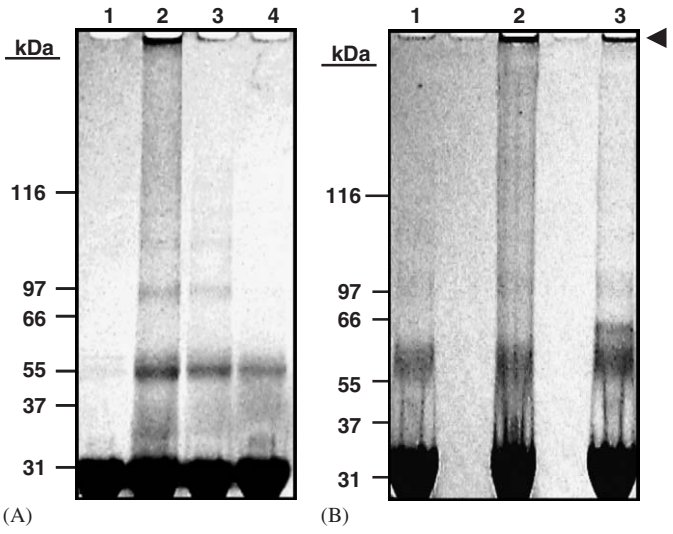

Fig. 11. Comparison of MsCP36 cross-linking in reactions containing fungal laccase, mushroom tyrosinase, and recombinant *M. sexta* laccase. (A) Analysis of tyrosinase-induced cross-linking of MsCP36. Lane 1, MsCP36 and NBAD; Lane 2, MsCP36, NBAD, and fungal laccase; Lane 3, MsCP36, NBAD, and tyrosinase; Lane 4, MsCP36 and tyrosinase. Reactions were carried out for 4 h at 30 °C with 7 μg/μl MsCP36, 10 mM NBAD, and 1.3 U/μl fungal laccase or tyrosinase in 50 mM sodium phosphate, pH 7. (B) Cross-linking of MsCP36 by recombinant *M. sexta* laccase-2 (MsLac2). Lane 1, MsCP36 and NBAD; Lane 2, MsCP36, NBAD, and fungal laccase; Lane 3, MsCP36, NBAD, and MsLac2. An arrowhead denotes protein that barely entered the gel after MsCP36 was incubated with NBAD and either fungal or insect laccase. The reactions were carried out for 24 h at 30 °C with 3.15 μg/μl MsCP36, 7.4 mM NBAD, and 1.0 U/μl fungal laccase or 70 ng/μl MsLac2 in 50 mM sodium phosphate, pH 7 containing 10% glycerol and 0.5 μl protease inhibitor cocktail.

produced very large polymers, which were not detected in the tyrosinase-catalyzed reaction. Laccase, therefore, was more effective than tyrosinase for cross-linking CPs.

We compared recombinant *M. sexta* laccase 2 (MsLac2) obtained by using a baculovirus-insect cell line protein expression system in our laboratory with fungal laccase to test whether insect laccase would also function to conjugate and polymerize MsCP36. MsLac2 induced oligomerization/polymerization of MsCP36, as indicated by the appearance of a ladder of oligomers and also polymers that would not migrate into the SDS–PAGE gel (Fig. 11B).

4. Discussion

We report here the use of recombinant insect cuticular proteins for model reactions to investigate mechanisms of protein modification that occur during sclerotization. cDNAs for the three most abundant proteins that could be extracted from *M. sexta* pharate pupal cuticle (Suderman et al., 2003) were cloned in an expression vector that includes sequence encoding an amino-terminal 6-His tag for purification of the recombinant proteins by nickel-affinity chromatography. After purification, the 6-His tags were removed through cleavage by a specific protease. The recombinant proteins were soluble and were recovered in good yield with high purity.

The CD spectra of the recombinant *M. sexta* CPs indicated that they lack a high proportion of typical secondary structure but that they may contain some β-sheet structure. The CD spectra of recombinant MsCP20 and MsCP27 are similar to the spectrum of a mixture of cuticular proteins extracted from flexible abdominal cuticle of *Hyalophora cecropia* (Iconomidou et al., 2001). This result suggests that the recombinant *M. sexta* proteins are folded in a manner similar to the natural proteins. The CD spectrum of MsCP36 indicates that it has a higher proportion of random coil than MsCP20 and MsCP27. This is reasonable, considering the high content of glycine in MsCP36. Analysis of intact cuticle and homogenates from *H. cecropia* flexible abdominal cuticle by several spectroscopic techniques indicated that the proteins in this cuticle consisted of predominantly antiparallel β-sheets (Iconomidou et al., 2001). Computer modeling has been used to envision hypothetical antiparallel β-sheet folds for cuticular proteins and to predict how such structures might form chitin-binding sites (Hamodrakas et al., 2002; Iconomidou et al., 2005). A thorough understanding of the secondary and tertiary structure of proteins from this family awaits future experimental structural determination by X-ray crystallography or NMR techniques.

MsCP36, which contains the RR1-type of motif that is usually found in proteins from soft pliable cuticle, was detected by immunoblotting in very sclerotized larval head

capsules. This observation is apparently the first report of an RR1-type protein being present in a very hard cuticle and indicates that both RR1- and RR2-types of proteins may occur in various types of cuticle and function perhaps by interacting with cuticular components in different ways.

We developed and optimized an in vitro sclerotization model using biologically relevant components, a recombinant cuticular protein, MsCP36, the *N*-acylcatecholamines, NBAD and NADA, which are present in *M. sexta* pharate pupal cuticle when sclerotization begins, and either a fungal or an insect laccase. MsCP36 mRNA and extractable protein increased to maximal levels in the pharate pupa (Hopkins et al., 2000; Suderman et al., 2003). After ecdysis, the amount of extractable MsCP36 from the sclerotizing cuticle decreased, with no MsCP36 extractable after 48 h (Hopkins et al., 2000). MsCP36 is very probably catecholated, cross-linked and polymerized during sclerotization of the pupal cuticle. Therefore, as the cuticle sclerotizes, MsCP36 becomes hydrophobic and inextractable, presumably by becoming conjugated with catechols and polymerized or being covalently bound and/or cross-linked to other cuticular components. The *N*-acylcatecholamines, NBAD, and to a lesser degree, NADA, are present in *Manduca* pupal cuticle during this time, with NBAD steadily increasing to maximal levels over several days as the cuticle becomes dark brown, hard, dehydrated and hydrophobic (Hopkins et al., 1984). Laccase transcripts (MsLac2) were shown to be upregulated in *Manduca* pharate pupal epidermis, suggesting that this enzyme is involved in sclerotization (Dittmer et al., 2004). Laccase has been extracted from pupal cuticles of *M. sexta* undergoing tanning (Thomas et al., 1989; N. Dittmer, unpublished data). Because NBAD and NADA are substrates for this enzyme and also are present in the cuticle, it is reasonable to assume that laccase would oxidize NBAD and NADA to their quinone or *p*-quinone-methide derivatives. Fungal laccase was utilized in establishing conditions for the model reaction experiments because it is well characterized and was commercially available in large amount. Results of similar experiments conducted using recombinant *M. sexta* laccase 2 (MsLac2) were comparable to those observed with fungal laccase.

Whereas all three of the recombinant proteins were demonstrated to form oligomeric and polymeric forms upon incubation with laccase and an *N*-acylcatecholamine, MsCP36 was cross-linked to a greater extent than either MsCP20 or MsCP27. This is somewhat surprising given that this protein contains only two histidine residues, whereas MsCP20 and 27 each contain five (Fig. 1). Nitrogens in the histidyl imidazole group are the strongest amino acid nucleophiles present in cuticular proteins at physiological pH; indeed, they have been identified as being bound to catecholamines in insect cuticle (Schaefer et al., 1987; Okot-Kotber et al., 1994, 1996; Xu et al., 1997; Kerwin et al., 1999). The nucleophilic side chains of Lys residues have also been postulated to form covalent linkages to *N*-acylcatecholamines (Schaefer, et al., 1987; Sugumaran et al., 1987). However, MsCP20, which contains no Lys residues, cross-links to approximately the same extent as MsCP27, which contains 4 lysines. It is interesting to note that MsCP36 contains 14 Tyr residues, while MsCP20 and MsCP27 contain 9 and 8, respectively. Sugumaran et al. (1982) reported that the residue left after extraction of unsclerotized flesh fly larval cuticle contains dityrosine in a peptide linkage, and that the dityrosine disappeared during puparial sclerotization. The presence of dityrosine, therefore, indicates that the larval cuticle of *Sarcophaga bullata* probably contains the rubber-like protein resilin in some regions, as first described by Andersen (1964), and it would accordingly not be involved in sclerotization.

Both an *N*-acylcatecholamine (NBAD) and laccase were required for oligomerization and polymerization of MsCP36 at physiological pH. However, oligomerization also occurred when the reaction was carried out at a higher pH (pH 8.3) in the absence of laccase. This result is probably due to the spontaneous oxidation of the *N*-acylcatecholamines at alkaline pH. Such oxidation was evident by the color change of the reaction mixture to orange, which also occurred at lower pH in the presence of laccase (data not shown). The reducing agent DTT effectively inhibited cross-linking when present in excess of NBAD, indicating that oxidation of the *N*-acylcatecholamine is necessary for oligomerization and polymerization of MsCP36.

The extent of protein oligomerization and polymerization was optimized by adjusting the concentration of MsCP36 in the reaction to 0.3 mM and adjusting the NBAD concentration to 7.4 mM, with the molar ratio of NBAD to MsCP36 of approximately 25:1 (Fig. 7B). Under these conditions the proportion of protein oligomerized and polymerized was maximized and the proportion remaining as monomer was minimized. At very high concentrations of NBAD (148 mM), the products formed an insoluble mass even in the absence of protein (data not shown). Therefore, that product may consist of polymeric *N*-acylcatecholamines, which were not detected by SDS–PAGE analysis.

As the cross-linking reaction proceeded, the monomeric protein became insoluble and began to form oligomers. Over time the soluble fraction was characterized by decreasing amounts of monomer and soluble oligomers. The insoluble fraction, however, exhibited a steadily increasing amount of oligomeric and polymeric proteins as well as the monomer. The proportion of monomer in the insoluble fraction peaked and then began to decline as more of the protein in this fraction became polymerized, an increasing proportion of which did not migrate into the SDS–PAGE gel. After 8 h, most of the protein became insoluble and the amount of protein that was solubilized by boiling in SDS sample buffer had declined significantly. At this point the protein had become polymerized to such a degree that the viscosity of the solution was too high to permit pipetting, even when suspended in boiling SDS sample buffer.

The insoluble product(s) formed to a greater extent and more quickly in the presence of NBAD than with NADA; however, both *N*-acylcatecholamines rendered the protein insoluble over time. Oligomers and polymers formed by cross-linking with NADA were generally more soluble than those formed by NBAD, a result that has been observed previously when using insect plasma proteins in similar cross-linking model reactions (Grün and Peter, 1983). MsCP27 formed oligomers that remained in the soluble fraction when cross-linked with NBAD, indicating that the degree of catecholation and cross-linking of individual cuticular proteins may differ during sclerotization, depending on differences in their sequence and tertiary structure.

The bonds forming the oligomers and polymers during the cross-linking reactions are very probably covalent, as disruption of hydrophobic interactions by the strong chaotropic agent, urea, had no effect on their stability, nor did extensive boiling in a solution of SDS. However, the nature of the covalent bonds is unknown, which will be the focus of a future investigation. MsCP36 also forms oligomers and polymers slowly and spontaneously during long-term storage and the nature of that bonding is also unknown.

Recently, laccase, not tyrosinase, was established as the phenoloxidase catalyzing cuticle tanning in the red flour beetle, *Tribolium castaneum* (Arakane et al., 2005). The present study is the first according to our knowledge to use laccase, a multicopper oxidase that has a very interesting enzymology (Mayer and Staples, 2002), as the phenoloxidase in an in vitro model system for quinone tanning of insect cuticle. Substitution of mushroom tyrosinase for laccase in the cross-linking reaction resulted in oligomerization of MsCP36 as well but to a lesser degree than that obtained with the same level of activity of fungal laccase. Several other studies have also used mushroom tyrosinase to oxidize different catechols for polymerizing proteins such as arylphorin, chicken lysozyme, ribonuclease, cytochrome c and bovine serum albumin (Grün and Peter, 1983; Sugumaran et al., 1987). Whereas tyrosinase produces *o*-quinones from *N*-acylcatecholamines, laccase produces these as well as *p*-quinone methides. Furthermore, tyrosinase oxidizes its substrates two electrons at a time and therefore produces no free radical intermediates. Laccase, on the other hand, oxidizes its substrates one electron at a time and does produce free radical intermediates (Thurston, 1994). It is possible that quinone methides, free radical intermediates and other unknown electrophilic products generated by the laccase-catalyzed reactions induced the higher degree of polymerization of MsCP36 relative to the tyrosinase-catalyzed reaction.

In summary, our results support the hypothesis that protein cross-linking during insect cuticle sclerotization consists of three processes: catecholation, oligomerization and polymerization of cuticular protein. We have shown that *N*-acylcatecholamines covalently bind to cuticular proteins (catecholation) upon oxidation by laccase, which causes some of the modified proteins to become insoluble. Concurrently, modified MsCP36 undergoes cross-linking, forming oligomers (oligomerization) and subsequently polymers (polymerization) that eventually become cross-linked to such a degree that the product becomes a viscous gel-like material with the cross-linked protein rendered insoluble even in chaotropic agents and detergents. The structures of the protein–catechol–protein cross-links are unknown, but research is currently in progress to identify the residues and types of bonding involved in these cross-links. The oxidized *N*-acylcatecholamines may also react with chitin, resulting in chitin–protein cross-links that further stabilize the cuticle. Our long-term goal is to obtain enough of the polymerized products from these model cuticle reactions so that we can evaluate their biomechanical properties for direct comparison with properties of the insect cuticle itself and other insect cuticle biomimetic materials (Miessner et al., 2001).

Acknowledgments

We thank Judith Willis and Stevin Gehrke for helpful comments on the manuscript. Support from the US National Science Foundation (MCB-0236039) is gratefully acknowledged. This is a cooperative investigation between Kansas State University (Contribution no. 05-216-J from the Kansas Agricultural Experiment Station) and the USDA. All programs and services of the USDA are offered on a nondiscriminatory basis without regard to race, color, national origin, religion, sex, age, marital status or handicap. Mention of a proprietary product does not constitute a recommendation by the USDA. We thank Judith Willis, Svend Andersen, and Stevin Gehrke for helpful comments on the manuscript.

References

Andersen, S.O., 1964. The cross-links in resilin identified as dityrosine and trityrosine. Biochim. Biophys. Acta 93, 213–215.

Andersen, S.O., 1985. Sclerotization and tanning of the cuticle. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 3. Pergamon Press, Oxford, pp. 59–74.

Andersen, S.O., 2005. Cuticular sclerotization and tanning. In: Gilbert, L.I., Iatrou, K., Gill, S. (Eds.), Comprehensive Molecular Insect Science, vol. 4. Elsevier Press, Oxford, UK, pp. 145–170 (Chapter 4).

Andersen, S.O., Højrup, P., Roepstorff, P., 1995. Insect cuticular proteins. Insect Biochem. Mol. Biol. 25, 153–176.

Arakane, Y., Muthukrishnan, S., Beeman, R.W., Kanost, M.R., Kramer, K.J., 2005. Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning. Proc. Natl. Acad. Sci. USA 102, 11337–11342.

Cox, D.L., Willis, J.H., 1987. Analysis of the cuticular proteins of *Hyalophora cecropia* with two-dimensional electrophoresis. Insect Biochem. 17, 457–468.

Dittmer, N.T., Suderman, R.J., Jiang, H., Zhu, Y., Gorman, M.J., Kramer, K.J., Kanost, M.R., 2004. Characterization of cDNAs encoding putative laccase-like multicopper oxidases and developmental expression in the tobacco hornworm, *Manduca sexta*, and the malaria mosquito, *Anopheles gambiae*. Insect Biochem. Mol. Biol. 34, 29–41.

Grün, L., Peter, M.G., 1983. Selective cross-linking of tyrosine-rich larval serum proteins and of soluble *Manduca sexta* cuticle proteins by

nascent *N*-acetyldopamine quinone and *N*-β-alanyldopamine quinone. In: Scheller, K. (Ed.), The Larval Serum Proteins of Insects. Thieme Verlag, Stuttgart, pp. 102–115.

Hamodrakas, S.J., Willis, J.H., Iconomidou, V.A., 2002. A structural model of the chitin-binding domain of cuticle proteins. Insect Biochem. Mol. Biol. 32, 1577–1583.

Hasson, C., Sugumaran, M., 1987. Protein cross-linking by peroxidase: possible mechanism for sclerotization of insect cuticle. Arch. Insect Biochem. Physiol. 5, 13–28.

Hopkins, T.L., Kramer, K.J., 1992. Insect cuticle sclerotization. Annu. Rev. Entomol. 37, 273–302.

Hopkins, T.L., Morgan, T.D., Kramer, K.J., 1984. Catecholamines in haemolymph and cuticle during larval, pupal and adult development of *Manduca sexta* (L.). Insect Biochem. 14, 533–540.

Hopkins, T.L., Krchma, L.J., Ahmad, S.A., Kramer, K.J., 2000. Pupal cuticle proteins of *Manduca sexta*: characterization and profiles during sclerotization. Insect Biochem. Mol. Biol. 30, 19–27.

Huang, X., Xu, R., Hawley, M.D., Hopkins, T.L., Kramer, K.J., 1998. Electrochemical oxidation of *N*-acyldopamines and regioselective reactions of their quinones with *N*-acetylcysteine and thiourea. Arch. Biochem. Biophys. 352, 19–30.

Iconomidou, V.A., Chryssikos, G.D., Gionis, V., Willis, J.H., Hamodrakas, S.J., 2001. "Soft"-cuticle protein secondary structure as revealed by FT-Raman, ATR FT-IR and CD spectroscopy. Insect Biochem. Mol. Biol. 31, 877–885.

Iconomidou, V.A., Willis, J.H., Hamodrakas, S.J., 2005. Unique features of the structural model of 'hard' cuticle proteins: implications for chitin-protein interactions and cross-linking in cuticle. Insect Biochem. Mol. Biol. 35, 553–560.

Kerwin, J.L., Turecek, F., Xu, R., Kramer, K.J., Hopkins, T.L., Gatlin, C.L., Yates, J.R., 1999. Mass spectrometric analysis of catechol-histidine adducts from insect cuticle. Anal. Biochem. 268, 229–237.

Kramer, K.J., Kanost, M.R., Hopkins, T.L., Jiang, H., Zhu, Y., Xu, R., Kerwin, J.L., Turecek, F., 2001. Oxidative conjugation of catechols with proteins in insect skeletal systems. Tetrahedron 57, 385–392.

Mayer, A.M., Staples, R.C., 2002. Laccase: new functions for an old enzyme. Phytochem 60, 551–565.

Miessner, M., Peter, M.G., Vincent, J.F.V., 2001. Preparation of insect-cuticle-like biomimetic materials. Biomacromolecules 2, 369–372.

Okot-Kotber, B.M., Morgan, T.D., Hopkins, T.L., Kramer, K.J., 1994. Characterization of two high molecular weight catechol-containing glycoproteins from pharate pupal cuticle of the tobacco hornworm, *Manduca sexta*. Insect Biochem. Mol. Biol. 24, 787–802.

Okot-Kotber, B.M., Morgan, T.D., Hopkins, T.L., Kramer, K.J., 1996. Characterization of two high molecular weight catechol-containing glycoproteins from pharate pupal cuticle of the tobacco hornworm, *Manduca sexta*. Insect Biochem. Mol. Biol. 24, 787–802.

Peter, M.G., Grittke, U., Grün, L., Schafer, D., 1988. Enzymatic oxidation of phenolic tanning agents and some analogues in sclerotizing insect cuticle. In: Sehnal, F., Zabza, A., Denlinger, D.L. (Eds.), Endocrinological Frontiers in Physiological Insect Ecology. Wroclaw Tech. Univ. Press, Wroclaw, pp. 519–530.

Pryor, M.G.M., 1940. On the hardening of the cuticle of insects. Proc. R. Soc. London Ser. B 128, 393–407.

Schaefer, J., Kramer, K.J., Garbow, J.R., Jacob, G.S., Stejskal, E.O., Hopkins, T.L., Speirs, R.D., 1987. Aromatic cross-links in insect cuticle: detection by solid-state ^{13}C and ^{15}N NMR. Science 235, 1200–1204.

Suderman, R.J., Andersen, S.O., Hopkins, T.L., Kanost, M.R., Kramer, K.J., 2003. Characterization and cDNA cloning of three major proteins from pharate pupal cuticle of *Manduca sexta*. Insect Biochem. Mol. Biol. 33, 331–343.

Sugumaran, M., Henzel, W.J., Mulligan, K., Lipke, H., 1982. Chitin-bound protein of *Sarcophagid* larvae: metabolism of covalently linked aromatic constituents. Biochemistry 21, 6509–6515.

Sugumaran, M., Hennigan, B., O'Brien, J., 1987. Tyrosinase catalyzed protein polymerization as an in vitro model for quinone tanning of insect cuticle. Arch. Insect Biochem. Physiol. 6, 9–25.

Thomas, B.R., Yonekura, M., Morgan, T.D., Czapla, T.H., Hopkins, T.L., Kramer, K.J., 1989. A trypsin-solubilized laccase from pharate pupal integument of the tobacco hornworm, *Manduca sexta*. Insect Biochem. 19, 611–622.

Thurston, C.F., 1994. The structure and function of fungal laccases. Microbiology 140, 19–26.

Vincent, J.F.V., Hillerton, J.E., 1979. The tanning of insect cuticle—a critical review and a revised mechanism. J. Insect Physiol. 25, 653–658.

Willis, J.H., Iconomidou, V.A., Smith, R.F., Hamodrakas, S.J., 2005. Cuticular proteins. In: Gilbert, L.I., Iatrou, K., Gill, S. (Eds.), Comprehensive Molecular Insect Science, vol. 4. Elsevier Press, Oxford, UK, pp. 79–110 (Chapter 2).

Yamasaki, N., Aso, Y., Tsukamoto, T., 1990. A convenient method for the preparation of *N*-beta-alanyldopamine as a substrate of phenoloxidase. Agric. Biol. Chem. 54, 833.

Xu, R., Huang, X., Hopkins, T.L., Kramer, K.J., 1997. Catecholamine and histidyl protein cross-linked structures in sclerotized insect cuticle. Insect Biochem. Mol. Biol. 27, 101–108.