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Model reactions for insect cuticle sclerotization: Participation of amino groups in the cross-linking of *Manduca sexta* cuticle protein MsCP36

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ABSTRACT

Current theories of sclerotization center on protein cross-linking and dehydration as major factors in the hardening and stability of the insect cuticle. Several studies have reported the identification of catechol-amino acid adducts from sclerotizing cuticle involving histidine, lysine, and tyrosine, though there have been no reports of a catechol linked between two amino acid residues. Previously, we reported an *in vitro* model system for sclerotization and observed that stable protein oligomers were formed, presumably through cross-links with oxidized catecholamines [Insect Biochem. Mol. Biol. (2006) 36, 353–365]. Using site-directed mutagenesis we created a mutant lacking histidine, rMsCP36(H65A/H178A), to investigate the possible involvement of the two histidine residues of MsCP36 in cross-linking. Surprisingly, this alteration had little or no effect on the formation of protein oligomers as determined by SDS-PAGE analysis. Blocking of the free amino groups in lysyl side chains and the amino-terminus by succinylation diminished, but did not eliminate, cross-linking of either rMsCP36 or rMsCP36(H65A/H178A). We also examined the possibility that cross-linking was due to intermolecular dityrosine linkages. Immunoblot analysis utilizing a monoclonal antibody known to recognize peptidyl dityrosine indicated that dityrosyl cross-links were present. Taken together, these results indicate that lysyl residues are important for the cross-linking of the cuticle protein rMsCP36, but that additional residues other than histidine can also contribute.

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1. Introduction

Whereas humans and other vertebrates have an internal mineralized skeleton, millions of different species of insects and other arthropods utilize an external non-mineralized exoskeleton or cuticle. This organic cuticle is a lightweight material that can vary in hydrophobicity, flexibility, stiffness, hardness and strength. This variation of physical properties can be seen in different developmental stages of the same species and even in different areas of a cuticle of one particular insect. Among the many roles attributed to the cuticle are protection from predators, pathogens, and environmental stresses (abrasion, UV radiation, desiccation), sensory perception (mechano- and chemoreception), attachment sites for underlying muscles and organs, and locomotion (both walking and flight).

The cuticle is composed of chitin fibrils embedded in a matrix of proteins, lipids, pigments and N-acylcatecholamines. The quinone tanning hypothesis predicts that sclerotization, or hardening of the cuticle, occurs through oxidation of the N-acylcatecholamines to highly reactive quinones, which can then form adducts and cross-links with nucleophilic amino acid side chains in proteins, stabilizing the structure (Xu et al., 1996; Huang et al., 1997; Sugumaran, 1998; Andersen, 2005, 2010; Bittner, 2006). Several reports have been published describing conjugates of proteins and N-acylcatecholamines in the insect cuticle (Schaefer et al., 1987; Okot-Kotber et al., 1994, 1996; Xu et al., 1997; Kerwin et al., 1999; Kramer et al., 2001; Andersen, 2007, 2008; Andersen and Roepstorff, 2007). However, there have been no reports of two peptides, or amino acids, linked together by a single catecholamine or its polymer.

An alternate hypothesis proposed that sclerotization is primarily a result of dehydration (Fraenkel and Rudall, 1940; Vincent and Hillerton, 1979). Newly synthesized cuticle is very pliable and has a high water content accounting for 60–80% of the weight, but subsequently drops to 20–50% during sclerotization, a loss of

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30–40% (Fraenkel and Rudall, 1940; Schaefer et al., 1987; Wappner and Quesada-Allué, 1996). Dehydration may be brought about, in part, via polymerization of the oxidized N-acylcatecholamines, which act as fillers to drive water out of the cuticle. In addition, conjugation of N-acylcatecholamines to proteins decreases the number of hydration sites resulting in an increase in the hydrophobicity of the cuticle. An active mechanism to pump water out of the cuticle is also likely important for dehydration (Wappner and Quesada-Allué, 1996). Cross-links between proteins, via oxidized N-acylcatecholamines, play a small role, if any, in the dehydration-based mechanism.

These two hypotheses, while differing on the primary cause of sclerotization, are mechanistically similar and not mutually exclusive; both dehydration and cross-linking may be critical to the sclerotization process. It is plausible that dehydration of the cuticle during sclerotization packs proteins close together allowing for cross-links to form, which in turn further stabilizes the cuticle. We noted in a previous report, detailing a model cross-linking reaction involving recombinant cuticular proteins and oxidized N-acylcatecholamines, that cuticle protein monomers became increasingly insoluble as they reacted with oxidized N-acetyldopamine (NADA) or N-β-alanyldopamine (NBAD) and then subsequently formed covalently linked multimers (Suderman et al., 2006). Here we further analyze that cross-linking model system utilizing site-directed mutagenesis and protein modification to more directly examine which of the cuticle protein's residues are involved in forming cross-links.

2. Materials and methods

2.1. Site-directed mutagenesis of MsCP36

Cloning of the cuticle protein MsCP36 into the bacterial expression vector pProEXHTa (Invitrogen) has been described previously (Suderman et al., 2006). This plasmid was then used to construct the His-to-Ala mutant using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). His65 was first changed to Ala with the sense primer 5′-GGAGGCGCTTTTGGCGCAGGAGGCGCGGGATCTGG-3′ and the antisense primer 5′-CCAGATCCCGCGCCTCCTGCGCCAAAAGCGCC TCC-3′ (the His-to-Ala codon change is underlined). Ten nanograms of the rMsCP36 plasmid was used as template for PCR with 125 ng of each of the H65A primers and 2.5 units of *Pfu* Turbo DNA Polymerase in a total volume of 50 μl. The PCR conditions consisted of an initial denaturation at 95 °C for 30 s, followed by 16 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min and extension at 68 °C for 12 min. The DNA was then digested with 10 units of *Dpn* I, an endonuclease specific for methylated and hemi-methylated DNA, for 1.2 h to digest the parental (non-mutated) DNA. Super competent *E. coli* strain XL1-blue cells (Stratagene) were then transformed and plated on LB/ampicillin (100 μg/ml) agar plates. Single colonies were picked and grown overnight in LB/ampicillin medium at 37 °C and 275 rpm. Plasmids were then purified using a Qiaprep Spin MiniPrep Kit (Qiagen) and sequenced to confirm the correct mutation. To create the double mutant H65A/H178A, the H65A plasmid was used as template with the same protocol using the primers (sense) 5′-CGTGCCCCAAGGCGCTGCACTCCCCACCCCGCCC-3′ and (antisense) 5′-GGGCGGGGTGGGGAGTGCAGCGCCTTGGGGCACG-3′; sequencing confirmed that the two codons for His had been successfully mutated to the codon for Ala.

2.2. Recombinant protein expression

The expression plasmid pProEXHTa adds a 6-His tag with a TEV protease cleavage site to the amino-terminus of the protein. Expression of recombinant proteins and removal of the tag followed the procedure described previously (Suderman et al., 2006). The strategy employed allowed for purification of the recombinant protein on a Ni-NTA column, removal of the tag by cleavage with a recombinant His-tagged TEV protease (AcTEV Protease; Invitrogen), and purification of the recombinant protein away from the tag and the protease by another Ni-NTA chromatography step in which the recombinant protein is collected in the flow through, while the tag, AcTEV protease, and any uncleaved recombinant protein remain bound to the column. Removal of the tag ensured that the histidine residues in the tag were not involved in subsequent cross-linking reactions.

2.3. Blocking of free amino groups

To block free amino groups and prevent them from forming cross-links with oxidized N-acylcatecholamines, 2 mg of purified recombinant protein was diluted in 1 ml of 0.1 M $NaHCO_3$, pH 8.3. A 20-fold molar excess of solid succinic anhydride (0.52 mg) was then slowly added to the protein while gently vortexing. The pH of the solution was held constant by measuring the pH of 1 μl aliquots on pH paper during mixing and adding 1 N NaOH as needed to keep the pH above 8. The pH was adjusted until no change was observed over 15 min after addition of succinic anhydride. Unreacted succinic anhydride was removed by filtration using Microcon YM-3 filters. Succinylation was verified by matrix assisted laser desorption ionization (MALDI) mass spectrometry.

2.4. Model cross-linking reactions

Cross-linking reactions were performed as described previously (Suderman et al., 2006). Briefly, each reaction contained 7.4 mM NBAD, 6 units of *Pyricularia oryzae* laccase (Sigma, catalog no. L-5510), 12-fold dilution of a protease inhibitor cocktail (Sigma, catalog no. P-8849), and 24–36 μg (0.13–0.20 mM) recombinant protein in either 50 mM sodium phosphate (pH 7) or 100 mM sodium bicarbonate (pH 8.3) and a total volume of 6 μl at 30 °C; variations in protein concentration and incubation times are noted in the text. Reactions using horseradish peroxidase (Sigma, catalog no. P-8375) instead of laccase were carried out under the same conditions except for the addition of hydrogen peroxide to a final concentration of 0.01%. For both laccase and peroxidase, ten units of enzyme produced a change in absorbance of 0.001 A_{390}/min as measured using an optical path length of 0.5 cm over a 10 min period, 1 mM NBAD as substrate in 50 mM sodium phosphate (pH 7) and a total reaction volume of 200 μl. Reaction products were analyzed by SDS-PAGE (Suderman et al., 2006).

2.5. Immunoblot analysis

Samples used for immunoblot analysis were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane using a Trans-Blot Semi-Dry Transfer Cell (BioRad). The membrane was incubated in a blocking solution consisting of 3% dry milk (w/v) in TBST (137 mM NaCl, 2.7 mM KCl, 25 mM Tris (pH 7.4), 0.05% (v/v) Tween 20 and 0.02% (w/v) NaN_3) on a rotary shaker at room temperature. After 1 h the blocking solution was replaced with fresh blocking solution containing a mouse monoclonal antibody to dityrosine (1C3, Kato et al., 2000) at a 1:500 dilution and incubated overnight. The monoclonal antibody was supplied by Dr. Yoji Kato, University of Hyogo, Japan. The following morning the membrane was washed 3× for 5 min in TBST and incubated in blocking solution for 1 h with the secondary antibody (goat anti-mouse IgG (H + L) alkaline phosphatase conjugated, BioRad; 1:3000 dilution). The membrane was washed again and developed for 20 min using the AP conjugate substrate solution kit (BioRad).

3. Results

3.1. Cross-linking reactions with rMsCP36 mutant lacking histidine

The quinone tanning hypothesis predicts that nucleophilic side chains of amino acids in cuticular proteins form adducts via covalent bonds to electrophilic quinones. The amino acid residues most likely to be involved in this mechanism are histidine, lysine and tyrosine, but not cysteine as most cuticular proteins lack this residue (Xu et al., 1996; Huang et al., 1997; Kerwin et al., 1999; Kramer et al., 2001; Willis et al., 2005; Andersen, 2005, 2010). Since MsCP36 has only two histidine residues (Fig. 1), we sought to address their role in cross-linking of rMsCP36 by changing both of those residues to alanine. The mutant protein rMsCP36(H65A/H178A) was created by changing H65 and H178 to alanines by site-directed mutagenesis, and the mutations were confirmed by cDNA sequencing. The observed mass of the mutant protein, as determined by mass spectrometry, was 30,256 Da, a value nearly identical to the expected mass of 30,254 Da. This indicated that alanine had replaced the histidines and that the N-terminal His-tag utilized for purification had been removed. The absence of histidine was also confirmed by amino acid analysis of the protein preparation (data not shown).

When used in cross-linking reactions with NBAD and laccase, rMsCP36(H65A/H178A) formed higher oligomers (Fig. 2), signifying that histidine residues are not essential for cross-linking in this model system. The rMsCP36(H65A/H178A) oligomers formed during a cross-linking reaction remained stable after incubation in 6 M urea for 1 h, followed by boiling in SDS sample buffer (Fig. 3), indicating that the bonds formed during the reaction with laccase and NBAD were covalent ones.

3.2. Cross-linking reactions of rMsCP36 with amino groups blocked

MsCP36 contains five free amino groups in the form of ε-nitrogens on four lysyl residues and the α-nitrogen on the amino-terminus (Fig. 1). To test whether these amino groups were involved in cross-linking, both rMsCP36 and rMsCP36(H65A/H78A) were treated with succinic anhydride to block the amino groups; reaction between the amino group and succinic anhydride yields a terminal carboxyl group instead of a nucleophilic amino group. Analysis of the protein by mass spectrometry after treatment with succinic anhydride detected an increase in mass by 500 Da, a value consistent with the addition of five succinic anhydride molecules, presumably to the four lysines and amino-terminus. Cross-linking reactions were carried out using the succinylated proteins rMsCP36Succ and rMsCP36(H65A/H178A)Succ, which contain no strong nucleophiles. As shown by SDS-PAGE, succinylation decreased, but did not eliminate, oligomerization of both recombinant proteins (Fig. 4, compare lane 1 with 3, and 5 with 7). The histidine-less mutant protein performed as well in the cross-linking experiments as the wild-type protein (Fig. 4, compare lane 1 with 5, and 3 with 7). This result demonstrates that rMsCP36 was able to become cross-linked to higher oligomers even after the mutation of histidines to alanines and also blockage of all of the amino groups. The use of [^{14}C]-labeled N-acetyldopamine ([^{14}C]-NADA) in place of NBAD in the cross-linking reaction verified that the N-acylcatecholamine was incorporated into both monomeric and oligomeric forms of rMsCP36(H65A/H178A) Succ (Fig. 5).

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GAMDPEFDRLDNKYLPPRGNAGAGFGPGFGGAGPKGGGSG   33
FGGAGSGFGGAGSGFGGAGSGFGGGAGGAFGHGGAGSGFG   73
GQAGGYSGAGGAGGYSGGASGQYSGRGGAASADANAQILR  113
LNNEVTAEGFAYDFETSNGIRADAQGVATNGVQSQGSFAY  153
KGDDGQDYSITYTADENGFVPQGAHLPTPPPIPEEILKSL  193
EQNARDEAAGIVDDGTYRGEGAGAGVAGGYSGAGGYSGGA  233
GGAGGFGAGGAGRAGGAGGFGTGGAGRAGGAGGFGAGGAG  273
GAGGAGGFGAGAGGFGGRGSGGFGGAASRQYLAPNAGGRG  313
SGSGNFNAQTGYQY                            327
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Fig. 1. Amino acid sequence of rMsCP36. The two histidyl residues mutated to alanine (H65A and H178A) are in bold. The four lysyl residues are highlighted in black. The fourteen tyrosyl residues are highlighted in grey. The portion of the tag remaining after cleavage (GAMDPEF) with AcTEV protease is denoted in heavy underline. The numbering on the right indicates the position of the last residue on that line and does not include the seven residues of the tag.

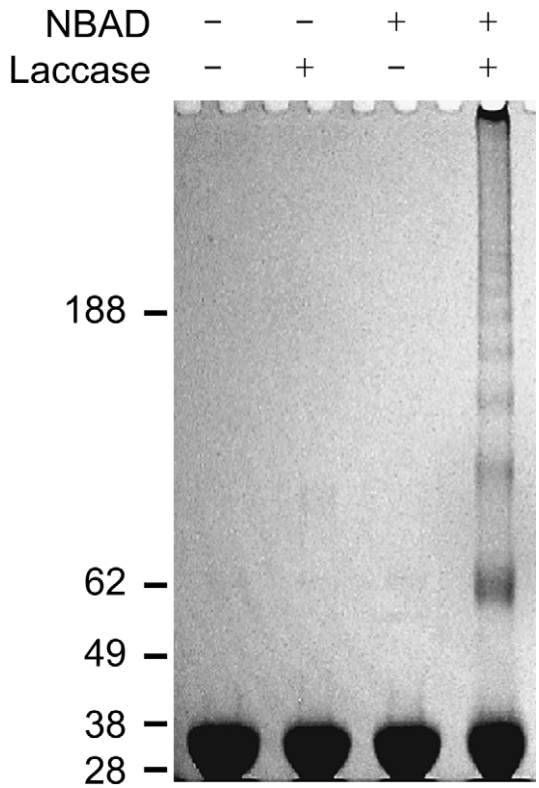

Fig. 2. SDS-PAGE analysis of the cross-linking reaction of rMsCP36(H65A/H178A). Recombinant protein (0.13 mM) was incubated in the presence (+) or absence (−) of 7.4 mM NBAD and fungal laccase in 50 mM sodium phosphate buffer (pH 7) for 1 h at 30 °C. Reaction mixtures were fractionated in a 3–8% polyacrylamide Tris–acetate gel (NuPAGE; Invitrogen). The molecular sizes of the standard marker proteins (SeeBlue (prestained); Invitrogen) in kDa are noted to the left of the gel.

3.3. Investigation of the presence of intermolecular dityrosine linkages

rMsCP36 contains fourteen tyrosyl residues (Fig. 1) that may be involved in cross-linking either through conjugation with quinones (or quinone polymers) or through intermolecular dityrosine or isodityrosine linkages, which form via tyrosyl radical intermediates (Eickhoff et al., 2001). Such intermediates can be produced either directly by peroxidase or indirectly by laccase via semiquinone radicals generated when laccase oxidizes the N-acylcatecholamines by a single electron transfer. We used both of these enzymes in cross-linking experiments with rMsCP36(H65A/H178A) and analyzed the results by SDS-PAGE and immunoblot analyses utilizing a monoclonal antibody known to recognize peptidyl dityrosine (Kato et al., 2000). Oligomers of the protein were observed only in the presence of NBAD (Fig. 6A, lanes 3 and 4), whereas the sample containing only peroxidase and H_2O_2 failed to generate multimers and was indistinguishable from the control reaction containing no enzyme (Fig. 6A, lanes 1 and 2). The anti-dityrosine antibody faintly recognized a band of

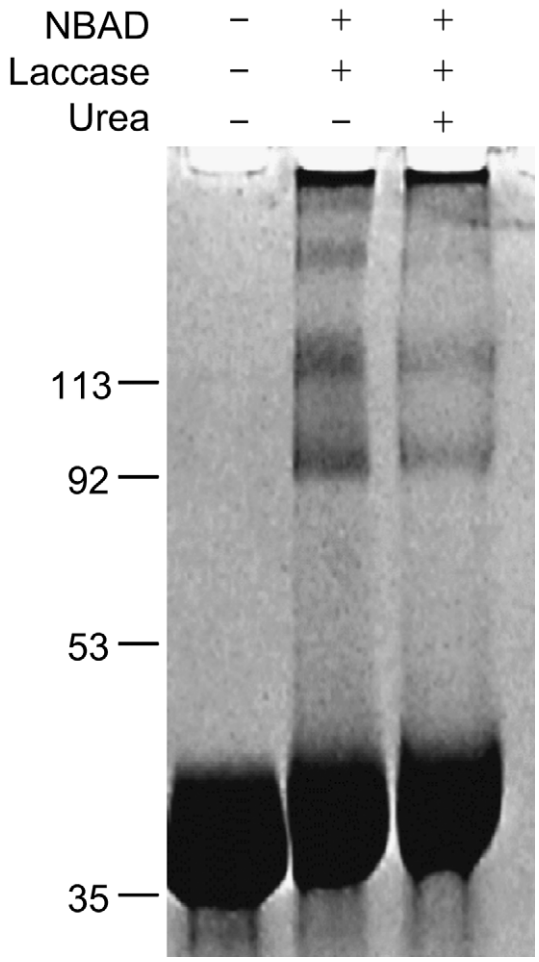

Fig. 3. Stability of rMsCP36 oligomers to treatment in 6 M urea. rMsCP36(H65A/H178A) (0.13 mM) was incubated in the presence (+) or absence (−) of 7.4 mM NBAD and fungal laccase in 50 mM sodium phosphate buffer (pH 7) for 1 h at 37 °C. Following incubation, 18 μl of 8 M urea was added (final concentration 6 M) to one of the cross-linking samples and incubated for another 1 h. Reaction mixtures were boiled in SDS sample buffer and fractionated in a 8–16% polyacrylamide gel (iGel; ISC BioExpress). The molecular sizes of the standard marker proteins (Low Range (prestained); BioRad) in kDa are noted to the left of the gel.

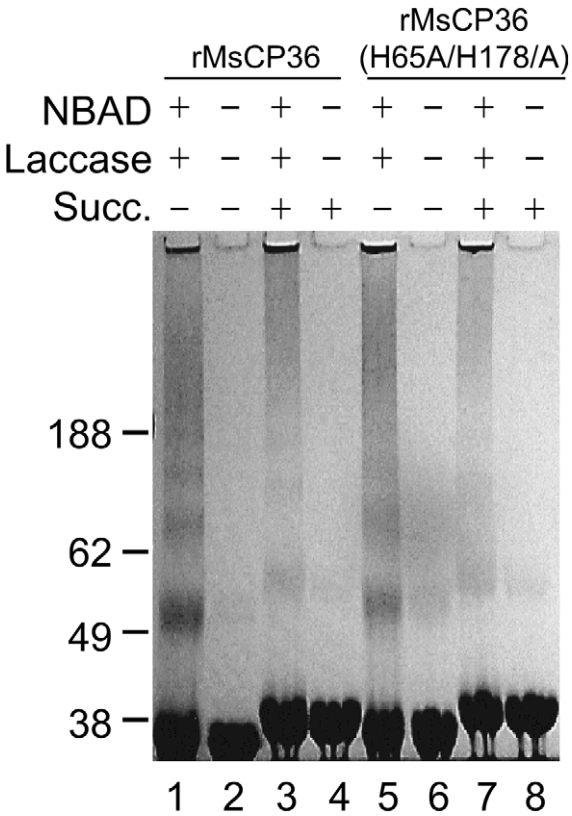

Fig. 4. SDS-PAGE analysis of cross-linking reactions using modified forms of rMsCP36. Cross-linking reactions were performed with 0.2 mM recombinant protein, 7.4 mM NBAD, and fungal laccase in 100 mM sodium bicarbonate (pH 8.3) for 1.5 h at 30 °C. N-Succinylation (Succ.) of proteins to block free amino groups was performed as described in the Materials and methods. Control samples containing no NBAD or laccase (lanes 2, 4, 6, and 8) were prepared at the time of gel electrophoresis and were not incubated as were the other samples. Samples were fractionated by SDS-PAGE in a 3–8% polyacrylamide Tris–acetate gel (NuPAGE; Invitrogen). Lane numbers are noted at the bottom of the gel. The molecular sizes of the standard marker proteins (SeeBlue (prestained); Invitrogen) in kDa are noted to the left of the gel.

approximately 66 kDa, the expected size of an MsCP36 dimer, and this recognition was strongest in the peroxidase only sample (Fig. 6B, lane 2). In cross-linking samples containing NBAD, proteins of higher molecular masses corresponding to oligomers were also recognized by the anti-dityrosine antibody (Fig. 6B, lanes 3 and 4). The fact that the antibody recognized only multimers of rMsCP36(H65A/H178A), but not the monomer, suggests that these linkages are intermolecular. Similar results were obtained when using the blocked protein rMsCP36(H65A/H178A)Succ (data not shown).

4. Discussion

Reactions between quinones and proteins are abundantly evident in nature, affecting such diverse processes as the color and taste of foods, wounding in plants, formation of humic compounds, and allergic inflammation (Bittner, 2006). In insect cuticle sclerotization, the combinatorial effects of protein cross-links and dehydration serve to harden and strengthen the cuticle, though elucidating the contribution of each method is complex. Mechanical analyses performed on the elytra of two beetle species offered some clues to this problem (Lomakin, 2009; Lomakin et al., 2009). The elytra from the beetles *Tribolium castaneum* and *Tenebrio molitor* were collected at 1 h (unsclerotized) and 7 d (fully sclerotized) post eclosion and their mechanical properties determined. For both species there was ~20-fold difference in Young's modulus, a measure of the stiffness of a material, between the 1 h and 7 d elytra. When the samples were thoroughly dried before the measurements were made, there still remained a ~4-fold difference in the modulus between 1 h and 7 d elytra in *T. castaneum* and a ~5-fold difference in *T. molitor*. Thus, the increase in stiffness of the elytra cannot be due entirely to dehydration.

Recently, Yamauchi et al. (2009) developed an enzyme-free system to mimic the quinone hardening of extracellular proteins in invertebrates. Their model included gelatin as the protein, hydroquinone as the cross-linking agent, and cuprous ion as a substitute for the copper ion-containing phenoloxidase. Hydrogels and dehydrated films were produced that were mechanically stable and flexible, a result consistent with the formation of gelatin that had been cross-linked via Michael addition reactions of lysyl, hydroxylysyl and histidyl residues with benzoquinone. Similarly, we had previously demonstrated a simplified *in vitro* model system for insect cuticle sclerotization consisting of three components: the enzyme laccase, the N-acylcatecholamine NBAD, and the cuticle structural protein MsCP36 (Suderman et al., 2006). In this system

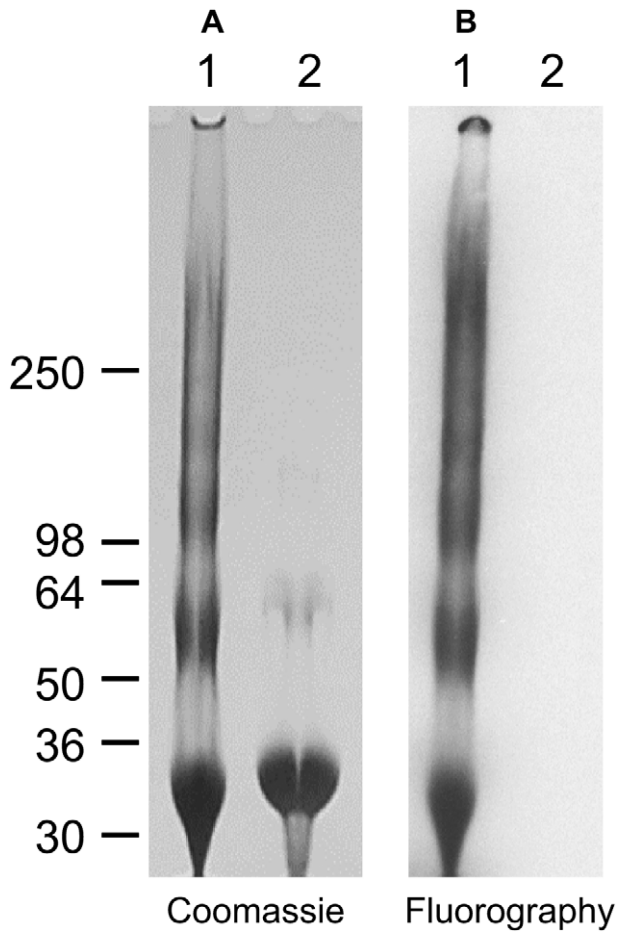

Fig. 5. SDS-PAGE analysis of rMsCP36(H65A/H178A)Succ labeled with [^{14}C]-NADA. Duplicate cross-linking reactions were performed with 0.2 mM of the succinylated form of rMsCP36(H65A/H178A), 7.4 mM [^{14}C]-NADA (Suderman et al., 2006), and fungal laccase in 100 mM sodium bicarbonate (pH 8.3) at 31 °C overnight. Reactions were fractionated in separate 3–8% polyacrylamide Tris–acetate gels (NuPAGE; Invitrogen). (A) Detection of proteins by staining with Coomassie blue. (B) Detection of proteins by fluorography (Skinner and Griswold, 1983); x-ray film was exposed to the treated gel for 2 h at −70 °C and developed. Lane 1, cross-linking reaction; lane 2, unreacted recombinant protein. The molecular sizes of the standard marker proteins ([^{14}C]-labeled Protein Molecular Weight Standards; Gibco BRL) in kDa are noted to the left of the gel.

we showed that oligomers of protein would form in the presence of both laccase and catecholamine, presumably through cross-linking with oxidized NBAD. However, the exact nature of the cross-links in our model system remained undefined.

Histidine, lysine, and tyrosine have all been previously identified as forming adducts with N-acylcatecholamines in insect cuticle (reviewed in Andersen, 2010). Linkages have also been found between catecholamines and the α-nitrogen of glycine, likely located at the amino-terminus of the mature protein (Andersen, 2007, 2008; Andersen and Roepstorff, 2007). The thiol group of cysteine is also a strong nucleophile and would presumably also form cross-links, however, cysteines appear to be extremely rare in insect cuticle structural proteins (Willis et al., 2005). Thus, we chose to focus on the possible involvement of histidine, lysine and tyrosine residues in cross-linking. For this study we have taken a systematic approach utilizing mutation and protein modification to further analyze the model cuticle protein cross-linking system.

MsCP36 contains only two histidyl residues, making mutational analysis a practical approach for examining their involvement in cross-linking. This analysis indicated that the histidyl residues were not essential for oligomerization of MsCP36. In cross-linking reactions with the histidine-less mutant rMsCP36H65A/H178A, oligomerization proceeded to the same extent as with the non-mutant rMsCP36. This was surprising given that histidine-dopamine adducts were the most abundant amino acid-catechol adducts detected in *Manduca sexta* pupal exuviae (Xu et al., 1997; Andersen, 2008). We have identified several cDNAs encoding putative histidine-rich cuticle proteins from a pharate pupal epidermal library and, therefore, histidines are likely important for the cross-linking of some cuticle proteins (Suderman, Dittmer and Kanost unpublished observation). It should be noted that this does not exclude the two histidine residues from participation in cross-linking, only that in the case of rMsCP36 they likely play only a minor role. It was probable then that additional amino acid residues were involved in cross-linking.

We next examined the potential role that free amino groups from lysyl residues and the amino-terminus might play in forming cross-links. The blocking of amino groups through N-succinylation had a significant effect as oligomeric cross-linking was measurably reduced. However, the protein lacking both histidines and free amino groups was still able to form oligomers, even to the extent that a high molecular weight complex was formed that was unable to migrate into the gel. Additionally, the use of [^{14}C]-labeled NADA confirmed the formation of protein-catecholamine adducts. These results indicate that rMsCP36 can form cross-links to the N-acyl-catecholamine through amino acid side chains other than histidine or lysine, possibly not utilizing a Michael addition mechanism.

It is also possible that the protein is cross-linked through a mechanism that does not involve the N-acylcatecholamines directly, such as the formation of dityrosine or isodityrosine cross-links. Such cross-links have been identified in the protein resilin from the prealar arm and wing-hinge ligament of the desert locust, *Schistocerca gregaria* (Andersen, 1963, 1964), and in the larval cuticles of the grey flesh fly, *Sarcophaga bullata* (Sugumaran et al., 1982). We examined this possibility by immunoblot analysis with a monoclonal antibody that recognizes peptidyl dityrosine linkages. The antibody detected rMsCP36(H65A/H178A) oligomers but not the monomer, suggesting that intermolecular dityrosine cross-links were formed; had intramolecular linkages been present, it was predicted that the antibody would have recognize the monomer as well.

Oligomers equivalent to trimers or greater were detected by the anti-dityrosine antibodies in reactions containing recombinant protein, enzyme (laccase or peroxidase) and NBAD. Conceivably, NBAD semiquinone radicals produced by laccase or peroxidase subsequently oxidized peptidyl tyrosine, which then formed dityrosine or isodityrosine linkages. We attempted to reproduce this type of cross-link with peroxidase and H_2O_2 in the absence of any catecholamine but were unsuccessful at generating any oligomers larger than a dimer. It is possible that the NBAD semiquinone radicals were more efficient at oxidizing peptidyl tyrosine than peroxidase alone and this allowed for the formation of larger oligomers. Alternatively, the failure to form larger oligomers with peroxidase only may suggest that the cross-links were a result of direct interaction of NBAD with tyrosine, a hypothesis consistent with the observation that [^{14}C]-NADA was incorporated into rMsCP36(H65A/H179A)Succ; it is likely that both intermolecular dityrosine and NBAD-tyrosine adducts were involved.

Interestingly, the dityrosine monoclonal antibody detected a protein of ~66 kDa, a size corresponding to a rMsCP36(H65A/H178A) dimer, in the control sample containing protein only. This is consistent with our previous report that dimers spontaneously formed during prolonged storage of rMsCP36 (Suderman et al., 2006) and indicates that they may have been formed by intermolecular dityrosine cross-links. The detection was stronger in the sample containing protein and peroxidase, suggesting that peroxidase enhanced the formation of intermolecular dityrosine linkage(s).

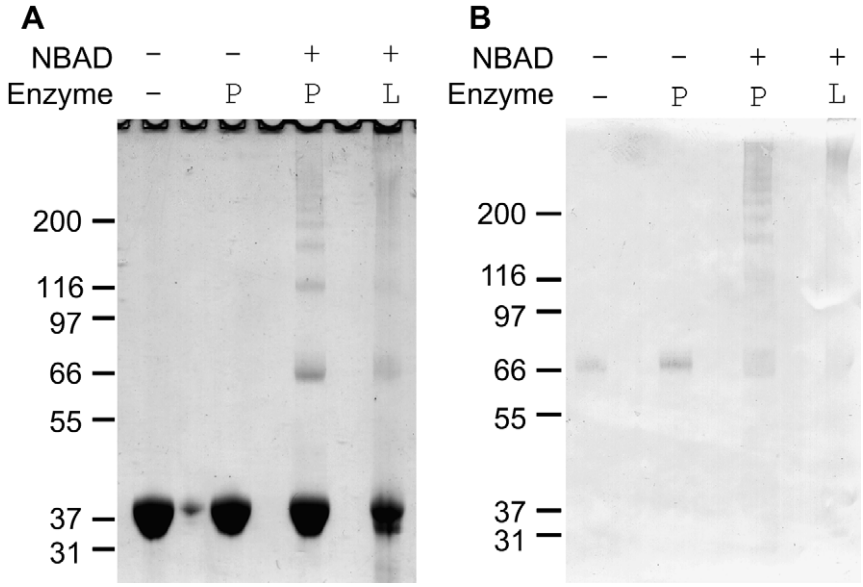

Fig. 6. SDS-PAGE and immunoblot analyses of rMsCP36(H65A/H178A) cross-linking reactions with peroxidase or laccase. Recombinant protein (0.2 mM) was incubated in the presence (+) or absence (−) of 7.4 mM NBAD and either horseradish peroxidase (P) or fungal laccase (L) in 50 mM sodium phosphate buffer (pH 7) for 8 h at 30 °C; reactions containing peroxidase also contained 0.01% hydrogen peroxide. Aliquots of each sample were fractionated in duplicate 4–12% polyacrylamide bis-Tris gels (NuPAGE; Invitrogen). (A) Gel stained with Coomassie blue. (B) Immunoblot using anti-dityrosine monoclonal antibody. Lane numbers are noted at the bottom of the figure. The molecular sizes of the standard marker proteins (Mark12; Invitrogen) in kDa are noted to the left of the gel.

We have demonstrated that for MsCP36, cross-linking is dependent in part on free amino groups of lysyl side chains and the amino-terminus, a result consistent with the quinone tanning hypothesis. However, cross-linking of the succinylated histidine-less mutant revealed that additional residues are involved. It is possible that some amino groups remained unblocked, even though the mass spectrometry analysis suggested that the protein preparation was a homogeneous population of fully succinylated proteins. If this were the case, our results would underestimate the contributions of lysines in forming cross-links in this system.

MsCP36 contains fourteen tyrosine residues and tyrosine-3,4-dihydroxyacetophenone adducts have been identified from the acid hydrolysates of cuticle from several insect species including *M. sexta* (Andersen, 2007, 2008). Additionally, immunoblot analysis utilizing an anti-dityrosine antibody suggests the presence of intermolecular dityrosine cross-links, although currently we do not know if the antibodies recognize isodityrosine cross-links as well. Unfortunately, the large number of tyrosine residues in MsCP36 allows for 196 possible combinations of cross-links between any two tyrosines in an MsCP36 dimer, greatly complicating the analysis.

Our results are consistent with a very complex mechanism for protein cross-linking in both our model system and insect cuticle. Much of the protein in the *in vitro* model remains as a monomer, suggesting that cross-linking in this system may not be as efficient as in the cuticle. This can be explained, in part, by the short incubation times used in the cross-linking reaction before analyzing the sample by SDS-PAGE. Previously, we noted that with increased reaction time, the sample became highly viscous and large complexes did not migrate into the gel (Suderman et al., 2006). To circumvent this problem, reaction times were decreased, and as a result much of the protein remained in monomeric form. Additionally, the protein concentration in the *in vitro* system is much lower than in the cuticle, and this likely also had an effect on cross-linking efficiency. Furthermore, the cuticle contains a more complex mixture of molecules than our simplified model which may facilitate the cross-linking process. Future investigations into the residues involved in cross-linking of MsCP36 and other cuticle proteins should contribute to a greater understanding of the involvement of additional amino acids, beyond histidine and lysine, in sclerotization.

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