

Expression, Cross-Linking, and Characterization of Recombinant Chitin Binding Resilin

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Resilin is a polymeric rubber-like protein secreted by insects to specialized cuticle regions, in areas where high resilience and low stiffness are required. Resilin binds to the cuticle polysaccharide chitin via a chitin binding domain and is further polymerized through oxidation of the tyrosine residues resulting in the formation of dityrosine bridges and assembly of a high-performance protein–carbohydrate composite material. We describe the mechanical, structural and biochemical function of chitin binding recombinant *Drosophila melanogaster* resilin. Various resilin constructs were cloned including the full length gene enabling Ni-NTA purification, as well as heat and salt precipitation for rapid and efficient purification. The binding isotherms and constants (K_d , B_{max}) of resilin to chitin via its chitin binding domain were determined and displayed high affinity to chitin, implying its important role in the assembly of the resilin-chitin composite. The structural and elastic properties were investigated using Fourier transform infrared spectroscopy, circular dichroism, and atomic force microscopy with peroxidase cross-linked solid resilin materials. Generally, little structural organization was found by these biophysical methods, suggesting structural order was not induced by the dityrosine cross-links. Further, the elastomeric properties found from the full length protein compared favorably with the shorter resilin generated previously from exon 1. The unusual elastomeric behavior of this protein suggests possible utility in biomaterials applications.

Introduction

Resilin was discovered in 1960 in studies on the flight mechanisms of desert locusts (*Schistocerca gregaria*) and dragonflies (*Aeshna juncea*).¹ The protein is found within structures where energy storage and long-range elasticity are needed, such as the flight system of locusts,¹ the jumping mechanism of fleas,² and the sound producing organ of cicadas.³ Resilin behaves like an entropic elastomer,¹ and was initially proposed to consist of randomly coiled protein chains linked by stable covalent cross-links, the elastic force being accounted for by a decrease in conformational entropy when the material was strained.^{4,5} Furthermore, it was reported that resilin could be stretched up to 3–4 times its original length before breaking, and immediately snap back to its resting length upon release of the tensile force showing no deformation.⁶ Resilin is insoluble in media which does not degrade peptide bonds and is stable up to 140 °C.⁵ Further, resilin possesses high resilience, 92% or more, and a very high fatigue lifetime,^{7,8} due to the covalent cross-linkage between tyrosine residues, generating di- and trityrosines.⁹

Earlier studies of resilin including electron microscopy and X-ray diffraction suggested that resilin is an unstructured amorphous protein matrix.^{8,10} Recent studies have provided compelling evidence that the crosslinking of resilin in insects

is mediated through the action of peroxidases.^{8,11} To date, structural studies of resilin are limited. A structural description of the synthetic construct resilin AN16 was completed by nuclear magnetic resonance spectroscopy (NMR) and Raman and it was suggested that the resilin chains are mobile.¹²

Ardell and Andersen identified the *Drosophila melanogaster* resilin gene and reported two significant elastic repeat motifs; an N-terminal domain comprising 18 pentadecapeptide repeats (GGRPSDSYGAPGGGN) and a C-terminal domain comprising 11 tridecapeptide repeats (GYSGRPPGGQDLG).¹³ Each repeat is found entirely on the first and third exons (exons 1 and 3), respectively. In addition the exon 2 gene comprises the typical cuticular chitin binding domain (ChBD) type R&R-2 that allows direct binding and strong interaction between the resilin and chitin during the process of resilin deposition and construction of the cuticle composite. Interestingly Ardell and Andersen as well as the authors of this paper found two splicing resilin mRNA variants CG15920-RA and CG15920-RB which differ in the presence of the ChBD (PAKYEFNYQVEDAPSGLSF-GHSEMRDGDFTTGQYNVLLPDGRKQIVEYEADQGGYR-PQIRYEGDANDGSGPSGP).¹³ Recently Elvin et al. expressed the first exon (exon 1) of *D. melanogaster* CG15920 gene in *E. coli*.¹¹ Cross-linking was used to form a solid biomaterial (Recl-resilin) via Ru(II)-mediated photo-cross-linking. This material had up to 90–92% resilience and could be stretched to over 300% of its original length before breaking, based on scanning probe microscopy and tensile testing.¹¹

In order to understand and assess the functional and structural properties of native resilin more completely, we cloned and expressed the full native resilin protein of the *Drosophilla* CG15920 gene, which encodes the three exons of the native

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MSYHHHHHHHDYDIPTTENLYFQGA
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 PPASGSGAGGAGSGPGADYDNDEPAKYEFNYQVEDAPSGLSFGHSEMR
DGDFTTGQYVLLPDRGRKQIVEYEADQQGYRPIRYEGDANDGSGPSGP

Figure 1. Amino acid sequence of 6H-resChBD. *Italics* mark the N-terminal tail; **bold** letters mark the ChBD that is missing in 6H-res protein.

protein (exon 1 + exon 2 + exon 3). The structural and elastic properties of un-cross-linked and cross-linked full length resilin were characterized by Fourier transform infrared spectroscopy (FTIR) and atomic force microscopy (AFM). The ChBD R&R consensus sequence was identified in 72% of cuticular proteins that were discovered from the arthropod kingdom. Nevertheless there is little experimental evidence showing that these sequences bind chitin. In this paper we present experimental data confirming that the peptide sequence identified as ChBD type R&R-2 in *D. melanogaster* resilin confers chitin binding to resilin, implying its role in the formation of the cuticle composite structure.

Materials and Methods

Gene and Plasmid Construction. *Cloning of 6H-res and 6H-resChBD Genes.* RNA was extracted from *Drosophila melanogaster* pupa using TRI Reagent (Sigma, St. Louis, MO).¹⁴ Reverse transcription of the resilin cDNA was performed with M-MLV RT (H-) (Promega corporation, Madison, WI) with oligo(dT)15 primer according to the manufacturers instructions (Supporting Information, part IV). The cDNA was used as template for production of 975 and 1200 base pair products coding for the first exon elastic repeats (6H-res, amino acids 18–324) and with the ChBD (6H-resChBD amino acids 19–415), respectively (Figure 1). Resfor, 5'-CCATGGGACCGGAGCCACAGTTAACTC-3', was used as the forward primer for the amplification of both constructs. Resbml1_rev, 5'-GGATCCCTCATCGTATATCGTAGTCAGCG-3', was used as the reverse primer for amplification of 6H-res. ResChBDRev, 5'-GGATCCTTAAGGACCGCTGGGACCACTG-3', was used as the reverse primer for amplification of 6H-resChBD. The genes were amplified using the following PCR method: 94 °C for 4 min, 35 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, 72 °C for 4 min. The genes were cloned using *NcoI* and *NotI* into pHis-parallel-3 vector (<http://wolfson.huji.ac.il/expression/local/parallel/pHis-Parallel3.jpg>), which contain an N-terminal His tag and a rTev cleavage site enabling purification of the protein on Ni-NTA column and removal of the His tag when desired.

Cloning of Nontagged Full Length Resilin. The full length resilin expression primers were 1PAF and 3PAR. Their sequences were 1PAF, 5'-GGAATATGTTCAAGTTACTCGCGTTGACGCTGCTCATGGC-3', and 3PAR, 5'-CTAGTACCATAACCGCTGCCATCGTTGT-CACCGTTCTGA-3'. These primers for PCR were designed to amplify the three exons of the proposed *D. melanogaster* resilin gene, CG 15920.¹³ Reagents were purchased from Invitrogen (Carlsbad, CA), and the following reaction mixture was used for amplification of all exons of resilin: 5 μ L *Pfx* reaction mix (22 U/mL *Thermococcus* species KOD thermostable polymerase complexed with anti-KOD antibodies, 66 mM Tris-SO₄ (pH 8.4), 30.8 mM (NH₄)₂SO₄, 11 mM KCl, 1.1 mM MgSO₄, 330 μ M dNTPs, AccuPrime proteins, stabilizers), 0.5 μ L (5 U/ μ L) AccuPrime platinum *Pfx* polymerase, 1 μ L (20 mM) forward primer, 1 μ L (20 mM) reverse primer, 37.5 μ L distilled water, and 5 μ L mixture of first strand cDNA synthesis reaction. The following thermocycler program was used to amplify full length resilin gene: denature at 95 °C for 2 min; 35 cycles of 95 °C for 15 s, 55 °C for 15 s, 68 °C for 1 min per kb to be amplified; and 68 °C for 10 min. The PCR products were analyzed using agarose gel electrophoresis and

purified by gel extraction. The PCR product (1818 bp) was inserted into the pCR-Blunt II-TOPO vector (Invitrogen Carlsbad, CA) and clones were selected for resistance to ampicillin (100 μ g/mL). The full length resilin DNA fragment was prepared for insertion into the bacterial T7-promoter expression vector pET22b (Novagen EMD Chemicals, Inc. CA) by partial digestion with *NcoI* and *NotI*. The *NcoI/NotI* fragment was inserted between the corresponding sites of pET22b; the recombinant expression plasmid, pET22b/Res, was isolated from *E. coli* NEB5 α cells (NEB, MA) with selection for ampicillin resistance (100 μ g/mL) and the correct construct was verified by DNA sequence analysis. Full details are provided in the Supporting Information including the DNA and amino acid sequence analysis of full length resilin.

Expression. All expression vectors were transformed into BL21 Star (DE3; Invitrogen Carlsbad, CA). Authenticity of the clones was confirmed by DNA sequencing. A total of 5 mL of overnight cultures were grown in LB medium with 100 μ g/mL ampicillin at 37 °C in a rotary shaker and were used for inoculation of 1 L of LB cultures with 100 μ g/mL ampicillin at a ratio of 1/100 of starter to culture volume. At O.D.600 of 0.8–0.9 expression was induced with 1 mM IPTG. Following 4–6 h from induction, bacteria were harvested by centrifugation (10000 g for 20 min at 4 °C). Pellets were stored at –80 °C.

Purification of Full Length Resilin. The cell pellets were thawed and resuspended in lysis buffer containing 1 \times BugBuster Protein Extraction Reagent (Novagen EMD Chemicals, Inc. CA), Lysonase Bioprocessing Reagent (Novagen EMD Chemicals, Inc. CA), and 1 \times phosphate-buffered saline. The cell suspension was lysed with shaking for 1 h. The soluble protein fraction was collected by centrifugation at 10000 g for 1 h at 4 °C. The resulting supernatant was heated for 30 min at 90 °C and denatured proteins were removed by centrifugation at 10000 g for 30 min at 20 °C. A total of 20% solid ammonium sulfate was added to remove bacterial proteins, and 30% salt was then used to precipitate the recombinant resilin. Precipitating proteins were resuspended in sterile phosphate-buffered saline (PBS), and dialyzed overnight at 4 °C in excess PBS. Purity and recovery rates were assessed by SDS-PAGE on 4–12% Bis-Tris precast gels (Invitrogen, CA).

Purification of Recombinant 6H-res and 6H-resChBD. Bacterial pellets recovered from 1 L fermentation broth were resuspended in 75 mL of 20 mM Na-phosphate, 0.5 M NaCl, 25 mM imidazole with Complete protease inhibitor (Roche, Switzerland) followed by sonication in ice bath. The soluble fraction was separated by centrifugation at 10000 g for 45 min at 4 °C. Cleared bacterial lysates were filtered with a 0.45 μ m syringe membrane followed by affinity chromatography (AC) on HisTrap HP Ni-NTA pre-equilibrated 5 mL column mounted to ÄKTAprius plus FPLC (GE, Sweden). The recombinant proteins were purified as follows: buffer A (binding), 20 mM NaHPO₄, 0.5 M NaCl, 10 mM imidazole; buffer B (elution), 20 mM NaHPO₄, 0.5 M NaCl, 0.5 M imidazole; (1) 2 column volumes (CV) of buffer A at 5 mL/min, (2) 75 mL injection of the lysate at 5 mL/min, (3) 5 CV wash with the buffer A, (4) 5 CV step of 10% buffer B, (5) 7 CV step of 100% buffer B, (6) equilibration with 5 CV of binding buffer at 1 mL/min. The eluted proteins were detected at O.D. 280. Approximately 400 μ L fractions were collected and 10 μ L of samples boiled with SAB were loaded on a 12.5% SDS-PAGE gel. Subsequently, the AC purified fractions were pooled and diluted with 20 mM Na-phosphate buffer to EC < 4 mS followed by ion exchange chromatography (IEC) on a 5 mL HiTrap Q FF column as follows: buffer A (binding), 20 mM NaHPO₄; buffer B (elution), 20 mM NaHPO₄, 0.5 M NaCl; (1) 2 column volumes (CV) of buffer A at 5 mL/min, (2) injection of the diluted pooled AC fractions at 5 mL/min, (3) 5 CV wash with the buffer A, (4) 5 CV step of 30% buffer B, (5) 8 CV step of 60% buffer B, (6) 5 CV of step of 100% buffer B, (7) equilibration with 5 CV of binding buffer at 1 mL/min. The purified proteins were analyzed by 12.5% SDS-PAGE and stained with Coomassie blue.

Chitin Binding. Qualitative binding assay was performed both with crude bacterial lysates and IEC purified proteins using chitin beads (NEB, MA). A total of 50 mg chitin beads were washed once with

PBS followed by addition of 50 μg recombinant proteins in 500 μL PBS. The mixture was incubated under gentle shaking for 30 min at RT followed by centrifugation. The supernatant was removed (unbound fraction) and the pellets were washed with 500 μL of PBS. Precipitated chitin pellets with bound proteins were boiled with 50 μL of 2 \times sample application buffer (SAB). Unbound and bound proteins were analyzed by SDS-PAGE. For quantitative chitin binding assay, 50 mg of chitin beads were added to 1.5 mL test tubes and washed once with PBS. Serial dilutions of IEC purified 6H-resChBD (0.4, 1.1, 2.2, 4.3, 8.6, 17.2, 21.5 μM) were incubated with the chitin beads (three replicates for each protein concentration) under gentle shaking for 30 min at room temperature. Subsequently, the supernatant was removed (unbound fraction) and the amount of bound proteins was determined using Lowry protein assay. The maximum amount of bound protein B_{max} and K_{d} values were determined by nonlinear regression of the binding isotherms using a modified model for saturation binding, comprising one binding site as developed by Swillens et al.³⁶

The equation $B = (F \times B_{\text{max}})/(K_{\text{d}} + F)$ used for the calculation, fits nonlinear regression for one binding site, where B is bound protein and F is free protein. The results were analyzed using GraphPad Prism 5 software (GraphPad software Inc., CA).

Cross-Linking of Recombinant Resilin. A total of 5 mg of lyophilized recombinant resilin was dissolved in 100 μL of 0.25 M sodium borate/boric acid buffer, pH 8.4, forming a solution of resilin with concentration of 50 mg/mL. As described by Malencik and Anderson,¹⁵ the solution was incubated at 37 $^{\circ}\text{C}$ for 1 h, and then 250 μL of a 1 mg/mL solution of horseradish peroxidase (Sigma, MI) was added. Subsequently, 10 mM hydrogen peroxide was added to initiate the cross-linking reaction. The solution was allowed to incubate at 37 $^{\circ}\text{C}$ for 1 h. Successful cross-linking was checked by gel electrophoresis and fluorescence detection with a microplate spectrofluorometer (Molecular Devices, CA).

Biomaterial Properties. Conformation. Resilin samples were freeze-dried and the structural characteristics were observed using Fourier transform infrared spectroscopy (FTIR) as previously reported by Hu et al.^{16,17} The fractions of secondary structural components including random coil, α -helices, β -strands, and turns were evaluated using Fourier self-deconvolution (FSD) of the infrared absorbance spectra. FSD of the infrared spectra covering the amide I region (1595–1705 cm^{-1}) was performed by Opus 5.0 software. The second derivative was first applied to the original spectra in the amide I region with a nine-point Savitsky-Golay smoothing filter. Deconvolution was performed using Lorentzian line shape with a half-bandwidth of 25 cm^{-1} and a noise reduction factor of 0.3.^{16,17} Circular dichroism (CD) spectra were also studied for the secondary structure of resilin samples as previously described.¹⁸ All spectra were recorded on an AVIV Model 410 spectrophotometer (AVIV Biomedical, Inc., Lakewood, NJ) at room temperature (25 $^{\circ}\text{C}$) using a 1 mm path length quartz cell with a 1 nm bandwidth. CD spectra were measured for un-cross-linked and cross-linked full length resilin in the 1 \times PBS (pH 7.4) with concentrations in the range of 3.0–5.0 mg/mL. CD data were analyzed using a DICHROWEB program (<http://dichroweb.cryst.bbk.ac.uk>).^{19,20}

Mechanical Properties. Resilience is defined as the energy recovered after removal of the stress divided by the total energy of deformation.^{11,21} The elastic properties of the full length and cross-linked resilins were performed using AFM as previously described.^{8,11,21} A force–distance curve was obtained for evaluation of material properties. Resilin samples were dried on the surface of mica. Measurements on the full length recombinant resilin and cross-linked resilin were conducted using AFM operated in force mode. A total of 10 independent trials were performed for resilin samples and mica was used as controls to calibrate the instrument. Resilience is given by calculating the ratio of the areas under the penetration and retraction curves in the force–distance curves.^{8,11,21}

Results

Gene Construction, Protein Purification, and Cross-Linking of Full Length Resilin. PCR amplification was used to successfully prepare the full length gene to construct the plasmid pET22b/Res containing the full length 1818 bp resilin gene (Figure 2A). After the plasmid pET22b/Res was transformed into *E. coli* strain BL21 Star (DE3), the expression was carried out for 6 h at 37 $^{\circ}\text{C}$. Using a heat and salting-out method, the resilin protein was purified (Figure 2B). The final purified yield was about 20 mg/L of culture. N-Terminal amino acid sequence analysis of the purified resilin revealed the expected amino acid sequence (MVRPEPPVNSYLPPSDSYGA).

Successful formation of cross-linked resilin was obtained using horseradish peroxidase. Cross-linked samples showed high molecular weight which barely entered the 4–12% SDS-PAGE gel, indicating significant polymerization (Figure 2C). Fluorometric analysis also indicated cross-links, with absorption and emission maxima at 320 and 400 nm, respectively. Based on the fluorometric analysis results (Figure 2D), the fluorescence of this cross-linked resilin was higher than that of full length resilin. During the purification, full length resilin exhibited heat stability up to 100 $^{\circ}\text{C}$ for 30 min. Generally, heat stability is dependent upon several factors, including time, temperature and antioxidants. Many structural proteins exhibit heat stability. In our studies, following heating of the culture supernatant at 60, 70, 80, 90, and 100 $^{\circ}\text{C}$ for 30 min, many proteins are denatured and precipitated, but the full length resilin proteins remained intact even after treatment at 100 $^{\circ}\text{C}$ for 30 min.

Construction, Expression, and Purification of 6H-resilin genes. Sequencing of cDNAs prepared from *D. melanogaster* pupas revealed the presence of the two spliced variants as reported previously (Supporting Information, part IV).¹³ Variant A that contains the ChBD was used for further work. The 975 and 1200 base pair products coding for resilin elastic repeats with or without the ChBD, respectively (6H-resChBD, amino acids 19–415; 6H-res, amino acids 18–324, Figure 1), were expressed in *E. coli*. 6H-res protein was produced as control to confirm that the chitin binding is conferred by the putative ChBD. In early experiments we discovered that the chitin binding ability of resilin was abolished due to the heat treatment (data not shown), therefore, a different purification approach was used for chitin binding experiments. Both proteins were expressed in *E. coli* and yielded approximately 100 mg/L of soluble protein, significantly more than the full length protein. Crude lysates were affinity purified by Ni-NTA chromatography (AC; Figure 3A,B) followed by IEC on Q-Sepharose column (Figure 3C,D). The affinity to chitin was assayed using both crude and pure proteins. Specific chitin binding was evident only for 6H-resChBD (Figure 4). Equilibrium adsorption isotherms were conducted and a K_{d} of 1.6 μM and B_{max} of 0.082 $\mu\text{mol/g}$ were calculated from a nonlinear regression of the adsorption isotherms (Figure 5).

Conformational Properties. Conformational properties of full length resilin have not been previously investigated. Both FTIR and CD spectroscopies were employed to probe the secondary structure of full length resilin before and after cross-linking. The FTIR spectra of un-cross-linked and cross-linked full length resilins are shown in Figure 6A,B, which includes an expansion of the amide I region for secondary structure analysis. The broad nature of the amide I bands (centered around 1650 cm^{-1}) indicates a wide range of heterogeneous conformations, and peak deconvolution suggested potential contributions from all known secondary structures (Table 1). The band between 1635 and 1655 cm^{-1} (centered around 1650 cm^{-1})

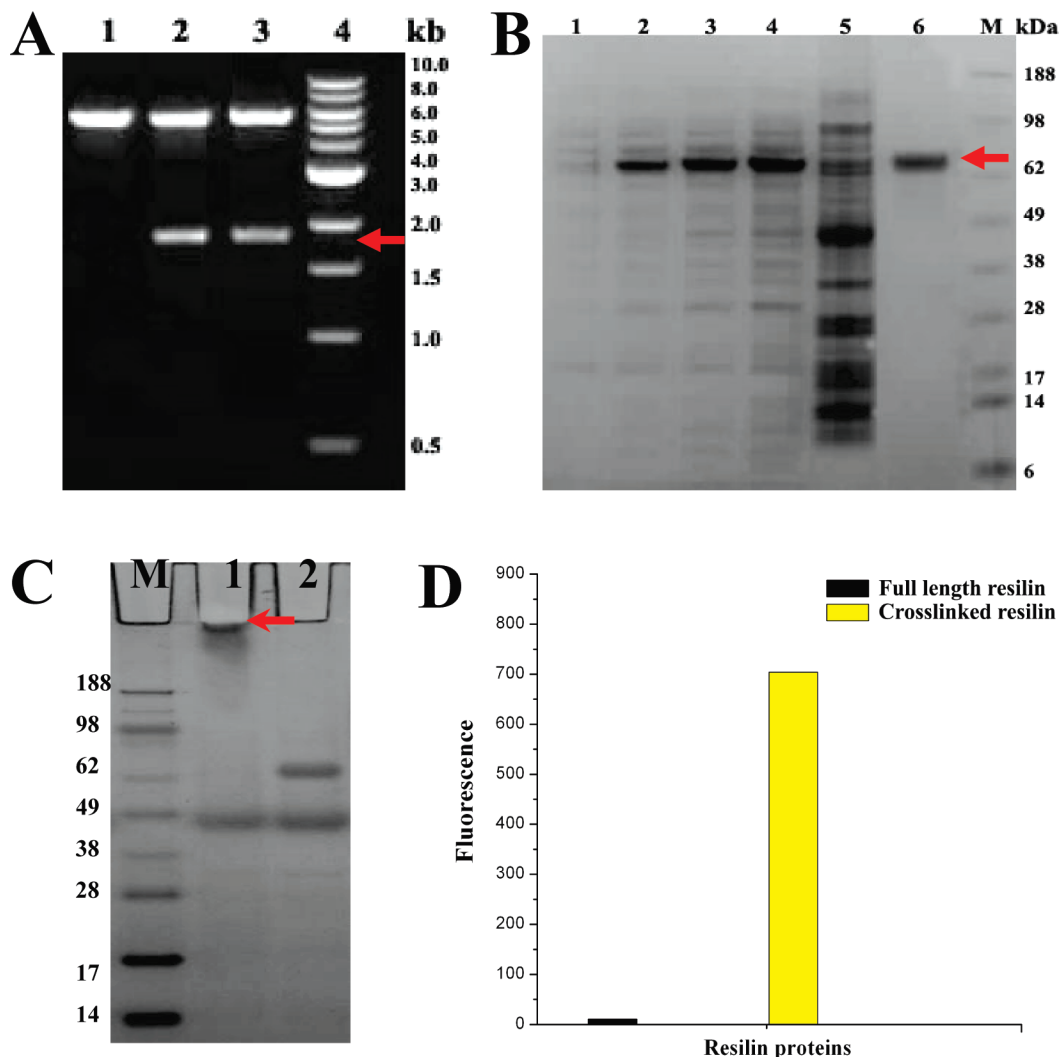


Figure 2. Gene construction, protein purification and cross-linking of soluble full length resilin. (A) Gene construction: lane 1, pET22b vector digested by *NcoI* and *NotI*; lanes 2–3, pET22b/Res plasmid digested by *NcoI* and *NotI*. Expected size of digestion product is 1818 bp (arrow). Size marker is Quick-load 1 kb DNA ladder (New England Biolabs) in lane 4. (B) 4–12% SDS-PAGE of samples from purification: lanes 1–4, cleared supernatant from lysed cells expressed for 0, 2, 4, 6 h; lane 5, precipitation from lysed cells expressed for 6 h; lane 6, soluble full length resilin purified by a heat and salting-out method (arrow). Seeblue plus2 prestained standard (Invitrogen, CA) was used as size markers. (C) HRP-cross-linking: The solution containing 50 mg/mL soluble full length resilin, 1 mg/mL HRP, and 10 mM hydrogen peroxide produces polymerized resilin, which barely enters the gel (arrow); lane 1, mixture with H₂O₂; lane 2, mixture without H₂O₂. Seeblue plus2 prestained standard (Invitrogen) was used as size markers. (D) Fluorescence of full length resilin. The absorption and emission maxima were 320 and 400 nm, respectively, for the full length and cross-linked resiliins.

exhibited characteristics of random coil configurations (more than 40%). This high degree of disorder is also consistent with the observed CD spectra (Figure 6C). Deconvolution of the CD spectrum suggests the minor contributions from coexisting secondary structures, indicating a largely unordered structure. The cross-linked resilin and the un-cross-linked full length resilin had similar FTIR and CD spectra, suggesting the similarity in the conformational properties of both samples (Table 1). These data indicate that the full length recombinant resilin chains are mobile, and sample a wide range of conformations, which is also the case for the cross-linked resilin.

Mechanical Properties of Full Length and Cross-Linked Resilin. Resilience is a measure of the ability of a material to deform and recover and it has become routine to use AFM to measure the modulus or stiffness of materials with resolution of nanometers, similar to a conventional compression tests.²¹ Force–distance curves for both samples are shown in Figure 7. Compared with 92% resilience of the partial resilin clone, rec1-resilin,¹¹ the full length resilin was $94 \pm 1\%$ and $96 \pm$

2% for the cross-linked material. Thus, the full length protein offered similar material functions to the partial clone. This resilience is superior to that of a known low-resilience rubbers, such as chlorobutyl rubber (56%), and even to high-resilience polybutadiene rubbers (80%).¹¹

Discussion

Elastic proteins are characterized by being able to undergo significant deformation, without rupture, before returning to their original state when the stress is removed. The sequences of elastic proteins contain repeated sequences of elastomeric domains, and additional domains that form intermolecular cross-links.⁵ Only a few elastomeric proteins, especially elastin, abductin, and flagelliform spider silks, have been studied for mechanical and biochemical properties, and their potential as biomaterials for industrial and biomedical applications have been documented.^{7,8,22,23} However, other elastic proteins, including resilin, have not been studied in such detail due to the difficulties in obtaining large amounts of pure resilin from natural sources.

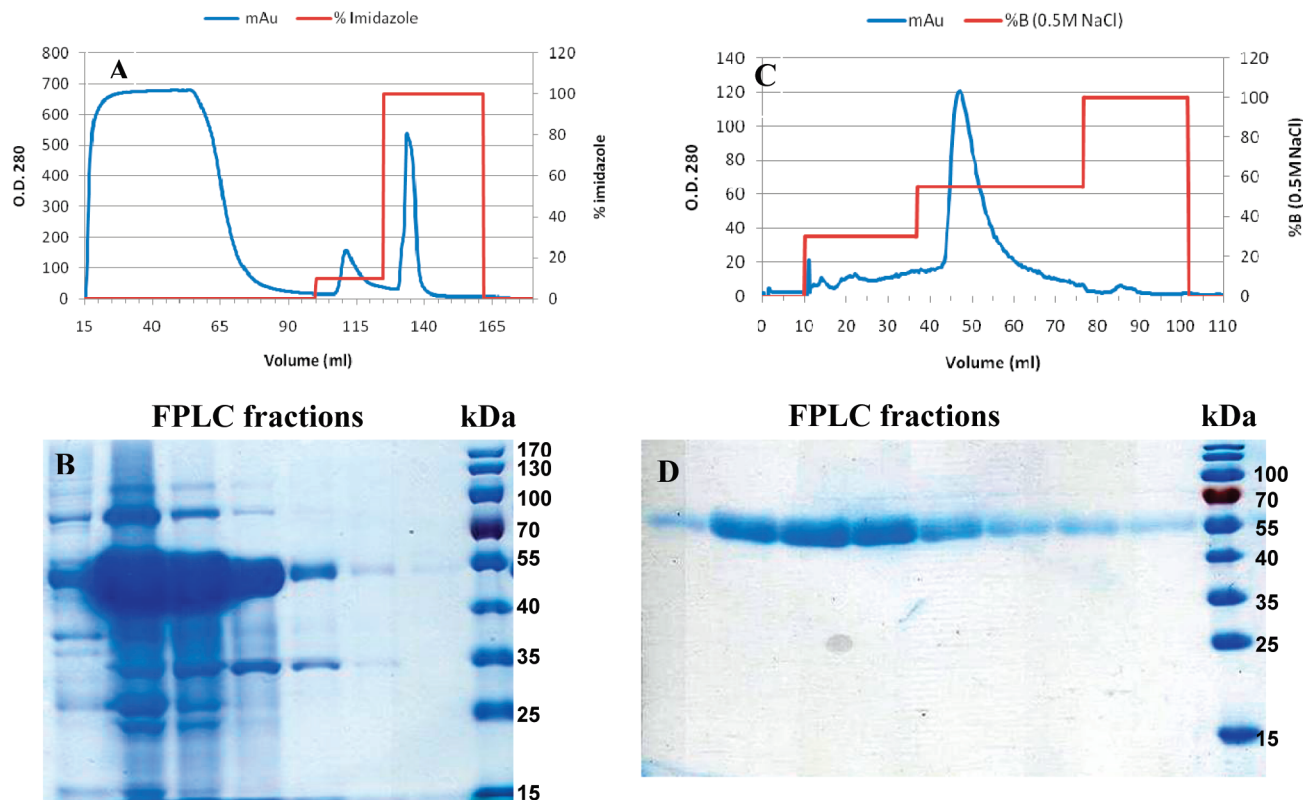


Figure 3. Purification of 6H-resChBD with ÄKTAprime plus FPLC. (A) Ni-NTA affinity FPLC chromatogram. (B) SDS-PAGE analysis of imidazole eluted protein (fractions 130–140 mL) stained with coomassie blue. (C) Ion exchange FPLC chromatogram. (D) SDS-PAGE analysis of the salt eluted protein (fractions 45–55 mL) stained with coomassie blue.

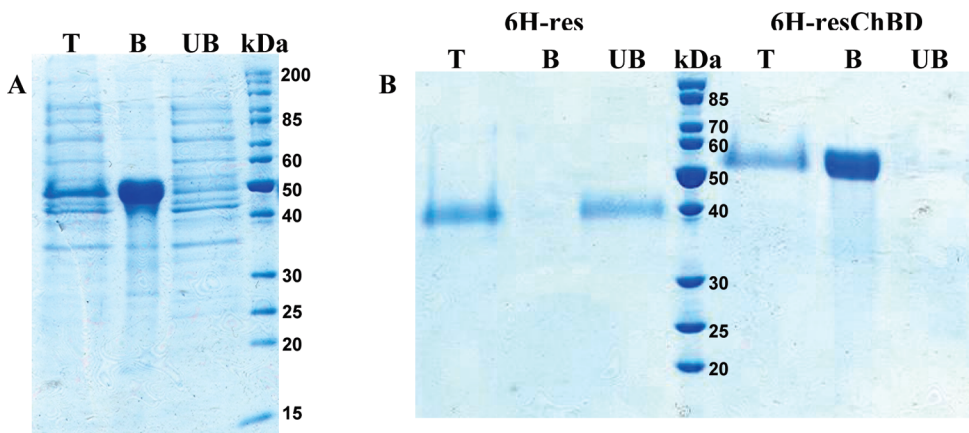


Figure 4. Chitin binding of 6H-resChBD. (A) SDS-PAGE analysis of chitin binding of 6H-resChBD crude lysate: **T**, total; **B**, 6H-resChBD affinity purified by chitin beads. Protein eluted by boiling in sample application buffer. **UB**, unbound fraction; **4B**, SDS-PAGE analysis of chitin binding of pure 6H-res (left) and 6H-resChBD (right); **T**: total protein post IEC purification; **B**, bound fraction eluted from chitin beads by boiling with sample application buffer; **UB**: unbound fraction.

Recently, a recombinant resilin-like protein (Rec1) was expressed, purified by an immobilized metal affinity chromatography (IMAC), and photochemically cross-linked to form an elastic biomaterial (Rec1-resilin) with exceptional resilience.^{8,11} Furthermore, the secondary structure of a synthetic proresilin (AN16) based upon 16 repeats from *Anopheles gambiae* (AQTPSSQYGAP), was investigated by Raman and NMR spectroscopy and showed that AN16 is an intrinsically unstructured and dynamic protein with no apparent α -helical or β -sheet features.¹²

Early attempts to produce the full length protein resulted in its expression mainly in the form of inclusion bodies.²⁴ In the present study, we cloned and expressed a soluble form of the full length resilin protein (approximately 60 kDa) containing

all three exons from *D. melanogaster*. The full length protein was purified based on heat and salting-out. Due to the loss of function of the ChBD in heat treatments we developed Ni-NTA AC followed by IEC methods that do not compromise the activity of the ChBD and allowed us to characterize binding to chitin. Over 70% of the cuticular proteins extracted from animals representing the entire arthropod kingdom contain the conserved R&R sequence. These findings indicate an important role of the ChBD (PAKYEFNYQVEDAPSGLSFGHSEMRDGDFTTGQYNVLLPDGRKQIVEYEADQQGYRQPQIRYEGDANDGSGPSGP) by anchoring the cuticular proteins to the chitin during the assembly of the protein-cuticle composite. Moreover, the fact that the R&R sequence is usually found in cuticle extracts is explained by the high degree of cross-linking of the

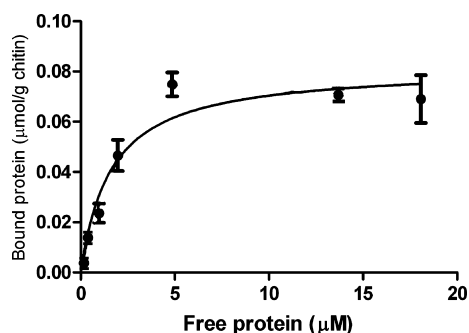


Figure 5. Nonlinear regression of 6H-resChBD binding to chitin. Different protein concentrations were incubated with 50 mg chitin beads as described in materials and methods. K_d (1.62 μM) and B_{max} (0.082 $\mu\text{mol/g}$) values were calculated from the nonlinear regression curve ($R^2 = 0.9232$).

other protein segments with the chitin that preclude their extraction.^{9,13,25,26} Consequently we suggest that the binding of the cuticular proteins occurs prior to their cross-linking by dityrosine formation, therefore the ChBD is protected from the oxidizing enzymes.

Furthermore the presence of two splice variants of resilin cDNA in *D. melanogaster* (found both by Ardell and Anderson¹³ and by us) can be explained by a requirement for a balance between chitin bound and free resilin molecules that assemble the final polymer composite in the cuticle. Furthermore, the limited available surface of chitin for binding resilin and other cuticular proteins may explain why these two splice variants evolved.

To produce high molecular weight cross-linked polymeric material, a peroxidase that is present in extracts of resilin from the adult desert locust (*Schistocerca gregaria*) was used to generate solid cross-linked material.^{8,25} Physical properties including structure and elasticity characteristics were measured for the recombinant full length resilin and the cross-linked version. Typically full length resilin contains 39 tyrosines of the 605 amino acids (6.4% composition), and resilin can be cross-linked because of the covalent cross-links between tyrosine residues. The amino acid cross-links were identified as di- and trityrosine by chemical analysis as previously reported.⁹ Because of these amino acids, resilin fluoresces with a bluish color with a maximum emission at 420 nm under ultraviolet light. The excitation maximum varies from 285 to 315 nm as the pH goes from acidic to alkaline. A maximum cross-linking near 21% dityrosines was formed in the cross-linked rec1-resilin.¹¹ By comparison, about 25% of tyrosines occur as dityrosines in natural locust wing hinge resilin.^{11,26} The full length resilin reported in this paper was cross-linked by HRP and similarly fluorescent, with the absorption and emission maxima at 320 and 400 nm, respectively.

Ardell and Andersen suggested that the amino acid composition of *D. melanogaster* resilin may induce the formation of β -turns that result in a β -spiral, which is composed of several repetitive β -turns which act as spacers between the turns of the spiral, suspending chain segments in a conformational free state.¹³ We investigated the secondary structure distribution in the full length recombinant resilin from *D. melanogaster*. The majority of the full length resilin backbone exhibited random coil configurations, indicating that the overall protein was dynamic and unstructured. This high degree of disorder was also consistent with the observed CD spectra. For cross-linked full length resilin, the structure characteristics were similar to the un-cross-linked version, which implied that a wide range

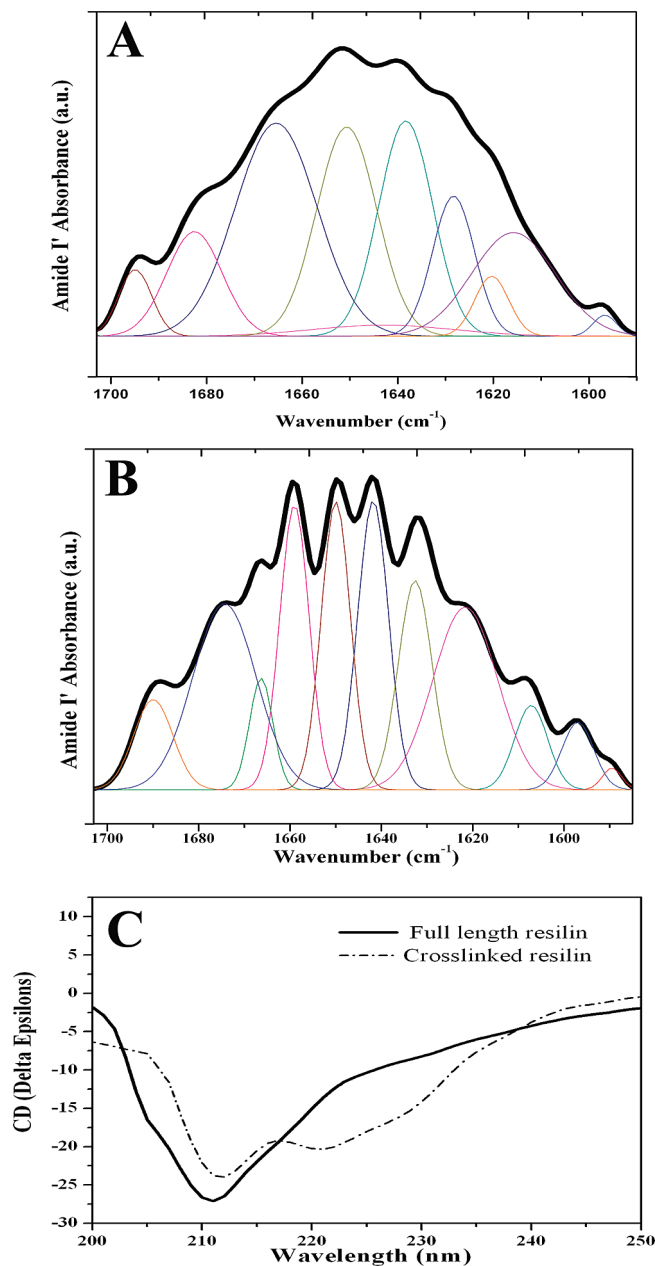


Figure 6. Secondary structure analysis of full length and cross-linked resilins. (A, B) Selected FTIR absorbance spectra of resilin film (A) and cross-linked resilin film (B) in the amide I' regions deduced after Fourier self-deconvolution. The heavy line represents the deduced absorbance band. The light lines represent the contributions to the amide I' band. (C) Far-UV CD spectra. All spectra were recorded at room temperature (25 °C) using a 1 mm path length quartz cell with resilin concentrations in the range of 1.0–3.0 mg/mL.

Table 1. Secondary Structure Analysis of Full Length and Cross-Linked Resilins by FTIR and CD

		helices (%)	strands (%)	turns (%)	random coils (%)
full length resilin	FTIR	11.9	15.3	29.1	43.8
	CD	9.8	16.1	17.5	41.6
cross-linked resilin	FTIR	14.2	19.9	24.4	41.4
	CD	9.3	15.7	17.9	42.7

of conformations were also present within the cross-linked proteins. No evidence was found for a β -spiral structure in resilin, and these results are consistent with the random-network elastomer model and the sliding β -turn model.^{27–31} The

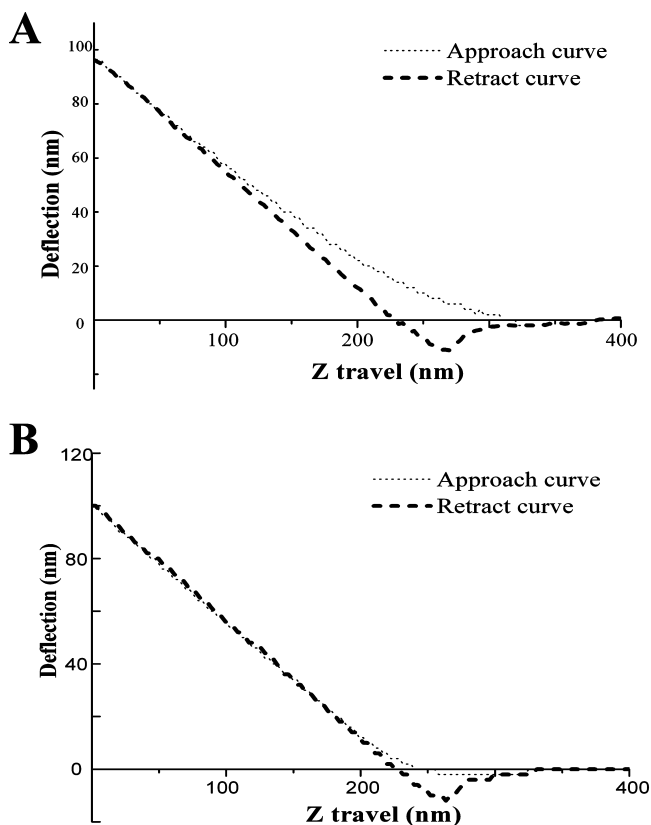


Figure 7. Elasticity of resilins by AFM. Force–distance curves were recorded for the full length resilin (A) and cross-linked resilin (B).

similarities in the contribution of disorder conformations in the un-cross-linked and cross-linked full length resilin suggest that cross-linked resilin retains sufficient chain mobility for elastomeric behavior (resilience), an important feature for some biomaterials applications.^{32–34}

Resilience is defined as the energy recovered after removal of the stress divided by the total energy of deformation. The energy of deformation/recovery is given by converting force-displacement curves into force-penetration/retraction curves and calculating the ratio of the areas under the penetration and retraction curves.^{11,21,31,32} Compared with the 92% resilience of rec1-resilin,¹¹ the full length recombinant resilin was 94% resilient, compared to 96% for the cross-linked resilin. These data suggest that both the full length and cross-linked resilins had high rubber efficiency (resilience).

In Nature, resilin must last the entire lifetime of the animal, extending and retracting millions of times. The unique elastic properties and the abundance of resilin in the jumping organs of arthropods such as froghopper and cat fleas led many researchers to the conclusion that resilin is the major constituent in the jumping mechanism of insects.³³ This assumption was recently brought to doubt by Burrows et al., that showed that resilin by itself is not sufficient to store so much energy and must act as constituent of a composite with the chitin for the purpose of jumping.²⁹ In the example given by Burrows et al., the jumping organ of the froghopper insect *Aphrophora* the pleural arch, is a composite structure containing approximately 80% chitinous cuticle and 20% resilin.²⁹

The high elasticity of resilin combined with the strength of the chitin in a composite manner enables insects to rapidly restore body shape as well as most of the energy required for the next mechanical movement either flying or jumping. Our experiments demonstrate for the first time that the putative ChBD confers specific anchoring of resilin to chitin.

The research findings provide several opportunities for future studies. Additional experiments are needed on the resilin solid material to gain further insight into the properties and potential applications for this elastomeric protein. Recently, the recombinant production of modular polypeptide materials based on the highly resilient protein resilin was reported and the proteins exhibited useful mechanical and cell adhesion behavior.³⁴ In particular, clarification of similarities and differences between resilin and elastin would be useful, in order to place each in a suitable context, such as for biomaterial-related applications. Composites of resilin and chitin may exhibit more resilient mechanical properties and may be suitable as a tissue engineering scaffolds. For example, composites of solubilized elastin with collagen and fibrin have been used as a scaffold for tissue repair of blood vessels.^{33,35} Similarly, elastin-silk copolymers have been studied for a range of biomaterial applications over the past decade.^{36–38} The stability and biocompatibility of resilin in vivo have yet to be reported. However, use of resilin as a scaffold may be of particular interest for the engineering tissues, which need to be subjected to elastic movements for proper differentiation, such as the pulsating movements of vascular tissues or skin.³⁹ In addition, the data demonstrate that the putative ChBD domain (PAKYEFNYQVEDAPSGLSFGH-SEMRDGDFTTGQYNVLLPDGRKQIVEYEADQQGYRQ-IRYEGDANDGSGPSGP) confers specific anchoring of resilin to chitin. This insight may be useful to introduce a ChBD-tag with recombinant resilin-like proteins to improve purification from crude lysates.

Conclusions

Full length recombinant resilin was generated and characterized. The material features of this protein were compared to previous studies where partial clones and proteins were generated and found to be comparable in both structure and function. Moreover, we showed that the chitin binding domain has high affinity to chitin implying for its role in the formation of the resilin-chitin composite in the cuticle.

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Supporting Information Available. DNA sequence used for expression of full length resilin; amino acid sequence of full length resilin; DNA sequence analysis of full length resilin; identification of repeat motifs; RT-PCR results of resilin gene from *D. melanogaster*. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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