Comparisons of Recombinant Resilin-like Proteins: Repetitive Domains Are Sufficient to Confer Resilin-like Properties

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Two novel recombinant proteins An16 and Dros16 have recently been generated. These recombinant proteins contain, respectively, sixteen copies of an 11 amino acid repetitive domain (AQTPSSQYGAP) observed in a resilin-like gene from *Anopheles gambiae* and sixteen copies of a 15 amino acid repetitive domain (GGRPSDSYGAPGGGN) observed in the first exon of the *Drosophila melanogaster* CG15920 gene. We compare structural characteristics of the proteins and material properties of resulting biopolymers relative to Rec1-resilin, a previously characterized resilin-like protein encoded by the first exon of the *Drosophila melanogaster* CG15920 gene. While the repetitive domains of natural resilins display significant variation both in terms of amino acid sequence and length, our synthetic polypeptides have been designed as perfect repeats. Using techniques including circular dichroism, atomic force microscopy, and tensile testing, we demonstrate that both An16 and Dros16 have similar material properties to those previously observed in insect and recombinant resilins. Modulus, elasticity, resilience, and dityrosine content in the cross-linked biomaterials were assessed. Despite the reduced complexity of the An16 and Dros16 proteins compared to natural resilins, we have been able to produce elastic and resilient biomaterials with similar properties to resilin.

Introduction

Resilin has long been recognized for its properties of high elasticity and resilience.[1] Resilin has a significant role in insect flight processes as demonstrated for insects including the desert locust (*Schistocerca gregaria*) and dragonflies of the *Aeshna* genus.[2] Resilin is also responsible for the remarkable jumping ability of the flea,[3,4] leg movement in arachnids[5] and vocalization in cicadas.[6] Recent studies suggest that resilin also possesses a very high fatigue lifetime. This was demonstrated by the observation in *Drosophila melanogaster* that the protein is expressed and localized only during the pupal stages of development, and must then remain functionally intact throughout the lifetime of the adult insect.[7] Elastin, the elastic protein found in mammalian artery walls and in skin, also displays very high fatigue lifetime, surviving for the entire lifetime of the animal.[8] These outstanding mechanical properties of high resilience and high fatigue lifetime make resilin or resilin-like materials attractive for a number of biomedical applications.

Recently our laboratory has been able to replicate many of these mechanical properties in Rec1-resilin.[7] The first exon of the Drosophila CG15920 gene, encoding a resilin-like protein[9] was cloned and expressed as a soluble protein in *Escherichia coli*. This recombinant protein was cast into a rubber-like biomaterial by rapid photochemical cross-linking. Photoinduced cross-linking of unmodified proteins (PICUP) has been demonstrated previously to be a powerful technique for cross-linking unmodified proteins.[10] Resilin-like proteins contain distinct repetitive domains containing tyrosine residues, and in nature, cross-linking occurs between tyrosine residues generating di- and trityrosine.[11,12] Using a recursive stepwise approach we have recently generated oligomeric expression constructs encoding periodic polypeptides based upon consensus repeat motifs of either the *D. melanogaster* resilin gene or a putative mosquito resilin gene.[13] These 16-repeat polypeptides were observed to exhibit heat stability and hydrophobic properties similar to that of recombinant Rec1-resilin.

In this paper, we investigate structural properties of these recombinant periodic polypeptides. We also generate cross-linked biopolymers from these proteins and compare their mechanical properties to that of Rec1-resilin to determine whether these consensus repeats are sufficient to generate highly resilient, elastic resilin-like biopolymers.

Materials and Methods

Recombinant Protein Expression and Purification. The An16 and Dros16 constructs were generated using a recursive cloning strategy described previously.[13] The Rec1-resilin construct was previously generated by cloning of the first exon of the *Drosophila melanogaster* CG15920 gene.[7] Recombinant An16 and Dros16 were initially obtained using the Studier method for auto induction of protein expression.[14] A simple nonchromatographic purification method of salt precipitation followed by heating[13] was then performed for complete and selective purification of the resilin-like proteins.

High yield expression of Rec1-resilin was recently optimized using a lactose-induced fermentation system, generating returns of up to 300 mg/mL Rec1-resilin.[15] A similar 100 L fermentation run was subsequently performed for An16 to rapidly generate bacterial cell biomass sufficient to obtain gram quantities of recombinant proteins.

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Circular Dichroism (CD) Studies. To ensure complete elimination of contaminants, An16, Dros16, and Rec1-resilin were further purified by affinity chromatography on a Ni-column and then dialyzed into a 10 mM sodium phosphate buffer, pH 7. A Jasco J-810 spectrometer was used with a 1 mm path-length quartz cell. Ten acquisitions per spectrum were run from 185−260 at 50 nm/min and the solution temperatures varied using a Jasco CDF-426S Peltier type CD/Fluorescence simultaneous measurement attachment. The measurements were taken once constant temperature was reached. All solutions, irrespective of solvent used (urea, trifluoroethanol) contained 100 μg/mL of protein in 10 mM sodium phosphate. In all cases, the buffer background was subtracted. The detector voltage remained below 600 mV for all spectral regions shown.

The fitting program interface CDPro (Sreerama, Colorado State University) was used to extract secondary structure information from the CD spectra. The reference spectra sets used were SDP42, SDP48, and SP37A. SDP42 and SDP48 contain some denatured proteins, along with soluble model proteins. SP37A includes model proteins for the polyproline II (PPII) structure. Three different fitting algorithms, ContinLL,[16,17] Selcon3,[18] and CDSSTR,[19] were used.

Hydrogel Formation. A photochemical method was used to cross-link soluble An16, Dros16, or Rec1-resilin into solid material.[7,13] The light source chosen for the present study was a 600 W tungsten-halide source (2 × 300 W lamps; GE no. 38476). The spectral output showed a broad peak from 300−1200 nm.

For production of solid samples of cross-linked recombinant An16, Dros16, and Rec1-resilin for materials testing, solutions of 200 mg/mL (20%) protein, 2 mM Ru^{2+} complex, and 20 mM of either ammonium sulfate or sodium sulfate were pipetted into clear polycarbonate molds that had been pretreated with Coatasil (1,1-dichloro-1-fluoroethane/dimethyldichlorosilane, 98:2 w/w) to prevent adhesion of the solid material to the molds. Samples were then routinely illuminated for 30 s at room temperature with the light source maintained at a distance of 15 cm from the sample.

Scanning Probe Microscopy (SPM). Disks of An16 and Dros16 were cast in clear polycarbonate molds (1.0 mm depth × 5 mm diameter). A Digital Instruments Dimension 3000 SPM was operated in force-volume mode using a probe with stiffness selected to match the stiffness of the sample. Standard silicon nitride probes with a nominal spring constant of 0.12 or 0.58 N/m were used for all samples. These samples were characterized in a phosphate-buffered saline bath at a strain rate of 1 Hz. Typically at least 3 force-volume plots (16 × 16 arrays of force-displacement curves taken over a 10 × 10 μm area) were recorded for each of the samples. Resilience is a measure of a material's ability to recover after deforming under an applied stress. It 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 the area under the force-penetration/retraction curves. Resilience was determined by converting force-displacement curves into force-penetration/retraction curves and calculating the ratio of the areas under the penetration and retraction curves.[20]

Tensile Testing. Dumbbell shaped, cross-linked samples were cast in clear polycarbonate molds using the photochemical cross-linking method previously described.[7,13] Because of the relatively soft nature of the cross-linked materials, it proved useful to embed a fine stainless steel mesh tab within the head of the dumbbell by which the samples could be easily gripped during testing.[21] Tensile tests were carried out on 9 An16 and 2 Rec1-resilin strips in PBS buffer on an Instron Tensile Tester (model 4500) at a rate of 5 mm/min and a temperature of 21 °C. The samples had a gauge length of 8 mm and were cycled initially up to a strain of 100% before testing to failure. Resilience was calculated from the cyclic test as the ratio of the area under the retraction curve to the area under the extension curve. The stress at 100% strain on the extension curve was taken as the secant modulus at 100%.

Analysis of Dityrosine in Samples. For determination of the formation of dityrosine from tyrosine in An16 and Dros16 samples, cross-linked solid materials were hydrolyzed in acid followed by HPLC as previously described.[7] In brief, hydrolyzed samples were diluted in 8% acetonitrile and 0.1% trifluoroacetic acid (TFA), and dityrosine and tyrosine amounts were calculated from the fluorescence ($\lambda_{excitation}$ = 300 nm, $\lambda_{emission}$ = 400 nm, and $\lambda_{absorption}$ = 210 nm) of the peaks detected with the series of synthesized dityrosine and authentic tyrosine standards, respectively. The solvent system was 8% acetonitrile containing 0.1% TFA in Waters HPLC system with an Alltech Alltima C18 column (4.6 × 150 mm), equipped with Waters 474 fluorescence and 486 UV detectors. The Waters Empower program was used for chromatography analysis.

Table 1. Fit Parameters for An16 and Rec1-Resilin in 10 mM Sodium Phosphate Buffer at 3°C, Obtained Using ContinLL with the SP37A Basis Set

	α-helix	β-sheet	turns	PPII	unordered
An16	0.05	0.10	0.11	0.10	0.64
Rec1-resilin	0.02	0.18	0.11	0.11	0.58

Results

Protein Expression. As shown previously,[13] when using auto induction for protein expression volumetric productivity (milligrams of recombinant protein per liter of culture) varied significantly for each protein at 220, 60, and 20 mg/L for An16, Rec1-resilin, and Dros16, respectively. To increase the productivity of An16 and to obtain larger quantities of the protein for analysis, a 100 L batch fermentation run was performed. Using the standard heat and salt purification method as described previously,[13,15] yield was increased to 450 mg/L and 45 g of the recombinant An16 was purified. An16 was maintained as a coacervate (230 mg/mL An16 in PBS) at −80 °C until required. Expression of Rec1-resilin has previously been optimized for small scale fermentation equipment using glycerol batch culture for initial growth and primary induction by IPTG at carbon source depletion, followed by new growth in lactose-induced culture,[15] resulting in yield of 300 mg/L. As Dros16 was comparatively poorly expressed, we chose not to pursue further expression of the protein.

Circular Dichroism. The CD spectra for An16 and Rec1-resilin in 10 mM sodium phosphate buffer, pH 7 are shown in Figures 1A,B respectively. Both sets of spectra exhibit minima at 195−200 nm. The minima are more intense in An16 than Rec1-resilin and also decrease in intensity as temperature increases. CDPro fit results (using ContinLL and the SP37a basis set) for the spectra at 3 °C are in Table 1, and results for all temperatures are in the Supporting Information (section S1). The secondary structure fractions obtained with ContinLL and CDSSTR were very similar. The calculations imply that the proteins are largely disordered with very little helical content and some contributions from sheets, turns, and PPII conformations.

However, Tamburro and colleagues[22] have pointed out that fitting CD spectra of peptides and nonglobular proteins such as elastin peptides and resilin using libraries derived from globular proteins is likely to give inaccurate results, especially for detection of PPII. To test this hypothesis, we have used CDPro to fit a spectrum of poly-L-proline[23] at 2 °C and neutral pH. At these conditions, poly-L-proline is known to have a predominantly PPII conformation. Even when the basis set SP37A (which includes a component for PPII) is used, a very low PPII content is calculated. The detailed results are in section S3 of the Supporting Information. This confirms Tamburro's hypothesis, and means that CDPro fitting will not detect substantial amounts of PPII; instead we must carefully examine our spectra for other indications of PPII conformations.

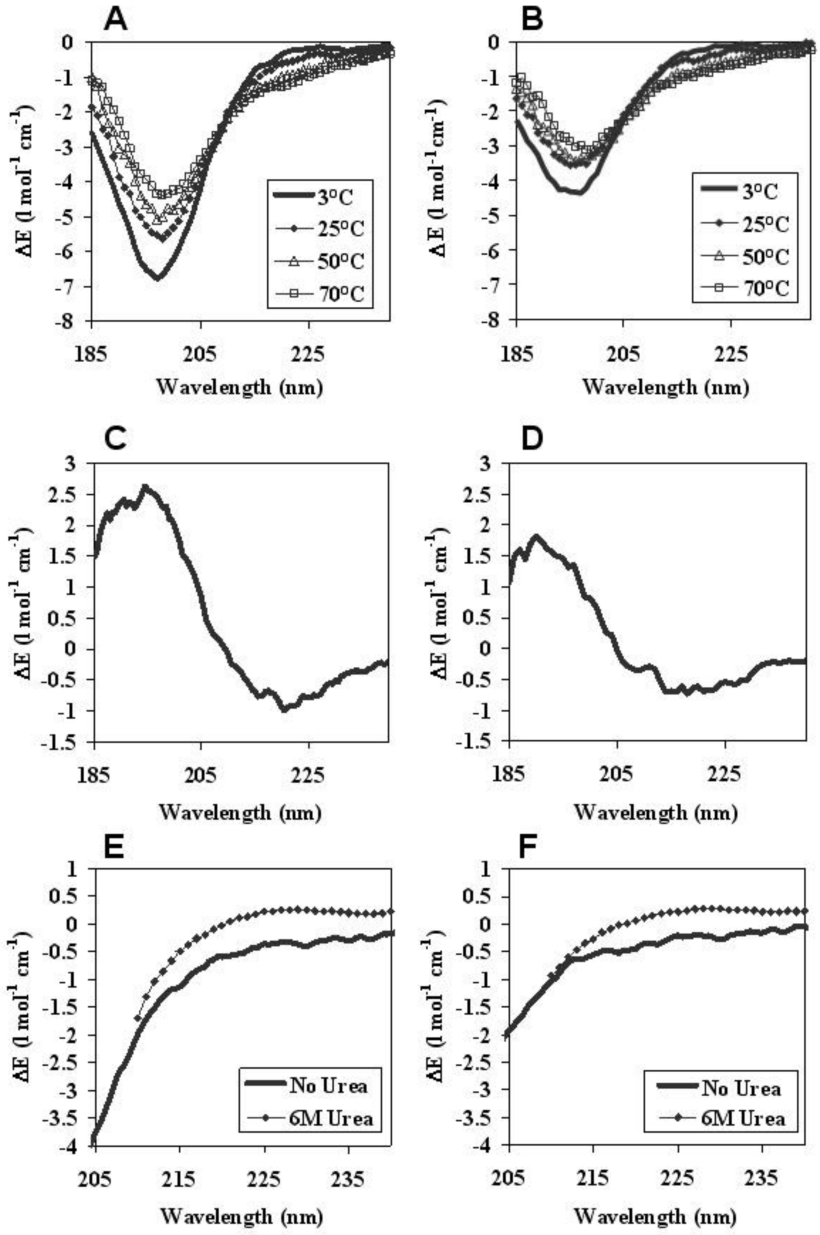

Figure 1. CD spectra of An16 (A) and Rec1-resilin (B) in 10 mM sodium phosphate buffer at a range of temperatures. Difference spectra (70−3 °C) for An16 (C) and Rec1-resilin (D) in 10 mM sodium phosphate buffer. The effect of 6 M urea on CD spectra of An16 (E) and Rec1-resilin (F) at 25 °C.

In general, PPII has several characteristics in CD spectra. A minimum is observed around 190−200 nm in a position similar to that attributed to "random coil". This minimum may be very intense (e.g., around −18 L mol^{-1} cm^{-1} for poly-L-proline) and becomes less intense as temperature increases.[23,24] A weak maximum is also observed around 220−230 nm but may not be seen in the presence of other conformations. This maximum becomes more prominent in the presence of urea,[25] as urea is known to stabilize PPII conformations.[26]

Both An16 and Rec1-resilin display minima around 195 nm. The intensities of these minima decrease with increasing temperature (Figure 1A,B), as expected for PPII structure. The greater intensity of the An16 spectrum suggests a higher PPII content in this protein. Maxima around 220−230 nm are not observed without urea, but small maxima become apparent in the presence of 6 M Urea (Figure 1E,F), again indicative of some PPII structure. This effect is not as strong as that observed with elastin polypeptides,[27] and hence suggests that the PPII content of resilins may be less than that of some elastin peptides.

Both An16 and Rec1-resilin also display isoelliptic points near 208 nm, suggesting an equilibrium between PPII and some other conformation.[24,28] Figure 1C,D are difference spectra (70−3 °C) for An16 and Rec1-resilin. CDPro fits of these difference spectra (Supporting Information section S1) indicate a conformation rich in β-sheets and turns. However, the low signal-to-noise of the spectra measured on 100ug/mL protein solutions makes identification of the positions of the minima in the difference spectra difficult. Hence spectra at higher protein and salt concentrations (250 ug/mL protein and 100 mM sodium phosphate buffer) have also been measured and fitted (Supporting Information section S2). These difference spectra show clear minima at approximately 220 nm. The CDPro fits of the spectra measured at 250ug/mL protein concentration are similar to those obtained at 100ug/mL. The deviations are mostly in the β-sheet content, which may be an artifact arising from the smaller wavelength range of the more concentrated spectra.

Others have observed spectra with similar shapes to the difference spectra of Figures 1C,D and Supporting Information

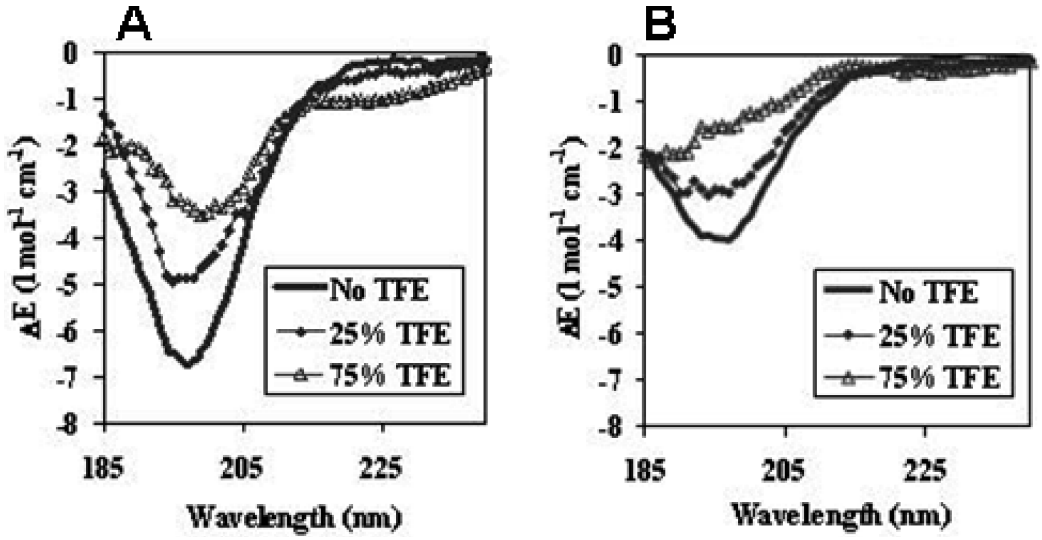

Figure 2. Effect of trifluoroethanol on An16 (A) and Rec1-resilin (B) in 10 mM sodium phosphate buffer at 3 °C.

S2C and S2D. For example, Eker and colleagues[29] attributed their results to an "extended β-strand" conformation, while Ladhokin and colleagues[30] attributed theirs to β-turns. Cann and colleagues[31] attributed a similar spectrum to a type VIa β-turn, involving cis-proline in position 3. As previous NMR studies of An16 in solution did not find evidence for cis-prolines,[32] a type VIa β-turn seems less likely than the extended β-strand or β-turn options.

The previous NMR studies of An16 in solution[32] also found that the NH groups of the "AG" sequence in An16 (AQTPSSQYG-AP) were less exposed to solvent than the other NH groups in the protein. This makes these residues potential locations for any extended β-strand or β-turn. Hutchinson and Thornton[33] have collated sequence preferences for the different types of β-turn. The highest "potentials" for type II β-turns are calculated for the QYGA sequence in An16 and the APGG sequence in Rec1-resilin. However, the observed difference spectra do not correspond to those suggested for type II β turns by Perczel and Fasman[34] (Perczel and Fasman's spectra do not have clear minima). Hence we are unable to clearly identify the conformation that arises at higher temperatures.

Trifluoroethanol (TFE) is able to induce more compact, folded conformations (such as α-helices and β-turns) in many peptides and less ordered proteins, including those based on elastin.[35] It also tends to destabilize PPII conformations.[22] The effect of adding TFE to An16 and Rec1-resilin is shown in Figure 2A,B respectively, and the fit parameters and difference spectra are in the Supporting Information, section S4. The difference spectra indicate an extended β-strand or β-turn conformation, although the fit results do not always give clear trends. The only fit method that gave a clear trend for An16 was ContinLL with the SP37a basis set, where an increase in β-sheet and α-helix and a corresponding decrease in PP2 and unordered structures were calculated. The fits were slightly more consistent for Rec1-resilin with an increase in β-sheet and decrease in unordered fractions for all fit methods. TFE did not induce a substantial increase in α-helix content, suggesting that An16 and Rec1-resilins are inherently unable to form α-helices.

The CD spectra for Dros16 are shown in Figure 3, and CDPro fit results at 3 and 70 °C are in the Supporting Information (section S5). Again, spectra and fits for higher protein concentrations are also in the Supporting Information (section S6). In contrast to the spectra for An16 and Rec1-resilin the CD spectrum for Dros16 is only weakly temperature dependent. The

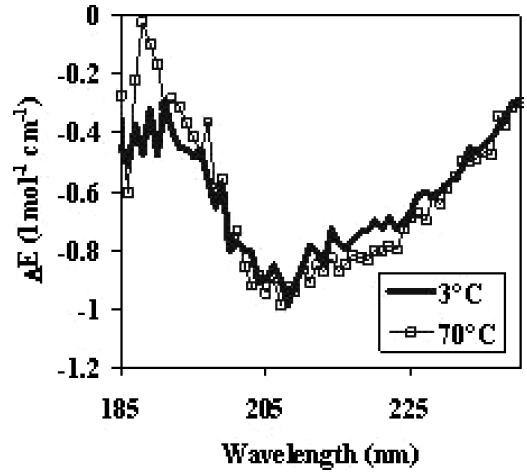

Figure 3. CD spectra of Dros16 in 10 mM sodium phosphate buffer at 3 and 70 °C. Curves at 25 and 50 °C are similar to those shown.

fit results indicate a more ordered protein with more β-structure than either Rec1-resilin or An16.

SPM. For SPM we prepared small disks (5 mm diameter by 5 mm deep) of each of An16 and Dros16, comparing results with Rec1-resilin. Elvin and colleagues[7] have previously demonstrated using an SPM operated in force mode that cross-linked Rec1-resilin displayed negligible hysteresis upon compression and was 92% resilient. Representative force−distance curves for An16 and Dros16 are shown in Figure 4. SPM suggests that cross-linked An16 is the softest material of the three materials with an average penetration of 495 (±199) nm, compared to 324 (±23) nm for Rec1-resilin and 235 (±39) nm for Dros16. Despite the significant variability across samples for penetration, resilience measurements were very similar for all samples. All cross-linked materials show negligible hysteresis upon compression with cross-linked An16 having a resilience of 98 (±4)%, compared to 97 (±3)% resilience for Rec1-resilin and 91 (±5)% resilience for Dros16.

Tensile Testing. Dumbbell shaped samples were prepared for An16 for comparison with Rec1-resilin. Dros16 was not analyzed by tensile testing as it required significant amounts of protein (140 mg) per sample, and with the comparatively poor expression of Dros16 it was deemed unsuitable for further analyses.

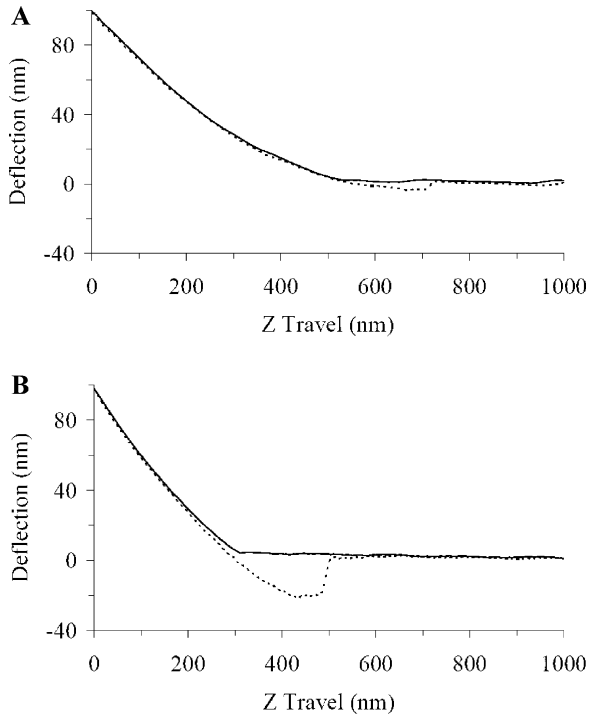

Figure 4. Representative force−distance curves recorded for cross-linked An16 (A) and Dros16 (B) are shown. The approach curve is represented by the solid line, and the retract curve is dotted.

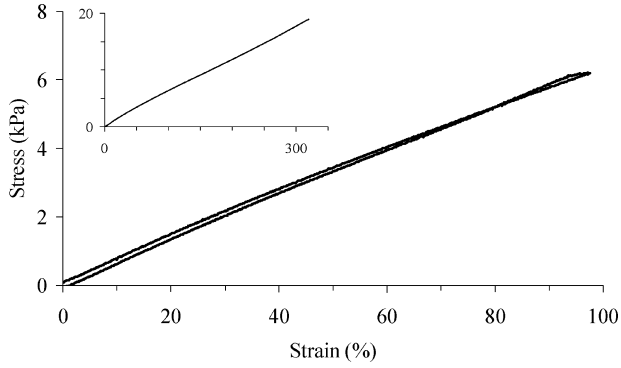

Figure 5. Typical stress strain plots for a strip of An16 tested in buffer. Sample cycled to 100%, showing resilience of 97%, and then tested to failure showing extension at break of 321% (smaller internal image).

The resilience of the cross-linked An16 in phosphate buffered saline was shown to be 94 (±3)%, consistent with SPM studies. Irrespective of whether 20 mM sodium sulfate or 20 mM ammonium sulfate was used as the catalyst, resilience properties were similar at 95.4 (±2.5)% and 93.3 (±3.4)%, respectively. The An16 samples were highly elastic with a strain at break of 347 (±61) %, a stress at break of 17.1 (±5.3) kPa, and a modulus of 5.7 (±2) kPa. A typical stress−strain curve for An16 is shown in Figure 5.

Within the limits of the experiment, Rec1-resilin was shown to be 100% resilient. This highly elastic material exhibited a strain at break of 250 (±43)%, a stress at break of 55 (±10) kPa and a modulus of 26 (±9) kPa.

Dityrosine Estimation. Photo-cross-linked disks of Rec1-resilin were determined to have 18.8% of available tyrosine residues cross-linked, compared to 14.3% for An16 and 46% for Dros16.

Discussion

Previous studies have demonstrated that An16 and Dros16, periodic polypeptides based upon the consensus repeat motifs of a putative mosquito resilin gene and the *D. melanogaster* resilin gene, respectively, exhibited properties very similar to that of the only previously expressed resilin-like gene, Rec1-resilin.[7] Both An16 and Dros16 are relatively heat stable and can be precipitated from solution at low ammonium sulfate concentrations (20%). These recombinant proteins also form coacervates at low temperature.[13] These properties in An16 and Dros16, as well as Rec1-resilin, have been exploited to develop a facile "heat and salting out" purification method.[18,20]

In the present study we have used CD for identifying secondary structure in our recombinant proteins. Electronic CD has been extensively used to investigate secondary structure in solutions of elastic protein precursors and peptides, and CD has been widely used as a reliable method for revealing PPII protein structure.[35–37] Despite having significantly different protein sequences, An16 and Rec1-resilin have similar spectra and behave in similar fashions when solvents such as urea or TFE are added. The resulting spectra have characteristics consistent with largely disordered proteins that contain little helical content and some contributions from sheets, turns, and PPII conformations. These results are consistent with a previous study of peptides based upon the repetitive sequences of resilin.[37] Indeed, both the peptide study and our own protein studies suggest resilin is structurally similar to other elastomeric proteins including elastin and titin and supports the hypothesis that PPII could have an important role in the promotion of elasticity.[36]

Surprisingly, the data from our study of Dros16 suggests a different structural model. Dros16 is predicted to be more ordered than Rec1-resilin and contains a high level of β-structure. This ordered structure was unexpected, since the repetitive sequence motif was based on one repetitive element found in Rec1-resilin and containing amino acid homology to at least one peptide used in the recent CD study by Bochicchio and colleagues.[37] Further characterization of this protein is required to confirm these preliminary data.

Despite this significant difference in secondary structure, Dros16 is observed to behave in a similar way to An16 and Rec1-resilin in mechanical properties. All three proteins can be photochemically cross-linked to form biopolymers, as was previously demonstrated for Rec1-resilin.[7] SPM analysis confirms that all three biopolymers are highly resilient and elastic, and penetration measurements suggest that they are all soft materials. Instron data from An16 and Rec1-resilin support the mechanical properties revealed by SPM analysis. Both An16 and Rec1-resilin are highly resilient (94 and 97%, respectively) but An16 has a lower modulus (5.7 kPa) than Rec1-resilin (25.5 kPa), as measured using SPM by the higher penetration of An16 versus Rec1-resilin. Dros16 is poorly expressed relative to the other two recombinant proteins, and ongoing optimization of expression is required before we can obtain sufficient protein to perform tensile testing. However, based upon SPM data, Dros16 is also highly resilient and is predicted to have a modulus greater than Rec1-resilin.

Measurement of the percentage of available tyrosine residues that are transformed into dityrosine during cross-linking demonstrates that An16 and Rec1-resilin have 14.3 and 18.8% dityrosine, respectively. This compares favorably with the previous measurement of 21% dityrosine in cross-linked Rec1-resilin[7] as well as 25% dityrosine in natural locust wing hinge resilin.[12] Other recombinant resilins generated in our laboratory also have similar dityrosine contents (Kim et al., in preparation).

The unexpectedly high estimation of dityrosine in cross-linked Dros16 suggests a high degree of intramolecular cross-linking, as no dramatic increase in modulus is observed relative to An16 and Rec1-resilin. This may be a reflection of the higher order observed by CD in Dros16.

Although difficult to extrapolate elasticity from SPM, Instron data suggests that elasticity is inversely related to dityrosine content, assuming that the dityrosine is intermolecular in nature. An16 has less dityrosine and more elasticity than Rec1-resilin. It is therefore tempting to assume that the increase in intermolecular dityrosine in Dros16 will result in a stiffer biomaterial than the other cross-linked recombinant resilins. Tensile testing is needed to confirm this prediction. The current data shows that photo-cross-linked Dros16 does not conform to the classic unordered structure and common PPII conformations present in An16, Rec1-resilin, and other bioelastomers such as elastin and titin. This work is ongoing.

As well as the YGAP repetitive motifs observed in the first exon of all characterized resilin genes, the third exon is also tyrosine rich, typically in the form of YGPP repetitive motifs. In our studies to date, transcripts isolated from a range of insect species always contain the first and third exons with the second exon being removed in some transcripts. At the present time, we do not have data regarding the roles of these YGPP repeats, although efforts are currently underway to express and characterize these different gene regions either individually or as full length transcripts. Conceivably the YGPP domains have roles in di- or trityrosine formation and hence biomaterial properties of resilin in insects, the effects of which are as yet unknown. What is clear from this and previous studies of the *Drosophila* and *Anopheles* resilin genes is that the repetitive domains observed in exon 1 of the resilin gene are sufficient to endow resilin-like properties to the resulting recombinant proteins and the biopolymers generated from them. In this paper, we investigated structural and material properties of novel recombinant periodic polypeptides. We generated biopolymers from these recombinant proteins and compared biomechanical properties to that of Rec1-resilin and other native resilins. Despite the loss of genetic diversity of the polymeric peptides relative to their natural resilin genes, these consensus repeats are sufficient to generate highly resilient, elastic, resilin-like biopolymers.

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Supporting Information Available. Tables and graphs showing CD fit parameters for An16 and Rec1-resilin in 10 mM sodium phosphate buffer; CD graphs and fits measured at higher protein concentrations; an evaluation of CD fitting for poly-L-proline; the effect of adding TFE on conformation of An16 and Rec1-resilin and fit parameters for Dros16. This material is available free of charge via the Internet at http://pubs.acs.org.

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