Expression, Crosslinking, and Developing Modulus

Master Curves of Recombinant Resilin

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1.1 Abstract

Resilin is a disordered elastomeric protein found in specialized regions of insect cuticles, where low stiffness and high resilience are required. Having a wide range of functions that vary among insect species, resilin operates across a wide frequency range, from 5 Hz for locomotion to 13 kHz for sound production. We synthesize and crosslink a recombinant resilin from clone-1 (exon-1 + exon-2) of the gene, and determine the water content (approximately 80 wt%) and dynamic mechanical properties, along with estimating surface energies relevant for adhesion. Dynamic moduli master curves have been developed, by applying the time-temperature superposition principle (TTSP) and time-temperature concentration superposition principle (TTCSP), and compared with reported master curves for natural resilin from locusts, dragonflies, and cockroaches. To our knowledge, this is the first time dynamic moduli master curves have been developed to explore the dynamic mechanical properties of recombinant resilin and compare with resilin behavior. The resulting master curves show that the synthetic resilin undergoes a pronounced transition with increasing ethanol concentrations, with the storage modulus increasing by approximately three orders of magnitude. Although possibly a glass transition, alternate explanations include the formation of intramolecular hydrogen bonds or that the chitin binding domain (ChBD) in exon-2 might change the secondary structure of the normally disordered exon-1 into more ordered conformations that limit deformation.

Keywords

Resilin; Doubly-shifted modulus master curves; Glass transition temperature; Recombinant resilin; Hydrogen bonds; Adhesive properties, Temperature dependence, Concentration dependence, Hydrogel.

1.2 Introduction

First discovered by Weis-Fogh in 1960¹, resilin is an elastomeric protein similar to elastin, gluten, gliadin, abductin, and spider silks. It has 50-60% water at neutral PH (drying over P₂O₅, microbalance). It reportedly suffers neither from creep nor stress relaxation even after weeks of straining and can be stretched to 300% of its resting length, though experimental details are unclear¹⁻². Furthermore, Lyon et al.³ reported that resilin has long fatigue life, sustaining millions of cycles in nature without failure. Multiple functions have been described⁴ for resilin, including roles in sound production (cicada tymbal mechanism⁵), feeding (salivary pump of assassin bugs⁶), and structural support in leg joints (cockroach⁷). Resilin has natural functions within a broad range of frequencies from 6 Hz in cockroach locomotion⁴ to 13 kHz for sound production in cicada⁸ and moth⁹.

Most insect resilin genes have three protein coding regions, or exons. Among the exons, both exon-1 and exon-3 contain elastomeric repeating units, while exon-2 is designated as the chitin binding domain (ChBD)¹⁰. However, some insects do not contain ChBD in their resilin, as in dragonfly tendons¹¹, which only have exon-1.

Identifiable under UV light exposure, resilin glows and emits blue fluorescence due to di-tyrosine crosslinking¹². Molecular cloning can be used to synthesize recombinant protein from the purified RNA of the insects. However, it's important to identify the stage of insect development when the protein is expressed at high levels to facilitate molecular cloning. Elvin et al.¹² discovered that expression levels of the *Drosophila* CG15920 resilin gene were a few thousand-fold higher in light colored pupae compared to any other stage of the *Drosophila* life cycle.

The amino acid sequences, composition and resulting mechanical properties differ depending on resilin function for various insect species, though there are some highly conserved regions in the protein sequences. The variations suggest that the sequence and properties may be tuned through natural selection. Characterizing recombinant resilins will aid in understanding the impact that these variations in sequence and composition have on the properties of resilin and could be important for future applications of synthetic resilin for biomedical or other purposes. Elvin et al.¹² were the first, followed by Qin et al.¹³, to synthesize synthetic resilin from recombinant protein. The mechanical properties of these synthesized resilins indicated that they were much weaker compared to natural resilin. Although Qin et al.¹³ characterized the chitin-binding properties of the recombinant resilin exon-1 with ChBD, equivalent to our clone-1, neither they nor others determined the dynamic mechanical properties of the recombinant resilin compared with natural resilin to understand the fundamental properties as functions of frequency, temperature, and diluent concentration, nor further explored the potential utility of recombinant resilin-ChBD fusions for creation of novel biomaterials. King¹⁴ and Choudhury¹⁵ conducted dynamic mechanical analysis (DMA) on natural dragonfly and cockroach resilins to determine the glass transition frequency and explore their dynamic mechanical properties using both time-temperature and time-concentration superposition.

During this work, recombinant resilin was synthesized from exon-1 and exon-2 (clone-1) of the gene. Although synthesis and characterization of the three exon sequence seen in many insects would be very desirable, we confirmed the finding by Qin et al.¹³ that yield is very poor compared to partial clones. We have continued to work with the same partial clone^a

^a Although purification under denaturing conditions could be used to concentrate low yield materials, it destroys chitin-binding activity. Thus, in order to work with protein with native

characterized in the work of Qin et al.¹³, extending measurements to include dynamic mechanical properties, water content, and adhesive properties not addressed by prior work. This choice of clone-1 is also believed to be an important first step in incorporating the influence of ChBD (exon-2), which is present in most insect resilins, though not all.

Dynamic mechanical properties were measured in water and at several different ethanol concentrations, the latter being used to shift the master curve to the left so that the prominent viscoelastic transition frequency moves into the measurable frequency range. Finally, master curves were developed by applying the time-temperature concentration superposition principle (TTCSP) to determine the storage modulus (E'), loss modulus (E''), and $\tan \delta$ over a wide range of frequencies for comparison with results reported for natural locust, dragonfly, and cockroach resilins.

1.3 Materials and Methods

1.3.1 Plasmid Construction. *Cloning of resilin genes.* Total RNA was extracted from light colored *Drosophila melanogaster* pupae of the Canton S fruit fly strain using TRIZOL Reagent (Life TechnologiesTM, USA). A reverse transcription of mRNA was conducted to obtain complementary DNA (cDNA) using an AccuScript High Fidelity 1st Stand cDNA synthesis kit (Agilent Technologies, USA) with oligo (dT) primer according to the manufacturer's instructions. Gene specific primers were used to amplify the target segment using the cDNA as a

properties and to advance on prior published work, we limited our research in the present work to clone-1, purified by non-denaturing nickel-chelate chromatography, which we believe to be more relevant to insect physiology.

template and the following Polymerase Chain Reaction (PCR) program: 94°C for 5 min, 32 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 1:30 min, 72°C for 5 min. The PCR product was ligated to the pGEM T-Easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions. Following transformation of *E. coli* with the resulting vectors (as described below), a clone was selected based on resistance to carbenicillin (100 µg/ml).

For sub-cloning into the expression vector, the following gene-specific primers were used to amplify clone-1 (exon-1 + exon-2): Forward primer 5'-CTCGAGCCGGAGCCACCAGTTAACTCGTA-3', Reverse primer 5'-CTGCAGCTAAGGACCGCTGGGACCACTGC-3'. The resulting amplified DNA fragment was prepared for insertion into the bacterial expression vector PColdI (TaKaRa Bio Inc., NJ, USA) by partial digestion with restriction enzymes *XhoI* and *PstI*. The expression vector PColdI was also digested with *XhoI* and *PstI*. A 10 µl ligation mixture was prepared combining T4 DNA ligase with the digested insert and PColdI vector.

Transformation. An aliquot of α -Select silver competent cell (Bioline Inc., USA) were transformed according to the manufacturer's instructions. The correct resilin clone was verified by DNA sequence analysis of the plasmid isolated from a single carbenicillin-resistant colony using a plasmid mini-prep kit (Qiagen, USA). The selection of the insects and cloning procedure were in accordance with accepted procedures.

1.3.2 Protein Expression. The PColdI expression vector encodes an N-terminal polyhistidine tag and a Factor Xa protease cleavage site for in-frame fusion with proteins. The former enables affinity purification of the protein on a nickel-nitrilotriacetic acid (Ni-NTA) affinity resin, while the latter allows removal of the polyhistidine tag, if desired. The expression vector was transformed into *E. Coli* strain BL21 (Bioline Inc., USA). The strain was grown overnight with shaking at 37°C in 2xTY with 100 µg/mL carbenicillin. The cultures were used for inoculation of

100mL 2xTY culture volume with 100 µg/mL carbenicillin to a 0.10 OD (A_{600}) and allowed to grow to mid-log phase, 0.45-0.50 OD at 37°C in a shaker (220 rpm for 3 hr). PColdI is a cold shock expression vector, meaning that it directs expression of the recombinant polyhistidine-tagged protein at 15°C, while expression of all the other cellular proteins is suppressed. Cultures were incubated at 15°C for 30 min and then induced by IPTG (Isopropyl β-D-1-thiogalactopyranoside) to final concentration of 1mM. The induced cultures were allowed to grow with vigorous aeration for 24hr at 15°C in a shaker. Bacteria were harvested by centrifugation (12000 g for 20 min at 4°C), and pellets were stored at -80°C.

1.3.3 Protein Purification. Cell pellets were thawed and re-suspended in 35mL extraction buffer (20mM Tris-HCl, pH 8.0, 5mM imidazole). Cells were lysed with a sonicator (15 sec continuous followed by 15 sec pause from sonication for 6 cycles) in an ice-water bath. Lysed cells were centrifuged at 15000 g at 4°C for 20 min to pellet the cell debris. The soluble protein fraction, consisting of the resulting clarified supernatant, was filtered with a 0.45 µM syringe membrane (Millex-HV Syringe Filter Unit, item # SLHV033RS). Recombinant resilin was purified by affinity chromatography on 5ml HisTrap High Performance (HP) Ni-NTA pre-packed column, AKTApurifier plus Fast Protein Liquid Chromatography (FPLC) (GE, Sweden), which was pre-equilibrated following the protocol provided by the manufacturer. The purification protocol used two buffers (extraction buffer; 20mM Tris-HCl, pH 8.0, 5mM imidazole and elution buffer; 20mM Tris-HCl, pH 8.0, 200mM imidazole) and the following steps: (1) 5-10 column volumes (CV) of extraction buffer at 5 mL/min, (2) 35 mL injection of the lysate in extraction buffer at 5 mL/min, (3) 5-10 CV wash with the extraction buffer, (4) elute the protein from the column using elution buffer, FPLC at 1 mL/ min. The column was regenerated for subsequent uses according to the manufacturer's protocol. Purity and recovery

levels of the protein were assessed by Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE). Protein identity was verified by peptide mass fingerprinting using mass spectrometry.

1.3.4 Scale-Up. The auto-induction method was applied to protein expression scale-up using the Studier protocol¹⁶ with some modification. Briefly, a total of 5 μ L of glycerol stock was added to 10mL 2xTY culture volume and grown overnight with 100 μ g carbenicillin at 37°C in a shaker, and this culture was used for inoculation of 500 ml ZYP 5052 to A_{600} of 0.015 OD. This culture was grown with vigorous aeration in a 2L baffled flask at 300 rpm for 6 hr at 37°C, long enough to obtain a high cell density (A_{600} = 4-5), before transferring to 15°C at 300 rpm for 24 hr for protein expression.

1.3.5 Freeze Drying of Protein. The recombinant resilin was dialyzed (dialysis tubing pore size 12 kDa) against H₂O overnight at 4°C. Water was replaced every 2-3 hrs. The dialyzed protein was lyophilized using a 4.5 Liter Freeze Dry System (Labconco model #7751020).

1.3.6 Crosslinking of Recombinant Resilin. Photo or chemical crosslinking methods were used to produce a solid resilin hydrogel. Solid hydrogel does not form if the protein concentration is less than 100 mg/ml for either method¹². Consequently, a resilin concentration of 200mg/ml was used for chemical crosslinking following the procedure described by Malencik and Anderson¹⁷ and photo crosslinking, following the method described by Fancy and Kodadek¹⁸ with slight modification. For chemical crosslinking, horseradish peroxidase (HRP) (50 μ g/mg of resilin) and superoxide dismutase (50 μ g/ml of resilin solution) were added followed by dropwise addition with stirring of 35% hydrogen peroxide to a final concentration of 5 mM to initiate crosslinking. For photo crosslinking, two 150 W tungsten-halide lamps were used. Lyophilized resilin was dissolved in phosphate buffered saline (PBS) to a final concentration of

200 mg/ml. Ammonium persulfate (APS) (20 mM final concentration) and $[\text{Ru}(\text{bpy})_3]^{2+}$ (2mM final concentration) were added to the resilin solution, which was then poured into an 8mm x 1.5mm x 1mm polytetrafluoroethylene (PTFE) mold and positioned approximately 15 cm away from the light source for 4-5 min at room temperature for photo crosslinking.

1.3.7.1 Thermogravimetric Analysis (TGA). Thermogravimetric analysis is a thermal analysis method to measure the water content of a sample through evaporation as a function of increasing temperature¹⁹. Thermogravimetric analysis was conducted using a TA Instruments TGA SA Q5000 to measure the water content in the crosslinked solid resilin-like hydrogel sample. During the process, temperature was increased to about 500°C, allowing any water to evaporate.

1.3.7.2 Dynamic Mechanical Analysis (DMA). Dynamic mechanical analysis is a technique to characterize the viscoelastic properties of polymers, where the complex modulus is calculated from measured displacement and load data, along with geometric properties of the specimen. During DMA tests, frequency and temperature can both be incremented to measure the resulting response, and a master curve can be generated from these results by applying the time temperature superposition principle (TTSP) to assess dynamic mechanical properties of polymers over a wide range of frequencies. In some cases, a complete temperature sweep cannot be carried out due to temperature and frequency limitations; for example, sub-zero temperature tests are not possible for some hydrogels requiring water immersion. In such cases, adding ethanol to the immersion water can be used to shift the curve to the left along the log frequency axis to bring the glass transition temperature into the measurable range, reportedly through effectively reducing the hydration level within the specimen²⁰. The reduced water concentration

due to ethanol in the test chamber draws water from the hydrogel. For example, according to Gosline and French²¹, an elastomeric material (elastin) stiffened even with only 10% water loss.

During the experiments, the dynamic mechanical testing was conducted using a custom-built DMA suitable for tensile tests on small, soft samples¹⁴. In the DMA, each resilin sample, synthesized from the recombinant proteins, was attached in a butt joint configuration to the pin head of an insect pin using a cyanoacrylate adhesive and accelerator (Loctite Super Glue and Loctite 712, Loctite, USA). The pin shaft was then clamped to the shaft of an electromagnetic oscillator (2160B, Data Physics Corporation, UK). The other end of the resilin sample was attached in a similar fashion to the head of a second pin attached to a force gauge (Futek, USA). Some nominal force was applied to avoid slackness during the dynamic testing. A custom-made transducer was used to measure the displacement, where the oscillator shaft was attached to a stainless steel cantilever beam instrumented with two strain gauges in a half-bridge configuration¹⁴. The dynamic mechanical properties of the resilin samples were measured by driving the oscillator with a custom, swept sine LabVIEW program (National Instruments, Austin, USA), and the inducing force and resulting displacements were recorded with a data acquisition card (PCI-4461, National Instruments, Austin, USA) capable of simultaneous sampling. Both the displacement gauge and force gauge signals were amplified through an amplifier (PA30E, Data Physics Corporation, UK) and sent to the data acquisition card. A schematic diagram of the experimental setup¹⁴ is shown in Figure 1. A small custom-made immersion chamber maintained specimen hydration (at levels dependent on ethanol concentration) and temperature control during testing. A slit was made on each end of this immersion cell, sufficiently wide so the insect pins could move without friction, yet narrow

enough so that surface energy would hold the water inside the chamber without leaking^b. Accuracy of the instrument was verified by conducting an experiment on a silicone rubber sample both in this custom made DMA and in a commercial DMA (TA Instruments Q800, New Castle, DE, USA). The storage modulus from the custom-made DMA was within 2.9% of the results from the commercial DMA at room temperature. Moreover, results from experiments conducted in both air and water for the silicone rubber using the custom made DMA with immersion chamber to test the water drag and the moduli were within 2.3% at room temperature. The effect of possible friction of the moving pin against the environmental chamber slit was tested by conducting experiments with and without the chamber in place. The resulting moduli were within 2.5%, suggesting that the effect of friction was negligible.

Data was collected under conditions of varying temperature, oscillation frequency, and ethanol concentration. Temperatures of the immersion liquid were recorded using a bead thermistor (Honeywell, USA) and controlled by circulating an antifreeze-based coolant using a LAUDA RE206 recirculating water bath and four stainless steel tubes passing through the immersion

^b Although the surface energy of water is normally sufficient to avoid leakage through slits less than 1.2 mm wide, the addition of ethanol reduced the surface energy, resulting in leakage. Adding high vacuum silicone grease (Dow Corning, USA) outside the chamber slits prevented leakage through the slits without introducing friction on the insect pins during the dynamic testing.

chamber. It would be useful to conduct the higher ethanol concentration experiments at lower temperature, but due to instrument limitations, temperatures below -5°C were not attempted.

Ethanol was diluted using distilled water to obtain the following six ethanol concentrations: 0%, 40%, 65%, 73%, 85% and 95%. During the water immersion experiment, temperature was increased from 2.3°C to 57.4°C for 0% ethanol and multi-frequency data was collected at $5\text{-}6^{\circ}\text{C}$ intervals. Experiments with 40% ethanol began at room temperature, after which the solution was allowed to reach thermal equilibrium at 57.4°C . Data was then collected while temperature was decreased towards -1°C . Similarly^c, temperature was changed from low to high for 65% ethanol, high to low for 73% ethanol and again low to high for 85% ethanol while multi-frequency data was collected at similar temperature intervals.

At each temperature, data was recorded at discrete frequencies ranging from 1 Hz to 120 Hz at 2 to 5 Hz intervals. Stresses were calculated by dividing the load output by the cross-sectional area (measured while immersed in water at room temperature) of each sample; measured actuator displacements were divided by the initial distance between pinheads to calculate the strains. Moduli were calculated from the ratio of stress and strain amplitudes during cyclic loading, and phase differences were obtained between displacement and load signals. Storage modulus, loss modulus, and $\tan \delta$ were calculated at each corresponding frequency and temperature. Master curves of the recombinant resilin were generated from the experimental data and compared with master curves obtained from the natural resilin of cockroaches¹⁵ and dragonflies¹⁴.

1.4 Results and Discussions

^c Though unconventional, this reduced test time; no anomalies were noted, but could be possible.

1.4.1 Protein Purification. Following affinity chromatography of polyhistidine-tagged clone-1 protein, analysis by SDS-PAGE yielded a single protein of approximately 50 kDa (Figure 2), which is slightly larger than that obtained by Elvin et al. for exon-1¹², and the same size as that found by Qin et al. for Exon-1 plus ChBD¹³, which is same protein used here. The purity level of recombinant clone-1 resilin was very high, as evident from the lack of substantial amounts of other proteins in lane 2.

1.4.2 Mass Spectrometry. Identity of purified recombinant resilin was verified using matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS). Analysis by MALDI-MS yielded detectable peaks corresponding to the peptide fragments comprised of the expected amino acid sequences (Supplementary information, Fig. A.1 and Table A.1).

1.4.3 Crosslinking. A pilot study of chemical crosslinking was conducted on the soluble protein using chemical crosslinking catalyzed by horseradish peroxidase. Crosslinking was verified by performing SDS-PAGE, as shown in Figure 3. Crosslinked protein was excluded from the gel due to an increased overall size of crosslinked protein, relative to soluble resilin (Fig. 2). Crosslinked proteins also fluoresced blue when exposed to UV light (data not shown), which was evidence of di-tyrosine crosslinking, according to Elvin et al¹². We tested crosslinking with and without superoxide dismutase, which usually increases the crosslinking density¹², also with different concentration of proteins, and for all cases we got similar results (Fig. 3). After the pilot study, hydrogels were casted in a 1mm x 1.5mm x 8mm PTFE mold.

1.4.4 Thermogravimetric Analysis (TGA). Thermogravimetric analysis was used to determine water content in the crosslinked resilin sample, which was approximately 80 wt% water based on the observed decrease in mass from 100 wt% to the 20 wt% with increasing

temperature. This value agrees with the theoretical calculations (weight of the solid hydrogel $\approx$ 20.56 mg, protein concentration = 200 mg/ml, volume of soluble protein used = 20 μ l, and amount of protein added for crosslinking = 200mg/mg * 20 μ l = 4 mg, resulting in a wt.% of protein = $(4 * 100 / 20.56) \approx 19.45 \%$).

1.4.5 Dynamic Mechanical Analysis. Using the DMA results obtained for different frequencies, temperatures, and ethanol concentrations, the storage modulus (E'), loss modulus (E'') and $\tan \delta$, were determined and plotted against the logarithm of frequency. The master curves, obtained from these calculated values using TTSP, showed the effect of ethanol concentration, temperature, and frequency on the viscoelastic properties of resilin (clone -1).

1.4.5.1 Temperature Shift. For 0% ethanol (100% water), we conducted the experiments every 5 to 6°C within the temperature range from 3 to 59°C, and plotted against the log of the frequencies. To develop the master curves, the data were shifted horizontally by distances corresponding to thermal shift factors, a_T, a_T , based on a reference temperature of 24.5°C, following the principle of TTSP according to the Ferry²², and the resulting master curves are shown in Figure 4. With less than perfect data due to the small delicate samples and instrument used, the E' and E'' curves were shifted simultaneously, visually monitoring both curves to obtain a shift factor that produced reasonable master curves for both. Density-based shifts would seem to be inappropriate, as the same number of chains pass through a given cross section, regardless of temperature²³. Entropic vertical shifts were considered using the absolute temperature ratios, though the master curves did not show significant difference with confidence. These vertical shifts would result in a relatively small 15% (less than 0.07 decade) differences at most, though the relatively flat slopes of the master curves could increase the sensitivity to such shifts.

It is clear from Figure 4 that the resilin sample did not show any significant transition at 0% ethanol concentration over the temperature and frequency ranges tested, and substantially lower temperatures could not be imposed without freezing the surrounding water. The storage modulus was on the order of 26 kPa, well within the rubbery plateau with the reference temperature at 24.5°C. Although low modulus would suggest tack²⁴, the work of adhesion in the presence of water was insufficient to allow polymer to wet the substrate²⁵. The Williams-Landel-Ferry (WLF) equation was used to fit a_T as a function of temperature and experimental results could be represented by the WLF equation with $C_1 = 12$, $C_2 = 200^\circ\text{C}$ (Fig. 5). We also applied the Arrhenius equation to fit the shift factors and found a similar quality fit with an activation energy, $E_a = 98 \text{ kJ/mol}$ (Figure 5). Although, as pointed out by a reviewer, the slight deviations of the theoretical curves from the subjectively measured shift factors could suggest biphasic behavior, it is likely that variability in these small shift factors resulting from visual shifting of the relatively flat curves could explain the perceived differences.

As water freezes below 0°C, and it was beyond the capacity of this custom made experimental setup to conduct experiments at sub-zero temperatures, the experiments could not be performed at sub-zero temperatures to characterize the transition from the rubbery to glassy state. Considering the temperature limitations, this superposition technique can reportedly be extended by varying the diluent concentration without lowering the temperature^{14, 20}. Thus to determine the transition, additional experiments were conducted on the same specimen^d as above, but immersed in 40%, 65%, 73%, 85% and 95 vol% ethanol, and TTSP was applied to each case.

³ Several specimens were used to debug the methods and once method was defined, complete frequency, thermal, and ethanol concentration scan experiments were conducted on the same sample. Thus, all DMA results presented herein correspond to data obtained from one single specimen.

The storage modulus data for 0%, 65%, 73% and 85% ethanol concentration cases are shown in Figure 6. Results indicate that the resilin sample became stiffer with increasing ethanol concentration and E' increased from 26 kPa at 0% ethanol to around 12.8 MPa at 85% ethanol, a change of nearly three orders of magnitude at 40 Hz.

From Figure 6, it can be seen that there was a significant change in viscoelastic properties (within the range of frequency and temperature tested) associated with the change in ethanol concentration. Data above 70 Hz for some tests were erratic, perhaps due to resonance limitations with the instrument. Consequently, these results were neither used nor retained in the shifted master curves. There were noticeable $\tan \delta$ peaks only for the 73% and 85% ethanol cases. No vertical shifts were used for reasons explained above, though potential volume changes associated with the varied ethanol content did raise potential concerns but these were expected to be small based on the relatively modest increases in static load levels, especially at higher test temperatures. Master curves for 85% ethanol are shown in Figure 7 with the reference temperature of 23.5°C, and $\tan \delta$ peak can be identified approximately at $10^{1.28}$ Hz with a storage modulus of 1.1 MPa. However, the modulus at the upper plateau seems two orders of magnitude lower than the expected glassy plateau for a solid polymer. Two possible interpretations will be discussed below.

Thus, it is clear from Figure 7 that high ethanol concentrations can drive the resilin sample to go through some transition. Master curves were generated for each ethanol-water combination using TTSP, and in every case the thermal shift factor was well fit with appropriate choices of constants for respective WLF and Arrhenius fits of the data. Table 1 shows the values of C_1 and C_2 for WLF equation fits and activation energies for Arrhenius fits of the thermal shift factors, along with frequencies at the approximate peak of the $\tan \delta$ plots.

The master curves, obtained from the different ethanol concentrations including the peak values determined by TTSP, were based on the ethanol concentration only. To determine the resilin properties similar to their native state, a doubly-shifted master curve was formed using all the ethanol concentration data and applying time concentration superposition²⁶ based on a reference concentration of 0% ethanol (i.e. 100% hydration in water) results with a reference temperature of 24°C (Figure 8). Combined shift factors ($a_{Tc} = a_T \cdot a_c$) for different ethanol concentrations are shown in Figure 9 where a_T corresponds to the temperature shift factor and a_c corresponds to concentration shift factor. From Figure 8, we can see that the $\tan \delta$ peak is approximately at 10^{15} Hz with a storage modulus of 1.1 MPa. The modulus at the stiffer plateau is much lower compared to the accepted modulus for this transition to be the glass transition²⁷. This transition occurs multiple decades beyond the natural frequencies of the resilin in insect usage, as shown in the doubly-shifted master curve using TTCSP at Figure 8. The highest frequency reported for resilin usage in insects was measured for Cicada at 13 kHz⁸, while for the clone-1 transition occurs at 10^{15} Hz in room temperature, which is consistent with the understanding that resilin operates at frequencies well below the measured transition frequency, at least at biologically relevant temperatures. Results indicate that the temperature has a noticeable effect on the mechanical properties of resilin. However, the glass transition frequency is about 11 decades beyond the highest operational frequency reported for natural resilin. According to Figure 5, the maximum shift from 24.5°C to 0°C would be about 2 decades. Therefore, even near freezing temperatures, the transition frequency would still be 9 decades beyond the use frequencies, assuming hydrated conditions, leaving resilin well within the rubbery regime and allowing it to function over a range of temperatures for insects without significant effect on the mechanical properties.

As there is a peak in the doubly-shifted $\tan \delta$ plot, the resilin sample was clearly going through a transition from one state to another. According to Muller et al.²⁸, materials with viscoelastic properties sometimes exhibit thermorheologically complex behavior due to some additional relaxation process caused by reversible hydrogen bonds, and can display a transition within the rubbery plateau. Also, during our previous molecular simulation study on the repeating motifs of resilin²⁹, it was observed that intramolecular hydrogen bonds might influence the properties of resilin in different functions³⁰. Adhikary et al.³¹ and Takano et al.³² also reported the contribution of the intramolecular hydrogen bonds on the stability of protein conformations. Thus, it is possible that the transition experienced by recombinant resilin during our experiments could be partially due to formation and disruption of intramolecular hydrogen bonds. On the other hand, even though the plateau modulus is much smaller than expected for glassy behavior, the transition involved a modulus increase on the order of three decades, which is typical of glass transitions. This would make this consistent with the transition corresponding to a T_g , and is considerably larger than the value mentioned by Muller et al.²⁸ for hydrogen bonds, where the storage modulus increased by only one order of magnitude. Another interpretation is that this transition corresponds to the glass transition frequency, with the abnormally low modulus resulting from the swelling due to the very high water content (ca. 80%). Diluent molecules can diffuse into a polymer and effectively plasticize the material by increasing the free volume, though in some cases diluents can act as an anti-plasticizer due to the strong interactions with specific functional groups³³. As the diluent concentration increases, however, the added effect of additional diluent is often reduced, as diluent molecules are partially shielded from the polymer molecules by the strongly bound diluent molecules and become loosely bound. At sufficiently high concentrations, as reported by Roy et al.³⁴ for proton exchange membranes, some water

molecules effectively act as free water, occupying free volume or even voids within materials. Thus, initial diluent molecules are often very effective at plasticizing a polymer, with less effect on swelling, whereas additional diluent at higher concentrations yields less plasticization but more efficient swelling.

With 80% water, these resilin-like hydrogels samples were highly swollen, thus reducing the number of polymer molecules per unit area, resulting in a lower modulus than would be expected for a solid polymer³⁵. According to Okay³⁶ and Schausberger et al.³⁷, the modulus of a diluted polymer (E_{dp}) can be described using a power law relationship between modulus of the undiluted polymer (E_{up}) and the volume fraction of the entangled or crosslinked polymer (v_p) within the rubbery state ($E_{dp}/E_{up} = v_p^x$, where the exponent x determines the dependence of modulus on polymer fraction. Okay³⁶ used, $x=2.1\pm0.1$ while Schausberger et al.³⁷, $x = 2$). For diluted polymers, the exponent was much higher than linear dependency ($x=1$) on the polymer fraction predicted according to linear elasticity³⁸. Considering 20% polymer fraction in the hydrogel, the modulus would be only 3.4% of the expected modulus if x is assumed to be 2.1, and this effectively closes the modulus discrepancy. It is not clear, however, how relevant the power law relationship and exponents are as one goes from the rubbery to the glassy state, and thus the validity of this extension is questionable. If one assumes the observed transition results from intramolecular hydrogen bonds within the rubbery plateau, then there would be another transition from the rubbery state to glassy state and shift, likely resulting in a much higher than expected modulus of a solid polymer in the glassy state (typically about 3GPa²⁷)).

ChBD (exon-2) usually forms β -pleated sheets³⁹, while exon-1 is mostly unstructured¹⁰. Another possible explanation for the observed transition is that, due to the presence of ethanol, the secondary structure of the chitin binding domain (ChBD) in clone-1 might change, taking on

more ordered conformations that could restrict molecular mobility as increasing crystallinity does in semi-crystalline polymers. Moreover, ethanol is less cohesive than water and could cause additional intramolecular hydrogen bonds in the hydrogel, changing the secondary structure from random coil to more ordered conformation⁴⁰. Consequently, the observed transition could possibly occur due to the effects of increased order within the hydrogel. The current study does not permit definitive determination of the mechanism(s) responsible for the observed transition and more study is needed to understand the very pronounced temperature and rate-dependent transition observed at increased ethanol concentrations.

We synthesized recombinant resilin from exon-1 + exon-2, which is arguably more relevant to previously unknown properties of the native protein, as most insects have ChBD (exon-2) and its influence was not previously studied. Moreover, none of the previous works explored the dynamic nature of recombinant resilin, though functions of resilin in insects are dynamic in nature. Even though we could not propose a definitive conclusion for the prominent master curve transition observed, our work not only provides the dynamic nature of recombinant resilin which is by itself novel work on recombinant resilin, but also opens the door for exploring its properties at a wide range of frequencies and temperatures. We believe our contribution represents a potentially useful addition to the literature by providing experimental properties associated with the presence of ChBD in consideration of how properties of this partial protein sequence can be extended to the full-length resilin.

1.4.5.2 Effect of Ethanol. During the experiments, we observed different sections of the master curves with an increase of ethanol concentration and consequently the sample became stiffer (Figure 8) within the experimental frequency and temperature window. In an attempt to better understand effects on specimen dimensions and mechanisms responsible for the retarding

effect of ethanol, static loads were measured at every temperature for each ethanol concentration. Figure 10 reveals that static loads increased with increasing ethanol concentration, apparently offsetting specimen shrinkage since the ends of the hydrogels were attached to fixed ends. (As the length was kept constant, the stress-free gauge length of the specimen could have decreased with ethanol-induced shrinkage, effectively increasing the actual strains beyond the nominal applied strain amplitude. These changes in stress-free length could not be measured which prevented any possible corrections in the reported moduli, but these are expected to be small based on the relatively modest increases in static load levels (Figure 10), especially at higher test temperatures. Moreover, the amplitudes for 73% and 85% ethanol concentrations at lower temperature were lower than the applied amplitude during the dynamic mechanical testing due to increased modulus of hydrogels and instrument capabilities, but these actual measured amplitudes were used to calculate the moduli. The experiments of ethanol effects have shown the behavior of recombinant resilin in polar solvent (Figure 10), and also provided information to use applied amplitudes for modulus calculation, rather to use actual measured amplitudes.

1.4.5.3 Comparison with Natural Resilin. The properties of recombinant resilin were compared with the properties of natural resilin obtained from locusts⁷ and dragonflies¹⁴ tested in water (Figure 11). Andersen et al.⁷ reported that resilience of the locust prealar arm resilin was 95% at 15 Hz, while according to King¹⁴, dragonflies resilin's resilience was 99% at 15 Hz. Here the resilience of recombinant resilin from clone-1 was found to be 90% at 15 Hz ($\tan \delta = 0.07$) in water at room temperature (According to Ferry²², Resilience, $R = e^{-(\pi/2)\tan\delta}$). The storage modulus of recombinant resilin from clone-1 was almost 1.5 decades lower than that of locust and dragonfly resilins. But, the loss modulus of clone-1 resilin was higher than dragonfly resilin but lower than locust resilin, while the lowest $\tan \delta$ was observed for dragonfly resilin. The

clone-1 resilin structure was more similar to locust than dragonfly resilin, because both clone-1 and locust resilin had ChBD within their amino acid sequence, whereas dragonfly resilin does not. The stiffness of ChBD is much higher than resilin, and the resulting resilin-like hydrogel behaves like a composite with ChBD¹³. The loss modulus (E'') and $\tan \delta$ for clone-1 resilin was constant over the measured frequency range in water, while they were increasing for locust resilin. The only structural difference among natural resilin and synthesized recombinant resilin was exon-3. During the photo chemical crosslinking it was not possible to control the site specific di-tyrosine crosslinking for recombinant resilin to mimic the natural resilin. These differences might be among the root causes for the variation in properties. We compared the master curve for the storage modulus of clone-1 resilin with the master curve of natural dragonfly resilin (both at 65% ethanol concentration¹⁴), and with the master curve of a cockroach's natural resilin (both at 73% ethanol concentration¹⁵). The storage modulus for the clone-1 resilin was almost 1.5 decades lower than that of natural dragonfly resilin (Figure 12(a)). The storage modulus of clone-1 was also about 1.5 decades lower than cockroach resilin at low frequencies, but at higher frequencies, the values became more similar (Figure 12(b)). The structure of clone-1 is similar with natural resilin from cockroaches and locusts resilin, in that all three have ChBD in the protein sequence.

1.5 Conclusion

Recombinant resilin from clone-1 (exon-1 + exon-2) was successfully synthesized, crosslinked, and characterized. TGA results showed that the crosslinked sample contained 80wt% water. The hydrophilic nature and high water content may have precluded appearance of tack, as surface energetics may not have favored wetting of a probe, even though the material was soft enough to satisfy the Dahlquist criterion. DMA was conducted on the recombinant resilin sample to determine the dynamic mechanical properties, comparing these results with

those of natural resilin from locust, dragonfly and cockroach. Doubly shifted master curves, based on application of the time temperature concentration principle (TTCSP), were formed with data collected over a frequency range at selected temperatures and ethanol concentrations. DMA results showed that the storage modulus was about 1.5 orders lower than that of natural resilin, and the master curves were more similar to locust and cockroach resilin properties, perhaps because the clone is more similar to these materials (which all contain ChBD) than to dragonfly resilin. The resilience of the resilin-like hydrogel sample was around 90% at 15Hz in water, and that was close to that reported for natural resilin from locust. ,

In the master curves developed from the DMA results, a pronounced transition was seen based on the peak of $\tan \delta$ plot and over 100-fold increase in the storage modulus. Such large transitions are typical of glass transition behavior, and prior DMA studies of resilin refer to the transition as such. An important contribution from the present study is to raise concerns with this explanation used in prior research by Gosline and his coauthors^{20, 21} and used by others^{14, 15}. This research suggests other possible mechanisms should also be considered, however, in light of the fact that the storage modulus at the stiffer plateau is much lower than the accepted modulus of glassy polymers. One possibility is that this transition is due in part to the formation of intramolecular hydrogen bonds which were predicted in molecular simulations of the repeat motif of resilin. Another very likely possibility is that due to the presence of the less cohesive ethanol absorbed in the specimen, ChBD and exon-1 might change their secondary structure, increasing the modulus through more organized conformations or formation of crystalline domains. More experiments are needed to conclusively determine the specific mechanism(s) responsible for the pronounced transition. Nonetheless, these findings based on recombinant resilin and comparable investigations with other natural resilins provided a better understanding

of the structure and properties for specific components of materials in the insect physiology. The use of the established procedure to use ethanol to effectively retard material response and acquire transition behavior can be questioned for biological relevance, and as shown herein, for possibly inducing unnatural structural organization. Nonetheless, the methods used and results obtained offer an improved understanding the elastomer itself, and could be of relevance for certain applications of future elastomers produced with such materials (including those with biomedical applications, though not limited to this).

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Author Contributions

MSKK, DMD, EPB and DAD wrote the manuscript. MSKK, DMD, and EPB designed experiments for production of recombinant resilin. MSKK, DMD, and DAD designed experiments for analysis of physical properties of resilin. MSKK prepared and analyzed composition and physical properties of recombinant resilin. All authors have given approval to the final version of the manuscript.

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Figures:

Figure 1. Schematic and top view of the custom dynamic mechanical analyzer setup. The LabVIEW program drives the oscillator and converts the outputs from force and displacement gauges to viscoelastic moduli. Here, the water bath is shifted out of the way to reveal the load train. A mounted resilin specimen is shown in the inset.

Figure 2. Ni-NTA affinity purified resilin. Molecular masses of standards (lane 1) are shown at left of gel. Lane 2, highly purified resilin (15 μ g) is visible at approximately 50 kDa.

Figure 3. Crosslinked resilin retained in wells of SDS-polyacrylamide gel. Lane 1, Resilin crosslinked without SOD (15 μ g). Lane 2, Resilin crosslinked with SOD (15 μ g). Lane 3, Resilin crosslinked without SOD (30 μ g). Lane 4, Resilin crosslinked with SOD (30 μ g). Arrow indicates the crosslinked resilin that could not enter the gel.

Figure 4. Master curve obtained using the TTSP for storage modulus, loss modulus and $\tan \delta$ at a reference temperature 24.5°C for data obtained at 0% ethanol.

Figure 5. Thermal shift factor plotted against temperature for 0% ethanol, WLF fit with the values $C_1 = 12$ and $C_2 = 200^{\circ}\text{C}$, and Arrhenius fit with activation energy $E_a = 98\text{ kJ/mol}$ at reference temperature 24.5°C .

Figure 6. Storage modulus against frequency plot for different ethanol concentrations in log-log scale for resilin-like hydrogel of clone-1.

Figure 7. Master curves of storage modulus, loss modulus and $\tan \delta$ for 85% ethanol concentration using TTSP with the reference temperature 23.5°C for clone-1.

Figure 8. Doubly shifted master curve of storage modulus, loss modulus and $\tan \delta$ for clone-1 of resilin based on 0% ethanol concentration (100% water) using TTCSP with the reference temperature 24°C .

Figure 9. Total shift factor $a_c a_T$ plotted against temperatures for different ethanol concentrations for clone-1 with $T_{ref} = 24^\circ\text{C}$.

Figure 10. Static loads at different temperature and ethanol concentration. Static load changed drastically at different ethanol concentrations.

Figure 11. Comparison of E' , E'' and $\tan \delta$ between the locust, dragonfly and recombinant resilin (Clone-1) between 5 to 100 Hz in water.

Figure 1. Comparison of the storage modulus master curves between (a) Clone-1 and dragonfly resilin¹⁴ at 65% ethanol concentration (b) Clone-1 and cockroach resilin¹⁵ at 73% ethanol concentration. Native dragonfly and cockroach resilin data are from King [14] and Choudhury [15], respectively.

Table:

Table 1. WLF constant values and activation energy for different ethanol concentrations and peak frequency from the $\tan \delta$ plots involving transitions from one state to another for resilin-like hydrogels of clone-1.

% Ethanol	Temp. range (°C)	C1	C2 (°C)	Arrhenius Ea (kJ/mol)	Ref Temp. (°C)	$\tan \delta$ peak (Hz)
0	2.3 to 57.4	12	200	98	24.5	
65	-1 to 48	25	175	217	23.9	
73	2 to 46.1	11.2	112.8	183	26.4	104.5
85	1.8 to 45.5	8.9	70.1	240	23.5	101.3

Supporting Information

Figure A1. Peptide mass spectrum from matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS).

Table A1. Protein sequence results from MALDI-MS. Range of amino acid residue indicates position within the resilin polypeptide. The corresponding mass/charge ratios and predicted amino acid sequences are shown in the center and right-hand columns, respectively. The dots indicate the cleavage site of trypsin.

Range of amino acid residues of resilin	Monoisotropic mass, mass/charge (u/e)	Amino Acid Sequence
168-182	1310.6	R.SSSSYGAPGGGNGGR.P
101-115	1320.6	R.PSSSYGAPGGGNGGR.P
214-228	1320.6	R.PSSSYGAPGGGNGGR.P
296-310	1333.6	R.PSSSYGAPGSGPGGR.P
266-278	1348.6	R.PSDSYGAPGQNQK.P
45-59	1348.6	R.PSDSYGAPGGGNGGR.P
116-130	1362.6	R.PSDTYGAPGGGNGGR.P
183-197	1362.6	R.PSDTYGAPGGGNGGR.P

Figure 8. Doubly shifted master curve of storage modulus, loss modulus and $\tan \delta$ for clone-1 of resilin based on 0% ethanol concentration (100% water) using TTCSP with the reference temperature 24°C .