Molecular and functional characterisation of resilin across three insect orders

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Resilin is an important elastomeric protein of insects, with roles in the storage and release of energy during a variety of different functional categories including flight and jumping. To date, resilin genes and protein function have been characterised only in a small number of flying insects; despite their importance in fleas and other jumping insects. Microscopy and immunostaining studies of resilin in flea demonstrate the presence of resilin pads in the pleural arch at the top of the hind legs, a region responsible for the flea’s jumping ability. A degenerate primer approach was used to amplify resilin gene transcripts from total RNA isolated from flea (Ctenocephalides felis), buffalo fly (Haematobia irritans exigua) and dragonfly (Aeshna sp.) pharate adults, and full-length transcripts were successfully isolated. Two isoforms (A and B) were amplified from each of flea and buffalo fly, and isoform B only in dragonfly. Flea and buffalo fly isoform B transcripts were expressed in an Escherichia coli expression system, yielding soluble recombinant proteins Cf-resB and Hi-resB respectively. Protein structure and mechanical properties of each protein before and after crosslinking were assessed. This study shows that resilin gene and protein sequences are broadly conserved and that crosslinked recombinant resilin proteins share similar mechanical properties from flying to jumping insects. A combined use of degenerate primers and polyclonal sera will likely facilitate characterisation of resilin genes from other insect and invertebrate orders.

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1. Introduction

Nature has, through hundreds of millions of years of evolution, developed materials that exceed the specifications of man-made substances. One such material is resilin, the most efficient elastomer known, with resilience (energy storage efficiency) exceeding 97% and fatigue lifetime in excess of 300 million cycles (Elvin et al., 2005). In order to develop new generation elastomeric materials for industry and medicine, we propose a biomimetic strategy by studying the structure and function of resilin genes and proteins from insects and adapting these native design insights for the fabrication of synthetic resilin mimics.

Elastic proteins are diverse and found in a wide variety of animals ranging from insects to humans (Tatham and Shewry, 2000). These proteins play pivotal roles in preventing material fatigue, serving mechanical functions such as storage of kinetic energy, functioning as shock absorbers and acting as antagonists for muscles (Andersen, 2003). Their varied roles have allowed them to be classified into different functional categories (Alexander, 2003), including: power amplifiers, energy stores for running, energy stores in flight and swimming, return springs, smoothing flow, cushioning impacts and force control.

Examples of power amplifiers include mechanical catapults in which a rubber is stretched prior to release, storing up the strain energy, and returning it as kinetic energy. The importance of the catapult in insect locomotion was first demonstrated in 1967, confirming that blocks of resilin found at the base of the flea hind legs were large enough to store energy for a jump (Bennet-Clark and Lucey, 1967). The energy stored in the resilin pad is released...
in less than 1 ms and the body accelerates at greater than 1000 m s\(^{-2}\), equivalent to 102 G. Subsequently, the involvement of resilin in the catapult mechanism has been described in a number of insects including click beetles, fleas, spittle bugs and froghoppers (Sannasi, 1969; Evans, 1973; Burrows, 2003; Burrows et al., 2008; Sutton and Burrows, 2011). Despite the importance of resilin in the insect catapult mechanism, to date resilin genes have only been characterised in flying insects. It would be informative to compare resilin sequences from flea or other jumping insects to determine whether there are significant differences in gene and protein sequences that relate to the different functional categories.

Knowledge of the gene and amino acid sequences of resilins from a range of insects would greatly contribute to our understanding of protein design for highly efficient elastomeric function. Arrell and Andersen (2001) identified a putative gene encoding resilin in *Drosophila melanogaster* by searching the *Drosophila* genome for gene products with similarities to tryptic peptides obtained from locust (*Schistocerca gregaria*) and cockroach (*Periplaneta americana*) resilin pads (Arrell and Andersen, 2001; Lombardi and Kaplan, 1993). An expressed sequence tag (EST) from the African malaria mosquito *Anopheles gambiae* (GenBank accession no. BX619161) was later identified by TBLASTN analysis of EST databases to have homology to the *D. melanogaster* gene (Lyons et al., 2007), based upon the presence of N-terminal YGAP repeats. More recently, advances in genomics and transcriptomics studies in medically or agriculturally important insect species have led to annotation of putative resilin or resilin-like proteins in a range of species including red flour beetle (*Tribolium castaneum*), honey bee (*Apis mellifera*), parasitic wasp (*Nasonia vitripennis*), body louse (*Acarus infestus*) and pea aphid (*Acyrthosiphon pisum*) (Andersen, 2001). The confidence in identifying these as resilin however is questionable with Andersen stating “At present, it appears not possible by means of gene product sequences to draw sharp borderlines between resilins and other structural insect proteins”.

Elvin et al. (2005) previously cloned and expressed the first exon of the resilin gene from *D. melanogaster* (cg15920) (Arrell and Andersen, 2001), and then fabricated a biomaterial by introduction of dityrosine crosslinks via a facile photochemical method (Elvin et al., 2005). The molecular design principles that underpin this performance might be usefully adapted into the fabrication of synthetic materials to mimic native resilin function. Furthermore, through the design and expression of multimeric constructs, we demonstrated that the repetitive motifs of exon 1 are sufficient to confer resilin-like biomaterial properties (Lyons et al., 2007, 2009). Extending these observations, we describe here the molecular cloning and comparative studies of flea and buffalo fly resilin genes, as well as preliminary analysis of a putative resilin gene sequence from dragonfly. Using a degenerate PCR approach (Telenius et al., 1992), followed by 5’ and 3’ RACE (rapid amplification of cDNA ends), we have obtained the full-length gene sequences for flea (*Ctenocephalides felis*), buffalo fly (*Haematobia irritans exigua*) and dragonfly (*Aeshna* sp.). In addition, we express isoform B pro-resilin from both the flea and buffalo fly resilin gene, examine structural characteristics of the recombinant proteins, and compare mechanical properties of resulting biomaterials to confirm these are indeed highly efficient elastic biomaterials.

2. Materials and methods

2.1. Collection of insect specimens

Pupae were used in this study as previous real time PCR experiments using *Drosophila* pupae showed that the CG15920 gene was expressed most strongly during the pupal stage of development (Elvin et al., 2005). Adult and pharate adult fleas (*C. felis*) were obtained from Novartis (Sydney). Buffalo fly (*Haematobia irritans exigua*) pharate adults were obtained from Dr David Kemp (CSIRO Livestock Industries) and were stored at −80 °C. Dragonfly (*Aeshna* sp) nymphs were a gift from Kevin Mulligan, Devonport, Tasmania. Specimens were sampled from wild river systems and identified as belonging to the genus *Aeshna* on the basis of size of nymph and morphological characteristics. Speciation has not been carried out at this stage. Nymphs have been stored within CSIRO Livestock Industries as voucher specimens.

2.2. Microscopic studies of adult structures in flea

Legs and resilin pads from flea were dissected from surrounding tissue and placed on a glass slide, then covered by a raised coverslip. PBS buffers at pH 7, 2 and 12 were sequentially added and removed through use of absorbent tissues. Specimens were viewed using a Zeiss Axioskop (transmitted light SNT 12V 100W fluorescence HBO100W) and imaged with a colour CCD camera (MTI 3CCD camera model DC330E) using Scion Image software.

2.3. Sample sectioning and antibody staining

Flea resilin pads were dissected and fixed in 4% neutral buffered paraformaldehyde for 4 h at room temperature or overnight at 4 °C. The cell samples were dehydrated and embedded in LR White resin (Polysciences; Warrington, PA). Briefly, three washes in phosphate buffer were followed by dehydration steps at 50%, 60%, 70%, 80%, 90% and finally twice at 100% alcohol. Each step was for 10 min at room temperature. Specimens were then infiltrated with LR white resin at room temperature by placing samples in a 1:1 ethanol:resin mixture for an hour, followed by 100% resin overnight. Infiltration was performed on a rotator at room temperature. Samples were cast into gelatin capsules and placed at 60 °C for 24–28 h to polymerise the resin. Capsules were stored with dessicant to absorb moisture.

Samples were sectioned with a glass knife on a rotary microtome (Leica, Milton Keynes, UK). Semi-thin sections of approximately 350 nm were removed from the water reservoir using a rounded tip glass Pasteur pipette, placed onto a slide with water and flattened onto the slide by placing on a heating block at 80 °C. Sections were placed on slides in a staining chamber, and washed using phosphate buffer with 0.4% Triton-X (PBT). Blocking was performed by adding 5% normal goat serum (NGS) in PBT for 2 h. Rabbit antiserum developed against the Rec1 resilin of *D. melanogaster* was used as a primary antibody, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit serum (Sigma–Aldrich, St Louis, MO) was used as a secondary antibody. The primary antibody was added at 1:100 dilution with 5% NGS in PBT and incubated overnight at room temperature. Following wash steps (3 × 10 s, then 3 × 5 min in PBT), secondary antibody (1:1000 dilution in PBT) was placed on the slide and incubated for an hour at room temperature. Wash steps followed as described previously. For detection, a 1:1 solution of 3,3’-diaminobenzidine (DAB) to PBT with 1 μl of 30% hydrogen peroxide was placed on the slide. The colour reaction was monitored under a stereo dissecting microscope and terminated by two quick washes with PBT followed by 2 × 2 min washes in PBT. Stained sections were imaged under a Zeiss Axioskop microscope as described above.

2.4. RNA extraction and cDNA synthesis

Total RNA from flea and buffalo fly pharate adults and dragonfly nymphs were isolated using a commercial RNA extraction kit (RNeasy Mini kit, Qiagen, Chatsworth, CA) according to the manufacturer's
instructions. First strand cDNA was synthesised using the Superscript III cDNA synthesis kit (Invitrogen, Carlsbad, CA) with Oligo(dT) primers, according to the manufacturer’s instructions.

2.5. Degenerate primer design

Degenerate primers were designed against conserved motifs identified in the alignment of primary amino acid sequences from *D. melanogaster* (CG15920) and *A. gambiae* (EAA07497.1). All primers are listed in Supplementary Table 1. PCRs were set up to determine the optimal conditions for amplification of specific products from the designed primer pairs (CF1 + CF4; CF1 + CF5; CF1 + CF6; CF2 + CF5; CF2 + CF6; CF3 + CF4; CF3 + CF6; CF7 + CF4; CF7 + CF6). All PCRs consisted of final concentration of 1× Qiagen reaction buffer, 1× Q buffer, 0.1–1 mM MgCl₂, 0.5 μM dNTP mix, 1 μM of each primer, 2.5 U of Taq polymerase (Qiagen, Chatsworth, CA) and 1 μl of template cDNA in 50 μl final volume. The cycling conditions were optimised for varying annealing temperature (gradient from 37 to 47 °C), cycle number (ranging from 35 or 40 cycles), or both. General PCR protocols are as follows: 94 °C for 2 min; 35 or 40 cycles of 94 °C for 30 s; 37–47 °C for 30 s; 72 °C for 1 min; final extension at 72 °C for 5 min. Alternatively, a two-staged PCR was carried out. This consisted of 5 cycles of 94 °C for 30 s; 37 °C for 30 s; 72 °C for 1 min, followed by 40 cycles of 94 °C for 30 s; 66 °C for 30 s; 72 °C for 1 min; final extension at 72 °C for 5 min. PCR products using these primers were cloned into pGEM-Teasy™ (Promega, Madison, WI) vector for transformation into E. coli DH5α cells and were sequenced with M13 primers.

Identification of flanking sequences was carried out by vector 5’ and 3’ RACE (Rapid Amplification of cDNA Ends). Using the BD SMART RACE cDNA amplification kit (BD Biosciences, San Jose, CA), first strand cDNA synthesis and RACE reactions were performed on flea and buffalo fly pupal RNA, according to manufacturer’s instructions. Specific primers used in the nested 5’ and 3’ RACE reactions are shown in Supplementary Table 1. PCR products were cloned and sequenced as above.

2.6. Amplification and cloning of full-length resilin genes

To confirm that the assembled sequence represented a single expressed gene transcript, full-length coding transcripts from flea, buffalo fly and dragonfly were amplified. In each case, amplification was carried out utilising primers designed to introduce restriction sites for downstream cloning into the expression vector pETMCS1 (Neylon et al., 2000). Primers are shown in Supplementary Table 1. PCR cycling conditions were as follows: 95 °C, 2 min; 8 cycles of 95 °C, 30 s; 55°C–1°C/cycle, 30 s; 72 °C, 1 min; 30 cycles of 95 °C, 30 s; 48 °C, 30 s; 72 °C, 1 min; final extension at 72 °C for 5 min. Bands were cloned and sequenced as described previously.

Following confirmation of sequence integrity, plasmid DNA containing isoform B transcript from flea and buffalo fly was purified using the QiAprep spin miniprep kit (Qiagen, Chatsworth, CA). The isolated plasmid DNA was double-digested with restriction enzymes SmalI and HindIII or MluI and Ncol for flea and buffalo fly clones respectively, and inserted into a modified pETMCS1 vector at corresponding sites. The recombinant expression plasmids were isolated from *E. coli* DH5α cells with selection for ampicillin resistance, and sequence confirmed.

2.7. Protein analysis and phylogeny

Sequence translation, isoelectric point (pI) and molecular weight (Mr) and domain identification were calculated for each putative resilin using publicly available software (http://www.ncbi.nlm.nih.gov/BLAST/; SignalIP 3.0). Multiple sequence alignments were created using the default parameters of the ClustalW method (Larkin et al., 2007). To determine the phylogenetic relationship of the putative resilin proteins, neighbour-joining trees were generated from multiple sequence alignments using the algorithm of Saitou and Nei (Saitou and Nei, 1987). Bootstrap analysis of 1000 replicates was carried out to determine the confidence of tree branch positions. Trees were generated using Treeview 1.6.6 (http://taxonomy.zooology.gla.ac.uk/rod/rod.html) software.

2.8. Expression and purification of Cf-resB and Hi-resB recombinant proteins

Putative isoform B pro-resilin gene transcripts from flea (CF-resB) and buffalo fly (Hi-resB) were cloned into the expression vector pETMCS1 (Neylon et al., 2000). Plasmids were transformed into the *E. coli* strain BL21Star™ (DE3)/plysS (Invitrogen, Carlsbad, CA) and selected on LB plates containing 20 mM glucose, 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. Overnight cultures of each construct were grown in LB medium containing antibiotics and glucose as previously described, and subsequently used to inoculate 1 L cultures of ZYP-5052 for auto induction of protein expression (Studier, 2005). Cells were collected by centrifugation (10,000 g for 20 min at 4 °C), and the cell pellets were frozen at −80 °C.

A “heat and salting out” method was applied to clarified soluble fractions of cell lysates as previously published (Kim et al., 2007; Lyons et al., 2007). Both recombinant proteins were heat stable at 80 °C for 10 min, and precipitated at 20% ammonium sulphate. Precipitated proteins were retained following centrifugation, resuspended in sterile phosphate-buffered saline (PBS), and dialysed overnight at 4 °C in excess PBS. Purified pro-resilins were analysed by SDS-PAGE analysis (Laemml, 1970) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS) as described previously (Lyons et al., 2007).

2.9. Western blots

Purified pro-resilin samples were separated by SDS-PAGE and transferred to nitrocellulose filters. Filters were dried, rinsed in TBST (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20), and blocked for 1 h at room temperature in 5% skim milk. Rabbit anti-serum raised against the Rec1 resilin of *D. melanogaster* was used as a primary antibody, and HRP-conjugated goat anti-rabbit serum was used as secondary antibody. Primary and secondary antibodies were used at dilutions of 1:1000 and 1:10,000 respectively. Interacting proteins were detected colorimetrically.

2.10. Secondary structure evaluation by circular dichroism

CF-resB and Hi-resB pro-resilins were dialysed into 10 mM sodium phosphate buffer pH 7 and concentrations adjusted to 100 μg/ml of protein for CD studies. Spectra were measured using a Jasco J-810 spectrometer with a 1 mm path-length quartz cell. Ten acquisitions per spectrum were run from 185 to 260 nm at 0.5 nm/min. The buffer background was subtracted. The Contin LL (Provencher and Glockner, 1981) algorithm within 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 and SP37A. SDP42 contains some denatured proteins, along with soluble model proteins. SP37A includes model proteins for the polyproline II (PPII) structure.

2.11. Photochemical crosslinking of recombinant proteins

Photochemical crosslinking of recombinant Cf-resB and Hi-resB pro-resilin was performed as previously described (Elvin et al.,...
2.12. Scanning probe microscopy

Mechanical properties were determined in a PBS bath, using a Digital Instruments Dimension 3000 SPM operated in force–volume mode. The SPM was fitted with a standard silicon nitride probe with a nominal spring constant of 0.12 N/m. At least 4 force–volume plots (16 × 16 arrays of force-displacement curves taken over a 5–10 square μm area) were recorded for each of the samples. Resilience was determined according to the method of Huson and Maxwell (Huson and Maxwell, 2006).

3. Results

3.1. Microscopic studies of resilin in fleas

Resilin pads were observed in fleas in the pleural arch at the top of the hind legs (Fig. 1A), a region concerned with the flea jump. Furthermore, fluorescence was quenched at pH 2 but intensity returned to normal at neutral and high pH (Fig. 1B). This pH-dependent blue fluorescence is consistent with previous studies of resilin in a range of insects, and is due to the presence of dityrosine as protein crosslinks (Andersen, 1966; Neff et al., 2000). Previous studies of the biomechanics of jumping in flea and frog-hopper (Philaenus spumarius) have revealed similar patches of fluorescence under UV in the pleural arches of these insects, and attributed this fluorescence to resilin (Burrows et al., 2008; Sutton and Burrows, 2011).

3.2. Cross-reactivity of anti-Rec1 resilin antibody

The Anti-Rec1 antibody is an affinity-purified, polyclonal antibody previously generated in rabbits using the Drosophila exon 1-derived recombinant protein Rec1 (Elvin et al., 2005). Using this antibody, we have been able to demonstrate cross-reactivity to resilin pads in dissected flea pads. The crescent of flea resilin cross-reacts with anti-Rec1 resilin (Fig. 2A), following detection with a secondary anti-rabbit HRP antibody. A control with no primary antibody produced no comparable staining (not shown). Surrounding tissues did not stain as shown by comparison with toluidine blue counterstained sections (Fig. 2B). The fact that this anti-Rec1 resilin polyclonal sera is cross-reactive with resilin from a distant insect order (Siphonaptera) suggests that the antibody represents a valuable resource for future identification of resilin-containing structures within a range of insects.

3.3. Amplification of resilin genes by PCR using degenerate primers

Degenerate PCR is a powerful method for isolation of sequences that are conserved between species. PCR was therefore performed using all the primer combinations outlined previously, initially using either flea or buffalo fly pupal cDNA as template. Bands were obtained in flea for primer pair CF2 + CF5 at 300 bp and 500 bp in size. Bands were obtained for buffalo fly using primer pairs CF1 + CF6 at approximately 500 bp, CF2 + CF6 at 300, 500 and 1 kb, CF3 + CF6 at 1 kb, CF1 + CF4 at approximately 1 kb and 3 + 4 at approximately 1 kb. Following cloning and sequencing of these amplicons, the 300 bp and 500 bp bands amplified from flea using primer set (CF2 + CF5), and the 1 kb fragment amplified from buffalo fly using primer set (CF3 + CF6) were observed to contain repetitive YGAP motifs characteristic of previous resilin genes.

The experiment was repeated using dragonfly pupal cDNA as template. Following cloning and sequencing of amplicons, a 270 bp clone amplified using primer set CF6 + CF7 was observed to contain repetitive YGAP motifs characteristic of resilin.

3.4. Isolation and characterisation of full-length cDNAs

Following successful 5’ and 3’ RACE, specific primers were designed within the 5’ and 3’ regions to amplify and confirm the

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**Fig. 1.** The resilin pad of the flea under ultraviolet illumination. (A) The resilin pad is found in the pleural arch at the top of the hind legs, as labelled by a white box above. (B) Demonstration of pH-dependent fluorescence of the flea resilin pads.
entire coding sequence of resilin transcripts in flea, buffalo fly and, most recently, dragonfly nymph cDNA. For flea and buffalo fly, 2 different transcripts were obtained. For flea, amplicons of 2074 bp [Genbank: JF968409] and 1714 bp [Genbank: JF968410] were cloned and sequenced. Both transcripts were identical in sequence, except for a 360 bp deletion in the smaller transcript, suggestive of splice variants. For buffalo fly, amplicons of 1933 bp [Genbank: JF968411] and 1788 bp [Genbank: JF968412] were cloned and sequenced. Again, both were identical except for a 135 bp deletion in the smaller transcript. A single transcript of 1390 bp [Genbank: JF968413] was cloned in the dragonfly.

Putative translation suggests overall features of the flea, buffalo fly and dragonfly transcripts are very similar to *Drosophila* CG15920 pro-resilin, and reveal a number of important conserved features common to previously characterised pro-resilin proteins. Firstly, all four proteins contain a 16–19 amino acid signal peptide (Nielsen et al., 1997), suggesting they are likely to be secreted proteins (Bendtsen et al., 2004; Iconomidou et al., 2001). Immediately after the predicted signal sequence cleavage site both the flea and buffalo fly pro-resilins contain a conserved N-terminal motif (EPPVNSYLPP), which has been demonstrated to be present within a number of putative pro-resilin proteins (Andersen, 2010). Dragonfly has a similar N-terminal motif (EPPVGGSQSYLPP). Secondly, all four proteins contain repetitive domains in the N-terminal region of the proteins, previously identified as Type A repeats in the *Drosophila* resilin protein (Ardell and Andersen, 2001). While these motifs vary in sequence and length both within and across the insects, it is striking that the tetrameric repeat YGAP is conserved within all proteins (Fig. 3A and Supplementary Information Fig. 1 for entire alignment). There are 17 copies of repetitive domains containing the YGAP motif in *Drosophila* and flea, and 14 and 18 copies in buffalo fly and dragonfly, respectively.

A phylogenetic analysis of putative proteins was conducted using ClustalW alignment. Similar trees were generated using both neighbour joining and maximum parsimony methods (Fig. 3B). As one would predict, *Drosophila* and buffalo fly resilins form branches within a clade representing the order Diptera, while flea (order Siphonaptera) and dragonfly (order Odonata) are on separate branches as expected given their divergence from Diptera.

Our studies in flea and buffalo fly also support the previous findings in *D. melanogaster* that there are at least two isoforms of the pro-resilin gene present in some insect species, with only the A isoform having a chitin-binding domain. Preliminary studies in dragonfly have resulted in the amplification of an isoform B transcript with many of the key features of resilin genes, although to date we have not found evidence of chitin-binding domains in any transcripts. This may be a reflection of differential expression patterns of these two isoforms at different stages in the insect’s development, although further analysis across a range of developmental stages is necessary to test this hypothesis. The C-terminal repetitive region appears to have less conservation between repeats in the buffalo fly and dragonfly sequences, suggesting the presence of type RR-2 domains and C-terminal repetitive motifs are not always suitable determinants of pro-resilin status.

Furthermore, the longer transcripts of the *Drosophila*, buffalo fly and flea resilin genes each contain a putative chitin-binding domain similar to the proven chitin-binding domain in *D. melanogaster* (Fig. 3A), and commonly present in cuticular proteins including resilin (Rebers and Riddiford, 1988; Cormnan, 2009). The domain belongs to the RR-2 family of consensus sequences (Andersen et al., 1997; Andersen, 1998), and is present in isoform A of *Drosophila* resilin (CG15920-PA). Hence it is likely that we have isolated both the isoform A and isoform B resilin homologues for both organisms, represented by the longer and shorter transcript respectively.

Flea resilin isoforms A and B contain 14 and 13 repetitive motifs respectively within the C-terminal region, dominated by the 9 amino acid consensus sequence GGAGGYPGG. A glycine-rich sequence (GYSGGRPGQGGDLG) is also repeated 11 times in the C-terminal region of the putative *Drosophila* resilin (Ardell and Andersen, 2001). Repetitive 5 amino acid motifs (GYSGG) were present 5 and 4 times respectively within the C-terminal region of the buffalo fly and dragonfly genes, although the amino acid sequence between motifs was not as highly conserved as in flea and *Drosophila*. Amino acid composition analysis of flea, buffalo fly and dragonfly pro-resilins revealed that they are all glycine-rich. Cf-resA and Cf-resB contain 39.4% and 42.2% glycine respectively, Hi-resA and Hi-resB contain 34.8% and 36.8% glycine respectively, and Df-resB contains 34% glycine. This is consistent with previous observation that glycine is highly abundant in resilin proteins (Bailey and Weis-Fogh, 1961; Andersen, 2010).

Despite the absence of a chitin-binding domain (putative exon 2) in the dragonfly transcript, the presence of repetitive YCAP motifs together with other features conserved across all resilin genes including N-terminal signal peptide, C-terminal repeats and high overall glycine content supports this gene's identification as a putative resilin isoform B transcript.
3.5. Expression, purification and structural analysis of recombinant proteins Cf-resB and Hi-resB

The structural and mechanical properties of putative pro-resilin protein of buffalo fly (Hi-res) and flea (Cf-res) and dragonfly (Df-res) were determined by cloning and expressing the shorter isoform B transcripts in bacteria. Although D. melanogaster isoform A transcripts have been expressed successfully (Qin et al., 2009), these longer transcripts do not express as well as the shorter transcripts in our laboratory (unpublished data). Furthermore, Qin et al. (2009) observed that the heat and salt purification method that we employ in the current paper abolished chitin-binding ability of the D. melanogaster isoform A recombinant proteins. As our goal was to generate recombinant proteins for biomaterial fabrication, we were most interested in the isoforms likely to give us greatest expression. For these reasons, we have decided initially to express the B isoforms. Both were expressed as soluble proteins which were amenable to the “heat and salting out” purification method as previously used for Rec1 resilin and another intrinsically-unstructured protein An16 (Kim et al., 2007; Lyons et al., 2007). Resulting recombinant proteins were pure as determined by SDS-PAGE analysis and sequence analysis by mass spectroscopy. Cf-resB and Hi-resB
migrated as 70 and 85 kDa bands respectively when run on SDS-PAGE gels (Fig. 4A), compared to predicted molecular weights of 46.8 and 52.1 kDa respectively. These predicted molecular weights were confirmed by mass spectroscopy. This aberrant migration of the recombinant pro-resilin proteins in SDS-PAGE gels had been previously identified in resilin-like proteins (Lyons et al., 2007), and is a property commonly observed in many cuticular proteins (Andersen et al., 1995).

It is worth noting that while Cf-resB contains an N-terminal G-His tag, Hi-resB does not have a tag. Therefore it would appear that the tag has negligible effect on either solubility or purification characteristics. Both Cf-resB and Hi-resB formed coacervate when stored on ice, a phenomenon previously noted for Rec1 and An16. Both Cf-resB and Hi-resB cross-reacted with the anti-Rec1 polyclonal sera as shown in Fig. 4B. While isoform B from dragonfly has recently been cloned, we have not yet expressed the gene transcript.

3.6. Circular dichroism studies of Cf-resB and Hi-resB

Previous studies of the secondary structure of resilin-like proteins suggest that they are unstructured with a high degree of disorder dominated by random coil configurations (Lyons et al., 2009; Nairn et al., 2008; Qin et al., 2009). To test that Cf-resB and Hi-resB have similar structural characteristics, we gathered secondary structure information using CD spectra and prediction of intrinsically-unstructured regions of proteins based on estimated energy content (Dosztányi et al., 2005) (http://iupred.enzim.hu/). CD spectra were similar to those generated for Rec1 resilin (Fig. 5A). Average disorder scores for Cf-resB and Hi-resB are 0.477 and 0.493 respectively, which is consistent with previously published data for other resilin-like proteins including resilin from D. melanogaster (Lyons et al., 2009; Qin et al., 2009) and the An16 protein (Nairn et al., 2008). No globular region was predicted in either protein.

All sets of spectra exhibit strong minima at 195–200 nm and, consistent with previous studies of elastomeric proteins and peptides including tropoelastin and resilin (Bochicchio et al., 2008), there is a trend for these minima to decrease in intensity as temperature is increased (Fig. 5B). Together with decreasing maxima values at 225 nm as temperature is increased, the data suggest the presence of random coil conformations predominant at higher temperatures. Isodichroic points at 202 nm, 208 nm and 201 nm for Cf-resB, Hi-resB and Rec1 resilin, respectively are indicative of a two-state equilibrium between a somewhat ordered structure (probably a combination of PPII and beta turns) and a more random coil conformation.

CD data suggest that the expressed recombinant proteins derived from buffalo fly and flea do indeed have secondary structure characteristics consistent with previously characterised resilins.

3.7. Mechanical properties of Cf-resB and Hi-resB hydrogels

Following the addition of 2 mM Ru(bpy)$_3$Cl$_2$ complex and 20 mM SPS to a 200 mg/ml (20%) protein solution of either Hi-resB or Cf-resB, illumination of the mixtures for 30 s with a high intensity 300 W tungsten-halide white light generated small disks of crosslinked recombinant resilin-like materials suitable for analysis by scanning probe microscopy (SPM). SPM has previously been operated in force mode to demonstrate that crosslinked Rec1 resilin and other resilin-like proteins displayed negligible hysteresis upon compression (Elvin et al., 2005; Lyons et al., 2009).
Representative force–distance curves for Hi-resB and Cf-resB are shown in Fig. 6. SPM indicates that crosslinked Cf-resB is the softer of the two materials with an average penetration of 654 (±42) nm, compared to 347 (±78) nm for Hi-resB. Rec1 resilin has previously been measured at 324 (±23) nm (Lyons et al., 2009). Both crosslinked materials showed minimal hysteresis upon compression with crosslinked Cf-resB and Hi-resB having resistances of 88% and 87% respectively, compared to 97% resilience for Rec1 resilin [1]. Dityrosine was present in each of crosslinked Cf-resB and Hi-resB resilins as demonstrated by HPLC analysis (Fig. 7A and B respectively). The yields of dityrosine relative to total tyrosine were 19.2% and 17.1% for Cf-resB and Hi-resB resilins respectively.

Cf-resB is softer relative to Hi-resB and Rec1 resilins as determined by SPM, with the probe penetrating 654 nm versus 347 and 324 nm in Hi-resB and Rec1 respectively. This is despite all three polymers having similar levels of dityrosine and resilience. One marked difference between the proteins is that Cf-resB contains fewer charged amino acid residues than either Hi-resB or Rec1. This difference is most pronounced for the negatively charged residues aspartic acid and glutamic acid, which together represent 0.9% of residues in Cf-resB compared to 5.5% and 4.2% in Hi-resB and Rec1 respectively. A similar trend is also observed for the positively charged residues arginine and lysine which represent 1.8% of residues in Cf-resB compared to 4.0% and 5.4% in Hi-resB and Rec1 respectively. Salt bridges play important roles in influencing protein folding (Donald et al., 2011). It is tempting to speculate that in the absence of salt bridge formation the propensity for pi-stacking of tyrosine residues is reduced in Cf-resB. Relatively few charged amino acid residues than either Hi-resB or Rec1. This is however required to confirm this hypothesis.

4. Discussion

Until now, comparison of putative pro-resilins from a range of insect species has relied upon mining of genomic and transcriptomic data. Putative resilin-like genes have recently been identified from the honey bee A. mellifera, red flour beetle T. castaneum, parasitic wasp N. vitripennis, body louse P. humanus, aphid A. pisum, and two mosquito species A. gambiae and C. quinquefasciatus (Andersen, 2010). While many of these gene sequences shared a number of characteristics with Drosophila pro-resilin, Andersen concluded that at present it is not possible to categorically identify a gene as a pro-resilin on the basis of sequence alone, with cuticle-binding domains and signal peptides being shared by a number of different insect gene families. The present study is the first application of a degenerate primer approach to specifically target and successfully amplify resilin homologues in other insect species, and together with the cross-reactive anti-Rec1 polyclonal antibody, provides tools and techniques for broader characterisation of these elastic proteins across a range of arthropods.

As well as isolating and characterising two different isoforms of resilin in flea and buffalo fly, we have expressed the B isoform of each. Both were soluble recombinant proteins that could be purified using a “heat and salting out” method previously described (Lyons et al., 2007). Aberrant migration of each recombinant protein in SDS-PAGE, cross-reactivity to anti-Rec1 resilin polyclonal sera, and CD spectra illustrating a high degree of disorder dominated by random coil configurations, all support the identification of these proteins as pro-resilins. Both recombinant proteins can be photochemically crosslinked via dityrosine formation to form hydrogels, with the resulting biomaterials displaying high resilience, as determined by SPM. Both proteins have relatively high dityrosine content, consistent with previously characterised biomaterials derived from resilin and resilin-like proteins. The combination of molecular and mechanical characterisation allows...
us to confidently identify these genes as resilin-encoding in flea and buffalo fly.

Many of the genes currently identified in the GenBank database as resilin have been characterised as part of large-scale transcriptomic or genome sequencing efforts. Generally the assignment of these as resilin homologues is based upon similarity to the D. melanogaster CG15920 gene, and particularly due to conservation within the chitin-binding domain known as the R&R consensus sequence (Cormman, 2009; Rebers and Riddiford, 1988). However, there are a large number of unrelated cuticular proteins found in the flexible cuticles of larvae and pupae of different insects that possess similar domains. Without two or more other diagnostic characteristics of resilin being observed, such as an N-terminal signal sequence, EPPVSYLPPS-like domain, repetitive N-terminal Type A domains containing YGAP or YGPP motifs and C-terminal repetitive Type B domains, the presence of an R&R domain at best suggests that these gene products have cuticle-binding potential. Furthermore, defining resilins as proteins containing a chitin-binding domain ignores the type B isoforms of resilin genes that lack this domain. It is tempting to speculate that both A and B isoforms of pro-resilin are present in the resilin pads and tendons of insects, with the chitin-binding isoform A being firstly laid-down to provide binding to cuticle and isoform B being used within the bulk of the pad or tendon. Early descriptions of resilin included both chitin-containing ligaments exposed to bending during flight and chitin-free tendons exposed to stretching during flight (Weis-Fogh, 1960). It was demonstrated that while the majority of the locust wing hinge consists of a combination of resilin and chitin, the innermost part of the ligament (the pad) in fully grown locusts does not contain chitin. Recent studies have shown that biomaterials derived from either full-length (isoform A) recombinant resilin from D. melanogaster (Qin et al., 2009) or derived from exon 1 only (Elvin et al., 2005) have similar mechanical properties, suggesting that the R&R domain does not contribute to the resilience and elasticity properties of resilin.

Clearly, further work is required in the understanding and definition of what a resilin gene or protein is. Key conserved features including an N-terminal signal sequence immediately followed by a conserved N-terminal region xEPPVSYLPPS, repetitive N-terminal Type A domains containing the YGAP or YGPP tetrameric domains, a chitin-binding domain, and a C-terminal repetitive Type B region generally containing GYSGG, GYSGG, GYPG or GYPSS motifs are shared by putative pro-resilin genes, but not all may be present all of the time. Other structural and elastic proteins including mucins share some motifs and domains including repetitive YGXP-containing domains and domains with resilin, confusing the classification of this group of elastic proteins in insects.

A case in point was our previous assignment of an A. gambiae EST (BX619161) as encoding a putative pro-resilin (Lyons et al., 2007) on the basis of N-terminal repetitive YGAP-containing domains, an N-terminal signal peptide and EPPVNSYLPPS domain. Furthermore, structural and mechanical properties (high resilience and elasticity) of the dityrosine-crosslinked recombinant An16 protein were consistent with resilin-like function. Others have since pointed out that many features of this transcript are not consistent with its assignment as an insect resilin (Andersen, 2010; Willis, 2010), most notably the absence of a chitin-binding domain and low glycine content of the predicted protein, and it is more similar to mucin91C in D. melanogaster (CG7709). On this basis, it is apparent that our earlier identification of BX619161 as a resilin-encoding gene was incorrect. However the identification of a resilin homologue in mosquitoes remains elusive, with none of the proposed candidates (AgamCPR152 and AgamCPR140 in Anopheles; XP_001843145.1 and XP_001844908.1 in Culex) meeting all criteria of a classic resilin gene (Andersen, 2010; Willis, 2010). The fact that neither of the sequenced mosquito genomes contains what is currently classified as a “true resilin” gene, suggests either that mosquitoes do not use resilin in flight and locomotion systems or, that they have adapted other genes to serve resilin-like mechanical functions.

While deformation of resilin can be relatively slow, as in the case of clypeo-labral springs in locusts and the abdominal springs in beetles (Andersen and Weis-Fogh, 1964), in other functional roles resilin may be required to release all of its stored energy within milliseconds such as sound production in the tymbal organ of a cicada, supporting flight in a fly or contributing to the mechanical catapult function in a flea (Elvin et al., 2005). Recent data supports this observation, including dynamic mechanical testing of dragonfly tendon that demonstrated dragonfly tendon to be 81% resilient at 13 kHz (King, 2010). This suggests rapid rates of protein chain movement at the molecular level in some functional categories. Consistent with this, comparison of the different resilins expressed in the current study revealed that they are largely unstructured with a high degree of disorder dominated by random coil configurations.

Despite resilins remarkable ability to store and return energy, the exact role of resilin remains elusive and its role in energy storage and release should not be over-emphasized. King (2010) concluded that resilin in the tendon in dragonflies does not improve the energetic efficiency of flight or act as a power amplifying spring, but passively controls and stabilizes the trailing edge of each wing during flight. Likewise in flea and locust, chitinous cuticle appears to be an important energy store (Sutton and Burrows, 2011; Bennet-Clark, 1975). Burrows and colleagues (Burrows et al., 2008) propose that resilin provides a flexible material that is employed together with the chitinous cuticle in a composite structure for efficient energy storage.

The research methodologies and resources generated in the current study, including degenerate primers to amplify resilin-like gene sequences, polyclonal sera for staining of insect sections and proteins, novel resilin-encoding genes, and biophysical and mechanical characterisation of recombinant proteins will be invaluable for future studies of this interesting class of genes and proteins. It is anticipated that synthetic resilin-inspired materials will find many applications in industry and medicine, where high resilience and high fatigue lifetime specifications are demanded.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ibmb.2011.08.002.

References


