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Design and facile production of recombinant resilin-like polypeptides: gene construction and a rapid protein purification method

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Resilin is an elastic protein found in specialized regions of the cuticle of insects, which displays unique resilience and fatigue lifetime properties. As is the case with many elastomeric proteins, including elastin, gliadin and spider silks, resilin contains distinct repetitive domains that appear to confer elastic properties to the protein. Recent work within our laboratory has demonstrated that cloning and expression of exon 1 of the *Drosophila melanogaster* CG15920 gene, encoding a putative resilin-like protein, results in a recombinant protein that can be photochemically crosslinked to form a highly resilient, elastic biomaterial (Rec1 resilin). The current study describes a recursive cloning strategy for generating synthetic genes encoding multiple copies of consensus polypeptides, based on the repetitive domains within resilin-like genes from *D. melanogaster* and *Anopheles gambiae*. A simple non-chromatographic purification method that can be applied to these synthetic proteins and Rec1 is also reported. These methods for the design and purification of resilin-like periodic polypeptides will facilitate the future investigation of structural and functional properties of resilin, and the development of novel highly resilient biomaterials.

Keywords: expression/purification/repetitive polypeptides/resilin/synthetic genes

Introduction

Resilin is an elastic protein found in specialized cuticular compartments in a range of arthropods. It was first demonstrated to play a significant role in flight for insects including the desert locust (*Schistocerca gregaria*) and dragonflies of the *Aeshna* genus (Weis-Fogh, 1960). Resilin has been implicated as having roles in other functions such as the jumping mechanism of the flea (Bennet-Clark and Lucey, 1967), leg movement in arachnids (Sensenig and Schultz, 2003) and vocalization in cicadas (Bennet-Clark, 1999). Early studies of the biomaterial properties of resilin in dragonflies suggest a model for elasticity in which randomly coiled, thermally agitated polypeptide chains are covalently crosslinked and undergo a decrease in entropy upon straining (Weis-Fogh, 1961a, b). Furthermore, it was demonstrated that resilin was a highly elastic material that could be stretched three to four times its resting length before breaking, and would return to its original resting shape with very little deformation, indicating its high resilience (Jensen and Weis-Fogh, 1962). Further research has demonstrated that resilin possesses two outstanding material properties: high resilience of 92% or more and a very high fatigue lifetime (Gosline *et al.*, 2002). Structurally, the protein is formed by crosslinking of tyrosine residues as di- and trityrosine complexes (Andersen, 1964). In nature, this crosslinking is probably facilitated by a peroxidase. Dityrosine has been synthesized *in vitro* through the action of a peroxidase enzyme (Gross and Sizer, 1959; Elvin *et al.*, 2005), and a peroxidase has been observed to be present in extracts of resilin from adult desert locust, *S. gregaria* (Coles, 1966). Together, these studies provide compelling evidence that the crosslinking of pro-resilin in insects is mediated through the action of peroxidases. To date, a lack of sequence data has limited structural studies of resilin, although early studies of resilin including electron microscopy and X-ray diffraction suggest that resilin is an unstructured amorphous protein matrix (Elliot *et al*., 1965).

Recent research within this laboratory has demonstrated that it is possible to generate a rubbery biomaterial by cloning and expressing the first exon of the *Drosophila melanogaster* CG15920 gene, encoding a putative resilin-like protein (Elvin *et al.*, 2005). The resulting recombinant protein was purified by an immobilized metal affinity chromatography (IMAC) and could be photochemically crosslinked to produce a rubbery biomaterial (Rec1 resilin) through dityrosine formation. Scanning probe microscopy and tensile testing showed that Rec1 resilin had high resilience (94%) and elastic properties, being stretched to over 300% of its original length without breaking. Exon 1 of the CG15920 gene comprises 17 copies of the putative elastic repeat motif, GGRPSDSYGAPGGGN (Ardell and Andersen, 2001). Repeat units are a common feature in many different elastic proteins including elastin, gliadin, abductin and spider silks (reviewed by Tatham and Shewry, 2002). Recursive methods have commonly been employed to generate periodic polypeptides based upon the repetitive motifs of these proteins (Prince *et al.*, 1995; Lewis *et al.*, 1996; Elmorjani *et al.*, 1997; Meyer and Chilkoti, 2002). These methods have been utilized as a means of understanding functional and structural aspects of many repetitive proteins (Topilina *et al.*, 2006; Meyer and Chilkoti, 2004). Advantages of recursive methods over alternative cloning strategies include being able to precisely control the number, order and direction of monomers inserted.

In an effort to further our understanding of the functional and structural properties of resilin, and to assess the relevance of the repetitive domains within resilin-like genes, we have developed a recursive stepwise approach for generating oligomeric expression constructs. These constructs encode periodic

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polypeptides based on the consensus repeat motifs of either the *D. melanogaster* resilin gene (GGRPSDSYGAPGGGN) or a putative mosquito resilin gene (AQTPSSQYGAP). Furthermore, on the basis of observed heat stability and hydrophobic properties of these recombinant resilin-like proteins including Rec1, we discuss a simple protocol that has enabled us to obtain these recombinant proteins at near homogeneity without the need for IMAC.

Materials and methods

Construct design and initial annealing of module

An *Anopheles gambiae* (African malaria mosquito) homologue (GenBank accession no. BX619161) of the *D. melanogaster* gene was identified by TBLASTN analysis of EST databases (Altschul *et al.*, 1997). Consensus amino acid motifs were determined for each protein based upon the most common amino acid at each position relative to the conserved YGAP motif. For *D. melanogaster*, alignment of the 17 repeats resulted in the 15 residue consensus sequence GGRPSDSYGAPGGGN, whereas for *A. gambiae* alignment of the partial cDNA resulted in an 11 residue consensus sequence AQTPSSQYGAP.

Construct design was developed by modification of a recursive method for generating periodic polypeptides of a gliadin repetitive motif (Elmorjani *et al.*, 1997). For each consensus sequence, 1 μM of the complementary oligonucleotides were combined in 1 × *Taq* polymerase reaction buffer (67 mM Tris HCl, pH 8.8, 16.6 mM $[NH_4]_2SO_4$, 0.45% Triton X-100 and 0.2 μg/ml gelatin; Fisher Biotec Australia), and annealed using a Peltier thermal cycler (PTC-200, MJ Research). The reactions involved initial denaturing at 94°C for 3 min, followed by cooling at 1°C per min for 30 min, then cooling at 2°C per min for 20 min. Primer sequence and primary structure of the *D. melanogaster A. gambiae* and modules following annealing are shown in Fig. 1A.

Annealed modules were cloned into compatible restriction endonuclease sites of the high copy number plasmid pCR®2.1TOPO (Invitrogen), transformed into competent M15pREP4 (Nal^s, Str^s Rif^s, Thi^-, Lac^-, Ara^+, Gal^+, Mtl^-, F^-, $RecA^+$, Uvr^+, Lon^+) *Escherichia coli* host strain as per manufacturers instructions (Qiagen), and grown on Luria–Bertani (LB) agar plates containing 20 mM glucose, 100 μg/ml Ampicillin and 34 μg/ml Kanamycin. Plasmid preparations were performed as per manufacturers instructions (Qiaprep® miniprep kit, Qiagen), and sequences were confirmed with M13 forward and reverse primers using BIG DYE 3.1 terminator mix on an ABI 377 Sequencer (PE Applied Biosystems). Consensus sequence files were created using Sequencher™ 4.1 (GeneCodes).

Recursive method for doubling of consensus motifs within constructs

Utilizing blunt-ended restriction endonuclease sites (*Sma*I and *Sna*BI) engineered into the modules at sites immediately before and after the repetitive motifs, together with the

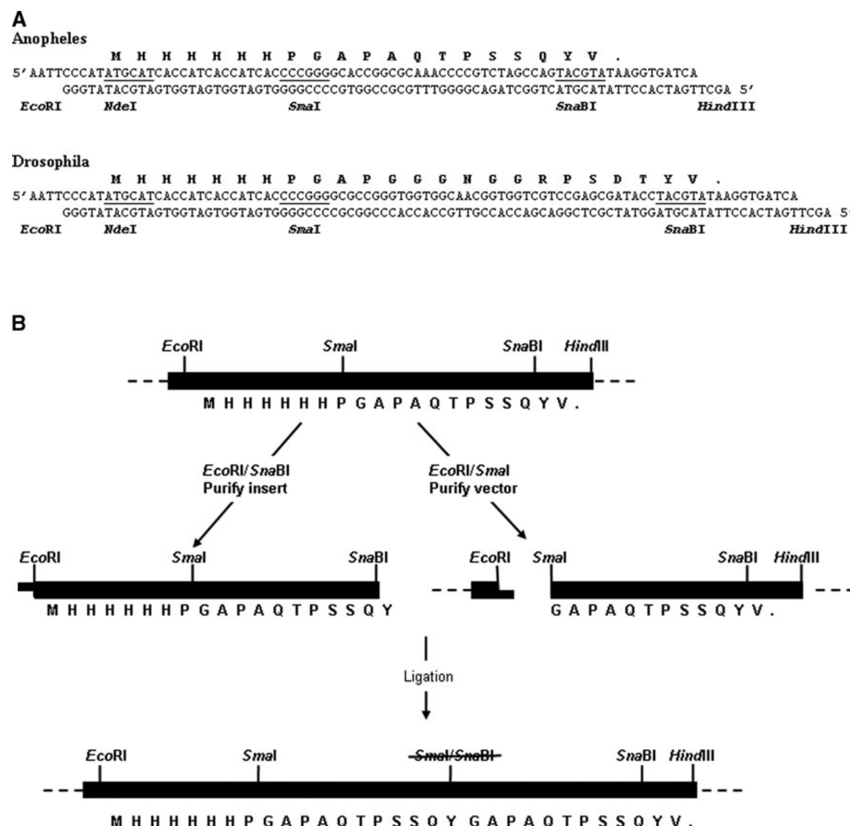

Fig. 1. (**A**) Sequences and primary structures of the designed double-stranded oligonucleotides encoding the repeat motifs of the putative *A. gambiae* resilin gene (AQTPSSQYGAP) and the *D. melanogaster* resilin gene (GGRPSDSYGAPGGGN). (**B**) Recursive procedure utilized to double the number of repeat motifs within the *A. gambiae* construct.

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*Eco*RI restriction endonuclease site at the beginning of the module, the consensus motif was doubled by a series of digestions and religations. Two digests were performed using the initial construct. Following digestion by *Eco*RI and *Sna*BI, the cleaved insert was purified by gel extraction. Simultaneously, the plasmid was digested with *Eco*RI and *Sma*I, and the vector was retained and purified. The *Eco*RI/*Sna*BI fragment and *Eco*RI/*Sma*I vector were then religated, resulting in a larger construct containing two copies of the consensus motif in tandem. Repetition of this procedure resulted in constructs containing 2, 4, 8, 16, 32 and 64 copies of the consensus motif in tandem. The methodology is shown schematically in Fig. 1B.

Expression of recombinant proteins

Inserts from the 16 copy constructs for each of *A. gambiae* (An16) and *D. melanogaster* (Dros16) were digested with *Nde*I and *Hind*III restriction endonucleases and cloned into compatible sites in the expression vector pETMCS1 (Neylon *et al.*, 2000). These plasmids were transformed into the *rne131 E. coli* strain BL21Star™ (DE3)/pLysS (Invitrogen) and selected on LB plates containing 20 mM glucose, 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The authenticity of the clones was confirmed by DNA sequencing. Overnight cultures of bacterial cells containing each construct (An16, Dros16 and Rec1) were grown in LB medium containing antibiotics and glucose as previously described, and subsequently used to inoculate 1L cultures of ZYP-5052 for auto induction of protein expression (Studier, 2005). Cells were collected by centrifugation (10000 *g* for 20 min at 4°C), and the cell pellets were frozen at −80°C.

Purification of soluble proteins

The cell pellets were thawed and resuspended in lysis buffer (50 mM Tris HCl, pH 7.2, 10 mM benzamidine HCl, 10 mM EDTA, 1% Triton X-100, 10 mM β-mercaptoethanol), and homogenized using an UltraTurrax T25 (IKA). Cell disruption was then carried out using an Ultrasonics™ (Melbourne, Australia) A180 sonicator (180 W maximum power output) with a 10 mm ultrasonic probe. The cell suspension (40 ml) was sonicated (30 s × 3) in an ice water bath. The soluble protein fraction was recovered in supernatant following centrifugation at 100 000 *g* for 1 h at 4°C. Polyethyleneimine (PEI) solution at pH 8.0 was added to the cleared supernatant to 0.5% final concentration, and the solution was then mixed and stored on ice for 30 min. The PEI-treated clear supernatant was collected by centrifugation at 8000 *g* for 15 min at 4°C. Recombinant proteins from An16 and Dros16 cultures were initially purified by IMAC as previously described (Elvin *et al.*, 2005).

Assessment of heat stability

To test the heat stability of the recombinant proteins, PEI-treated supernatant of each recombinant protein (An16, Dros16 and Rec1) was heated at 50, 70, 80 or 95°C for 10 min, and at 95°C only for 30 min, 1 and 4 h. Heated samples were immediately centrifuged at 12 000 *g* for 15 min at room temperature. Heat stability and purity were assessed by SDS-PAGE analysis (Laemmli, 1970) using precast 12% Bis-Tris gels (Invitrogen). Gels were stained with Coomassie Brilliant Blue R-250 for 1 h, and destained in 45% methanol, 10% acetic acid for no more than 1 h at room temperature.

Ammonium sulphate precipitation

To assess the optimal concentration of ammonium sulphate for purification and concentration of recombinant resilin-like proteins, PEI-treated supernatant of each recombinant protein (An16, Dros16 and Rec1) was precipitated in ammonium sulphate at final concentrations of 10, 20, 30 or 40% saturation (56, 114, 176 and 243 g/l respectively). Ammonium sulphate (SigmaUltra, Sigma-Aldrich) was added slowly to the supernatant with mixing, and the resulting mixture was kept on ice for 30 min prior to centrifugation at 6000 *g* for 30 min at 4°C. Purity and recovery rates were assessed by SDS-PAGE analysis.

Final purification regime

Following clarification of the soluble fraction, ammonium sulphate was added to the PEI-treated supernatant to a final concentration of 20% for both An16 and Rec1, and 30% for Dros16. Precipitating proteins were retained following centrifugation, resuspended in sterile phosphate-buffered saline (PBS), and dialysed overnight at 4°C in excess PBS. The resuspended proteins were heated at 80°C for 10 min with stirring, and denatured proteins were removed by centrifugation at 12 000 *g* for 15 min at 20°C. The supernatant was stored at −80°C. Purity and recovery rates were assessed by SDS-PAGE analysis and mass spectroscopy, and final protein concentrations were estimated spectrophotometrically.

Mass spectroscopy

For matrix-assisted laser desorption/ionization mass spectrometry time-of-flight mass spectroscopy (MALDI-TOF MS), 0.5 μl of supernatant was thoroughly mixed with 0.5 μl of the matrix consisting of a saturated solution of sinapinic acid in 1:1 acetonitrile, 0.1% v/v trifluoroacetic acid. Then, 0.5 μL of the mixture was spotted onto a 2 × 96 spot plate and dried at room temperature. All solutions were analysed on a Voyager DESTR mass spectrometer (Applied Biosystems) operating in linear mode. Representative spectra for all samples were acquired from 2000 shots. The laser intensity was set to 2425 V, the accelerating voltage was set to 20 000 V; the grid voltage set to 64% of the accelerating voltage and the delay time was 165 ns. The low mass gate was set to 500 Da. Data were collected between 5000 and 80 000 Da. Data acquisition was performed using Voyager Instrument Control Panel v5.10 and analysed using Voyager Data Explorer v4.0 software.

Biomaterials properties of Rec1 resilin

Comparison of the biomaterial properties of crosslinked Rec1-resilin derived by IMAC or the salt and heat purification method was performed using scanning probe microscopy as previously described (Elvin *et al.*, 2005).

Results

In early approaches, we utilized PCR amplification to increase the number of repeats within the constructs. However, when these constructs were sequenced, we invariably found that they contained deleterious mutations that encoded truncated or modified recombinant proteins.

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To tightly control the repeat length and prevent PCR-generated mutations, we therefore selected an alternate construction approach based upon a similar recursive method developed by Elmorjani *et al.* (1997) to generate periodic gliadin constructs. This approach proved highly successful in our laboratory. The structure of the initial modules based upon both *A. gambiae* and *D. melanogaster* repeat motifs are shown in Fig. 1A. Important features incorporated into the design include 5′ overhanging sequences compatible with *Eco*RI and *Hind*III restriction endonuclease sites, a transcription initiation codon (ATG) as part of an *Nde*I restriction site followed by a region encoding a hexahistidine amino acid tag, internal blunt-ended restriction endonuclease sites (*Sma*I and *Sna*BI) and a termination codon. Wherever possible, nucleotide sequence was chosen to match *E. coli* codon usage preferences for optimal expression. The overhangs engineered into the module allowed for convenient ligation into *Eco*RI/*Hind*III-digested maintenance vector.

The strategy for increasing the number of repeat motifs within constructs is shown in Fig. 1B. The presence of suitable blunt-ended restriction sites within or surrounding the repeat motifs is critical for this strategy to be successful. The initial construct was subjected to double digestion with a combination of either *Eco*RI and *Sna*BI or *Eco*RI and *Sma*I. For the *Eco*RI/*Sna*BI digest, the insert was purified, whereas for the *Eco*RI/*Sma*I digest the linearized vector, lacking a small insert corresponding to the *Eco*RI/*Sma*I section of the module, was retained. The vector and insert were then religated, resulting in a doubling of the repeat motif of interest with retention of the *Eco*RI, *Sna*BI and *Sma*I restriction sites, but importantly the restriction sites at the position where the blunt ends religated are lost. All other features as previously described were retained, and the new construct encoded two tandem repeats of the consensus sequence including the YGAP tetrapeptide motif. Repetition of this process allowed us to generate up to 64 repeats in tandem as shown in Fig. 2. Due to problems with introduced errors, possibly due to the toxicity of the expressed protein, all colonies and cultures were maintained on LB agar plates with 20 mM glucose to repress expression. Sequencing of constructs confirmed that under these conditions, mutation of the constructs did not occur.

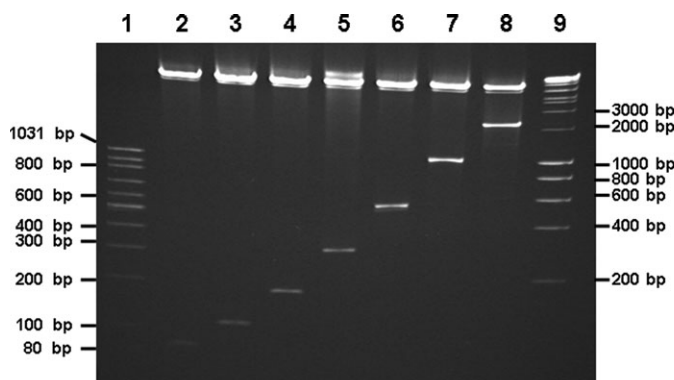

Fig. 2. Insert size of *A. gambiae* constructs following *Nde*I and *Hind*III digestion. Lanes 2–8 correspond to constructs resulting from each doubling process, containing 1, 2, 4, 8, 16, 32 and 64 repeats, respectively. Expected sizes of digestion products are 72, 105, 171, 303, 567, 1095 and 2153 bp, respectively. Size markers are the MassRuler^{TM} low Range DNA ladder (Fermentas) in lane 1 and Hyperladder I (Bioline) in lane 9.

Initially, we chose to express the *A. gambiae* and *D. melanogaster* 16 repeat constructs, as these reflect the numbers of repeats observed in native genes. Due to the engineered *Nde*I restriction site, we were able to digest the constructs with *Nde*I and *Hind*III, and directionally clone them in-frame into the expression vector pETMCS1. Initial expression demonstrated that both of the An16 and Dros16 constructs expressed soluble recombinant protein, and could be purified by IMAC. The purified An16, Rec1 and Dros16 proteins had apparent molecular weights of approximately 42 000, 38 000 and 33 000 Da, respectively, as determined by mobility on SDS-PAGE. This was larger than expected with theoretical molecular weights of 18 917, 28 463 and 23 016 Da for An16, Rec1 and Dros16, respectively. This discrepancy in apparent versus predicted molecular weight has previously been observed for the Rec1 recombinant protein (Elvin *et al.*, 2005). Amino-terminal amino acid sequence analysis (Procise) was carried out on purified recombinant proteins. The first 12 amino acid residues of An16 and Dros16 at 120 pmol yield were MHHHHHHPGAPA and MHHHHHHPGAPG, respectively, as predicted by DNA sequence analysis. Rec1 amino acid sequence has previously been confirmed using the same technique (Elvin *et al.*, 2005).

Crosslinked resilin from insects has previously been demonstrated to be unaffected by heating up to at least 125°C in water (Weis-Fogh, 1960). Similarly, recombinant spider silk protein has been demonstrated to be extremely heat stable. Heating of the supernatant followed by fractional ammonium sulphate precipitation has been applied successfully for the purification of recombinant spider silk protein (Scheller *et al.*, 2001). We also investigated whether heat and/or fractional ammonium sulphate precipitation could be applied for the purification of the resilin-like proteins.

Both the native form (Rec1) and the synthetic proteins (An16 and Dros16) exhibited extreme heat stability. As shown in Fig. 3A, following heating of supernatants at 50, 70, 80 and 95°C for 10 min, many proteins are denatured and removed. However, even after treatment at 95°C for 10 min, the resilin-like recombinant proteins remain intact. The heat stability was further tested by heating An16, Dros16 and Rec1 for 10 min, 30 min, 1 h and 4 h at 95°C. Even after 1 h at 95°C, An16 was not significantly degraded as determined by SDS-PAGE analysis (Fig. 3B, lanes 1–5), although partial degradation is noted after 4 h. Rec1 remained intact at 1 h incubation, but degrades by 4 h (Fig. 3B, lanes 6–10). Although not initially degraded by heating, gel mobility of Rec1 appears to be retarded by increased time of heating at 95°C (Fig. 3B, lanes 6–10). No effect on protein mobility was observed in either An16 or Dros16 (Fig. 3B). Dros16 was most susceptible to heating with partial degradation apparent at 1 h, and the protein was completely degraded after 4 h (Fig. 3B, lanes 11–15). To limit the effect, we routinely heat it for 10 min, and as there appeared to be little difference between heating at 80 and 95°C, we have chosen the lower temperature as a standard for all heating of PEI-treated lysates. Although it is possible to heat supernatants prior to ammonium sulphate precipitation and dialysis, performing the precipitation step first has the added advantage that smaller volumes can be more rapidly and accurately heated.

The hydrophobic nature of resilin-like proteins lends itself to salting out of the recombinant protein. Indeed, all three

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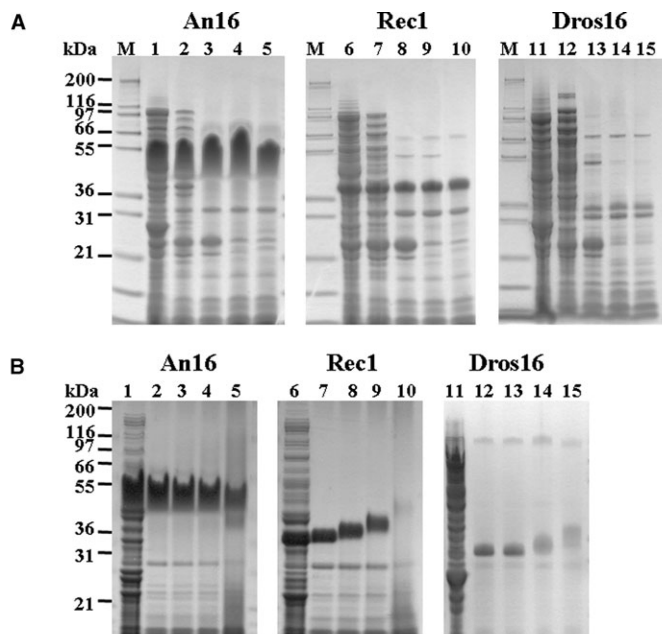

Fig. 3. Effect of heating of soluble lysates of An16, Rec1 and Dros16. Following heating, denatured proteins were removed by centrifugation and supernatants analysed by SDS-PAGE. (**A**) Effect of different temperatures for heating of soluble lysates of An16 (lanes1–5), Rec1 (lanes 6–10) and Dros16 (lanes 11–15). Lanes 1, 6 and 11 correspond to unheated lysates, lanes 2, 7 and 12 correspond to supernatants following heating at 50°C; lanes 3, 8 and 13 correspond to supernatants following heating at 70°C for 10 min; lanes 4, 9 and 14 correspond to supernatants following heating at 80°C for 10 min; and lanes 5, 10 and 15 correspond to supernatants following heating at 95°C for 10 min. (**B**) Effect of prolonged heating at 95°C on soluble lysates of An16 (lanes1–5), Rec1 (lanes 6–10) and Dros16 (lanes 11–15). Following heating, denatured proteins were removed by centrifugation and supernatants analysed by SDS-PAGE. Lanes 1, 6 and 11 correspond to unheated lysates; lanes 2, 7 and 12 correspond to supernatants following heating at 95°C for 10 min; lanes 3, 8 and 13 correspond to supernatants following heating at 95°C for 30 min; lanes 4, 9 and 14 correspond to supernatants following heating at 95°C for 1 h; and lanes 5, 10 and 15 correspond to supernatants following heating at 95°C for 4 h.

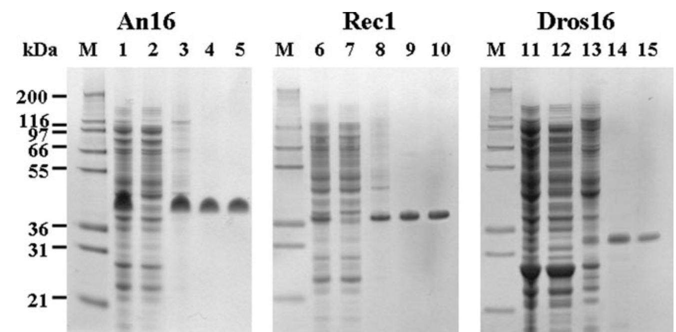

Fig. 4. Comparison of recovery and purity of An16 (lanes 1–5), Rec1 (lanes 6–10) and Dros16 (lanes 11–15) using the non-chromatographic method versus IMAC. Each lane corresponds to equivalent volumes of starting material and were analysed by SDS-PAGE. Lanes 1, 6 and 11 correspond to soluble lysates; lanes 2, 7 and 12 correspond to remaining soluble protein following precipitation in ammonium sulphate; lanes 3, 8 and 13 correspond to precipitates following resuspending and dialysis in PBS; lanes 4, 9 and 14 correspond to resuspended soluble proteins following heating at 80°C for 10 min and removal of denatured protein by centrifugation, and lanes 5, 10 and 15 correspond to recombinant proteins isolated by IMAC.

recombinant proteins precipitated out at low concentrations of ammonium sulphate. For An16 and Rec1, 20% saturation was optimal for precipitation, whereas 30% saturation was optimal for Dros16 (data not shown). Ammonium sulphate precipitation was therefore applied as a first step to partially purify all three recombinant proteins, as demonstrated in Fig. 4.

By combining these two features of hydrophobicity and heat stability, we developed a rapid purification protocol. This method employs three important steps: precipitation of recombinant proteins from PEI-treated soluble lysates by the addition of ammonium sulphate at predetermined optimal concentrations, resuspending of precipitated proteins in a small volume of PBS followed by dialysis in PBS to remove excess salt, and heating of the resuspended proteins at 80°C to further remove undesirable proteins. This non-chromatographic method has proven to be extremely efficient in the purification of recombinant resilin-like proteins, giving full recovery of the recombinant protein from soluble fractions. For each protein, complete recovery of recombinant protein was observed following precipitation in ammonium sulphate. This is demonstrated in Fig. 4 by the absence of the recombinant protein in soluble lysates following precipitation (lanes 2, 7 and 12 for An16, Rec1 and Dros16, respectively), and retention of the recombinant proteins in the resuspended pellets (lanes 3, 8 and 13 for An16, Rec1 and Dros16, respectively) with no significant loss of band intensity. Precipitation of Dros16 in 30% ammonium sulphate was necessary for full recovery, resulting in a comparatively dirtier resuspension than for An16 and Rec1 that were precipitated in 20% ammonium sulphate (lanes 3, 8 and 13 for An16, Rec1 and Dros16, respectively). Heating of the lysates at 80°C for 10 min resulted in no significant loss of recombinant protein but removed all remaining visible bacterial contaminants, as demonstrated in Fig. 4 (lanes 4, 9 and 14 for An16, Rec1 and Dros16, respectively). Fig. 4 also highlights that the end-products of this purification regime (lanes 4, 9 and 14 for An16, Rec1 and Dros16, respectively) were of similar size, purity and concentration to corresponding proteins purified by IMAC (lanes 5, 10 and 15 for An16, Rec1 and Dros16, respectively). Volumetric productivity (milligrams of recombinant protein per liter of culture) varied significantly for each protein at 220, 60 and 20 mg/l for An16, Rec1 and Dros16, respectively.

In an attempt to understand the aberrant gel mobility of the recombinant proteins, especially the changes in gel mobility of Rec1 by increased time of heating at 95°C (Fig. 3B, lanes 6–10), MALDI-TOF MS was used to compare the molecular weights of non-chromatographically purified resilin-like proteins to the same proteins purified by IMAC. Recombinant proteins heated at 95°C for 10 min, 30 min and 1 h were also compared. Results revealed that the molecular weights of all three recombinant proteins were unchanged irrespective of which purification method was used, and that heating at 95°C for up to 1 h had no effect on molecular weight (Table I). Molecular weights of An16, Rec1 and Dros16 as determined by MS are similar to their theoretical molecular weights. Importantly, the MALDI-TOF MS spectra of IMAC-purified Rec1 and Rec1 purified by the non-chromatographic method are similar in both molecular weights and purity (Fig. 5).

To determine whether heating had any effect on the material properties of Rec1 resilin, Rec1 was purified by either IMAC or the non-chromatographic method, cross-linked, and the resulting Rec1 resilin was analysed using

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Table I. MALDI-TOF MS analysis of An16, Rec1 and Dros16

Treatment	AN16	Rec1	Dros16
IMAC	18 294	28 052	22 332
AS + 80°C for 10 min	18 301	28 033	22 304
AS + 95°C for 10 min	18 305	28 027	22 307
AS + 95°C for 30 min	18 306	28 029	22 297
AS + 95°C for 30 min	18 305	28 025	22 310

Comparison of molecular weights of recombinant proteins purified by IMAC, the standard nonchromatographic method, and extended heating at 95°C for 10, 30 and 60 min.

SPM as previously described (Elvin *et al.*, 2005). Force–distance curves for each sample were identical (Fig. 6) irrespective of purification method, displaying similar levels of penetration and negligible hysteresis upon compression. These results indicate that both materials have similar levels of stiffness and very high resilience (92%).

It should be noted that the resilin-like proteins do not retain the Coomassie Brilliant Blue well during destaining in methanol and acetic acid, hence the shortened destaining time of 1 h was followed by further destaining in water. While it is unclear why this is the case, the inability to retain Coomassie Brilliant Blue R-250 has been noted in other unrelated proteins including some herpes simplex virus structural proteins (Gibson and Roizman, 1974). To date, no consensus has been reached as to the reasons for observed differences in binding and retention of Coomassie Brilliant Blue R-250, although preferential binding of basic amino acids by the stain, hydrophobic properties and steric effects of the proteins have been proposed as potential contributing factors (Tal *et al.*, 1985; Rabilloud and Charmont, 2000; Fievet *et al.*, 2004). At this point of time, we are unable to ascertain the cause of the noted destaining characteristics in resilin-like proteins.

Discussion

Elastomeric proteins have been identified in a range of biological systems. The mechanical and biochemical properties of some have been extensively studied, especially elastin, abductin and flagelliform spider silks (Tatham and

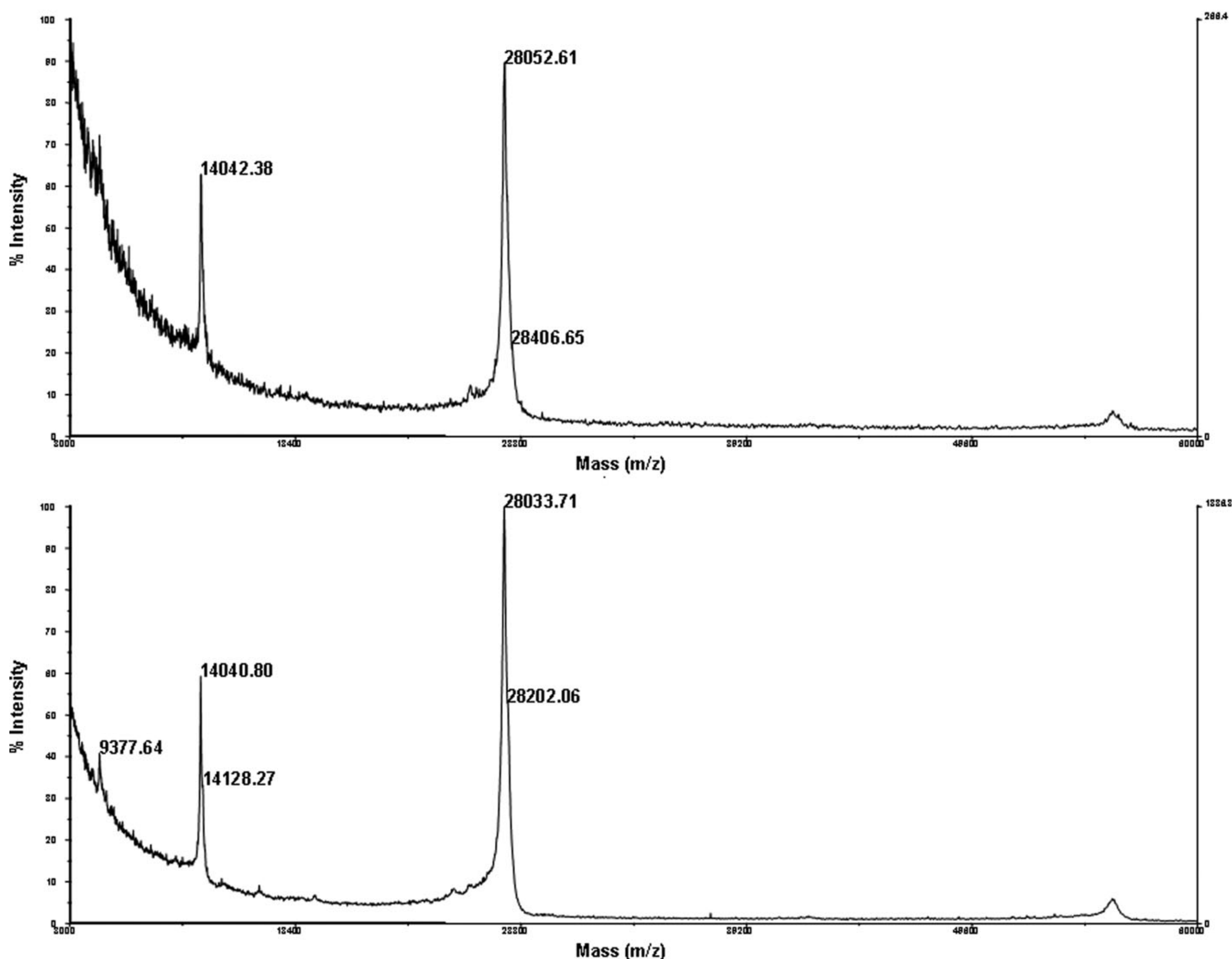

Fig 5. Representative MALDI-TOF MS spectra for comparison of purity and molecular weight of Rec1 recombinant protein purified by IMAC (upper) versus the nonchromatographic method (lower).

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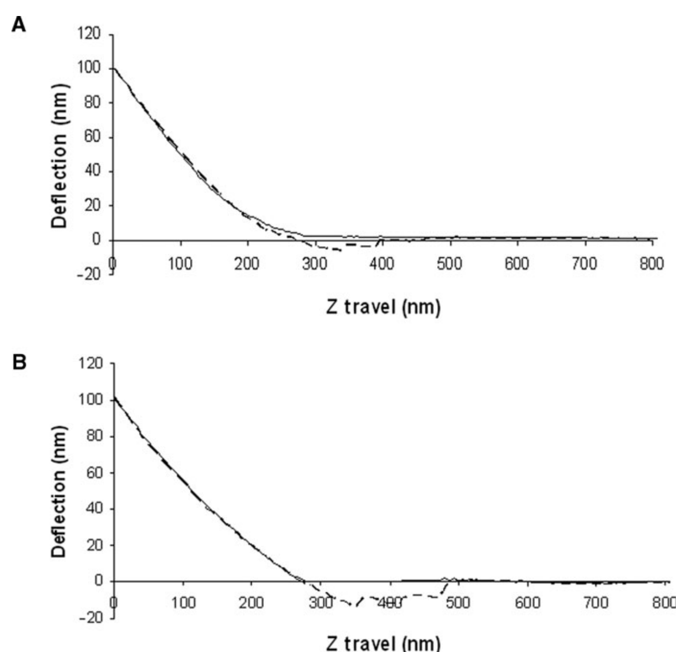

Fig. 6. Force–distance curves recorded for crosslinked Rec-1 resilin purified by either the non-chromatographic method (**A**) or IMAC (**B**). The approach curve is represented as an uninterrupted line, and the retract curve by the broken line.

Shewry, 2002; Gosline *et al.*, 2002), and their potential as biomaterials for industrial and biomedical applications are well documented (Yoda, 1998; Martino *et al.*, 2002). However, other proteins including resilin have not been studied in such detail, partly because of difficulties in obtaining large amounts of pure resilin from natural sources. Recently, a recombinant resilin-like protein (Rec1) has been expressed, purified by IMAC, and photochemically crosslinked to form an elastic biomaterial (Rec1 resilin) with exceptional resilience properties (Elvin *et al.*, 2005).

To capitalize on this research, we have developed a recursive cloning strategy, similar to that used for other elastomeric proteins including gliadin, silk and elastin (Elmorjani *et al.*, 1997; Prince *et al.*, 1995; Lewis *et al.*, 1996; Meyer and Chilkoti, 2002), to generate periodic polypeptides based upon the repetitive domains of resilin-like genes in *D. melanogaster* and *A. gambiae*. Utilizing this method, we have been able to precisely control the sequence integrity, number, order and direction of monomers inserted into the vector through a simple stepwise series of digestions and religations. To date, we have produced constructs up to 64 repeats long, and we are able to generate chimeric constructs consisting of combinations of the *Anopheles* and *Drosophila* motifs. We are currently employing this technique to generate constructs from other arthropods.

The development of a rapid protocol for complete and selective purification of resilin-like proteins has also been an invaluable step in allowing us to economically purify the recombinant proteins without the need for IMAC, and is currently being used within our laboratory to evaluate and improve expression systems for Rec1 and related proteins (Kim *et al.*, in press). Importantly, the recombinant An16 and Dros16 proteins behave in a similar manner to that of Rec-1, suggesting that this purification protocol may be useful for many resilin-like proteins. As there is limited sequence homology between the *Drosophila* and *Anopheles* motifs other than the conserved tetrapeptide YGAP, one may infer that this motif is critical in conferring heat resistance and hydrophobic properties to resilin and related proteins. As noted previously for Rec1, a characteristic feature of these resilin-like proteins is a pronounced discrepancy between molecular masses as determined by mobility in SDS-PAGE and molecular masses as predicted based upon amino acid sequence or HPLC. The recombinant proteins ranged from 1.3 to 2.2 times larger by electrophoresis than the predicted sizes. For all three resilin-like proteins, molecular weights as determined by MALDI-TOF MS were in agreement with predicted molecular weights. Such discrepancies have previously been noted in cuticle proteins of insects (Andersen *et al.*, 1995). Atypical SDS-binding has been proposed as the cause, and has been noted previously in glycoproteins and in proteins having unusual amino acid composition (Weber *et al.*, 1972).

Importantly, although heating of Rec1 affects its mobility in acrylamide gels, heating does not affect the molecular weight or biomaterial properties of the recombinant protein. Like elastin (Rousseau *et al.*, 2004), structural analyses of Rec1 and An16 using conventional techniques such as circular dichroism spectroscopy, X-ray crystallography and nuclear magnetic resonance have thus far failed to identify secondary structures (unpublished observations from our laboratory), and analysis of these proteins using the IUPred web server (http://iupred.enzim.hu/) for the prediction of intrinsically unstructured regions of proteins (Dosztányi *et al.*, 2005) suggests they are unstructured. However, it is tempting to suggest that changes in the mobility of Rec1 following extended heating implies the loss upon heating of some secondary structure not identified by the techniques used to date. Efforts are currently underway to further characterize this phenomenon.

It should also be noted that concentrated aqueous solutions of all resilin-like recombinant proteins tested (An16, Dros16 and Rec1) have a tendency to form a concentrated protein lower phase at 4°C. Empirically, at equilibrium, the upper phase contains 10–20 mg/ml resilin-like recombinant protein, whereas the lower phase contains 200–300 mg/ml resilin-like recombinant protein. The enriched protein phase can be readily diluted in PBS. The upper phase can appear as an opaque solution at 4°C, suggesting a micellar structure, and heating of the cloudy solution results in clearing of these solutions. On the basis of these observations, it is likely that the resilin-like proteins have self-associating propensity, a property that has previously been identified in elastin (Urry *et al.*, 1969). However, unlike our observations for resilin-like proteins, elastin coacervation is an entropically driven reversible process in which elastin and related proteins are soluble at low temperatures, and only upon heating to a specified inverse transition temperature do the solutions become cloudy as self-aggregation and ordering of hydrophobic domains occur (Vrhovski and Weiss, 1998). It is anticipated that future characterization of this property in the resilin-like proteins will provide valuable insights into the ordering and crosslinking of resilin. Although we do not yet understand the molecular mechanisms of phase separation in the resilin-like proteins, the property has proven to be extremely useful in the purification of concentrated resilin-like protein.

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We are currently using the techniques described here to synthesize and express a number of periodic peptides of varying sizes and amino acid composition. It is anticipated that these developments will accelerate our ability to characterize and compare resilience, modulus of elasticity, and other functional attributes of the resulting biomaterials, as a first step in understanding the structural and mechanical properties of resilin. In the longer term, we anticipate these or similar customized biomaterials may have biomedical and/ or industrial applications.

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