INVESTIGATION OF *TRIBOLIUM CASTANEUM* RESILIN, A RUBBER-LIKE INSECT CUTICULAR PROTEIN

by

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Abstract

Resilin is a rubber-like cuticular protein found in many insect species. Resilin is important for jumping and flying of those insects due to the properties of high elasticity and efficient energy storage. Some recombinant proteins or peptides derived from resilin sequences have been synthesized to produce biomaterials that mimic the remarkable properties of resilin. This research focused on resilin in the red flour beetle, *Tribolium castaneum*. A cDNA for *T. castaneum* resilin was inserted into plasmid vectors for expression of resilin in *Escherichia coli* or *Bacillus subtilus*. Resilin produced in *E. coli* was used as antigen to produce a rabbit antiserum. Resilin synthesized by *B. subtilis* as a secreted protein was purified and used for biochemical studies. Resilin is highly expressed in the late pupal stage, and in hind wings, but not found in elytra of pharate adults, indicated by RT-PCR and immunoblot analysis. Recombinant resilin could be cross-linked in the presence of horseradish peroxidase and hydrogen peroxide, detected by appearance of a high molecular weight band on SDS-PAGE, which had blue fluorescence under ultraviolet light, presumably due to dityrosine linkages. RNA interference was used to knock down resilin expression in *T. castaneum*. Immunoblot and RT-PCR analyses indicated that resilin expression was successfully decreased by RNAi. However, the knockdown adults exhibited no apparent differences in morphology, behavior or life span from control beetles. Blue fluorescence under ultraviolet illumination has frequently been used as an indication of the presence of resilin containing dityrosine cross-links in insect tissues such as wings, wing tendons and leg joints. A similar blue fluorescence was observed in hind wings of *T. castaneum*. However, this fluorescence was not decreased in hind wings of beetles in which resilin expression was knocked down by RNA interference. There was a blue fluorescence in the hind wings of knockdown beetles, which was similar in distribution to that in wings of control insects. This result suggests that the observed blue fluorescence in *T. castaneum* hind wings is derived not only from cross-linked resilin but also from components other than resilin, perhaps other cuticular proteins that contain dityrosine cross-links.

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Chapter 1 - Introduction

Elastic proteins

Materials with special properties for different functions are formed during evolution. Elastic proteins have a natural property of returning to their original state after the shape is changed by an external force; in other words, they can stretch and relax (Gosline et al., 2002). Different elastic proteins can have diverse properties in terms of resilience, stiffness, strength and extensibility. Different combinations of these properties provide some essential functions for an organism (Gosline et al., 2002; Rauscher and Pomes, 2012). Some well-known examples of elastic proteins include elastin, resilin, silk, abductin and gluten. These proteins share chemical similarities, such as repeated amino acid sequences, a high content of glycine and alanine residues, cross-links and disordered structures (Rauscher and Pomes, 2012; Grove and Regan, 2012; Fernandez and Ingber, 2012). What follows is a brief introdution of all of these proteins except for resilin, which will be described in greater detail in the later sections of this thesis.

In the human body, elastic fibers are found in the skin, lung, aorta and other tissues which need elasticity and resilience for their essential functions (Christiano and Uitto, 1994). The two main components of mammalian elastic fibers are elastin, which is the major one and forms the core structure, and microfibrils, which surround the core and consist mainly of fibrillin (Kielty et al., 2002; Wagenseil and Mecham, 2007). Human elastin is encoded by a single gene. The precursor unit of elastin is called tropoelastin, which is a soluble protein of about 70 kDa. Tropoelastin contains alternating hydrophobic and cross-linking domains and can form covalent cross-links with the help of microfibrils (Wagenseil and Mecham, 2007). The cross-linkages are formed between about five lysine residues from the crosslinking domains, catalyzed by lysyl oxidase(s) (Wagenseil and Mecham, 2007). Compared to tropoelastin, the crosslinked mature elastin is insoluble. Elastin fibers swell in water and are hydrated, so they do not exhibit elasticity in the absence of water (Duca et al., 2004). Fibrillins have 3 group members: fibrillin-1, fibrillin-2 and fibrillin-3, which are large glycoproteins encoded by different genes (Kielty et al., 2002; Jensen et al., 2012). Fibrillins further polymerize into microfibrils through both disulfide bond formation and transglutaminase-catalyzed cross-links (Kielty et al., 2002) in a head-to-tail manner (Ramirez and Sakai, 2010) and form a “beads-on-string” structure (Ramirez et al., 2004).

Calcium binding also plays an important role in stabilizing microfibrils (Ramirez and Sakai, 2010). Microfibrils form first and act as a scaffold for elastin to deposit on (Christiano and Uitto, 1994), but they are also found in some tissues without elastin and nevertheless provide the elastic property (Wagenseil and Mecham, 2007). Elastic fibers are not replaced during the whole lifetime after being synthesized, and they are related to some aging issues and diseases such as Marfan syndrome (Sherratt, 2009). Moreover, what makes elastic fibers more interesting is that they are promising biomaterial templates for tissue engineering. For example, some elastin-derived peptides and recombinant tropoelastin were synthesized, and some of the different artificial materials produced were used in dermal, vascular applications (Daamen et al., 2007; Almine et al., 2010).

Spiders are not liked by many people, but they attract the interest of a lot of researchers because they can produce the fascinating silk used to make the web and other functions. Spider silk is an excellent material that is better than any of the man-made fibers due to its high strength and extensibility as light weight fiber (Hu et al., 2006; Omenetto and Kaplan, 2010). The entire orb-shaped spider web is composed of several different types of silks (Lewis, 2006) that are not similar to silkworm silks which mainly have one protein (Omenetto and Kaplan, 2010). In fact, a spider can produce up to six different silks (such as dragline silk, flagelliform silk, eggcase silk and others) and the silk glue, each of which is generated in a specific gland and function in different ways. For instance, dragline silks act as the frame of the orb-web and the lifeline of the spider, while flagelliform silk serves as the catching spiral, whereas eggcase silks are used to protect the offspring (Lewis, 2006; Bittencourt et al., 2012). Proteins that make up spider silks are called spidroins. Most spider silks are composed of more than one spidroin, with the exception of, for example, flagelliform silk containing only one major protein (Rising et al., 2011). The spidroins are encoded by different genes but they share a common architecture with the protein molecules composed of three parts: a highly repetitive polyalanine/glycine rich core region and highly conserved non-repetitive N-terminal and C-terminal domains (Heim et al., 2009; Rising et al., 2011). There could be more than one hundred repeats in a single protein, and the repeats can comprise more than 90 percent of the entire protein (Romer and Scheibel, 2008).

Among all the silks, the dragline silk is the most studied, probably because it is the strongest one, whereas the flagelliform silk is the most elastic (Hu et al., 2006; Bittencourt et al., 2012). Dragline silk has two main similar components: major ampullate spidroin 1 and 2 (MaSp

1 and MaSp2, respectively), which differ in the proline content of the repeated region of MaSp2 (about 15 percent proline), whereas MaSp1 has no proline residues (Hu et al., 2006). The repeated core of the two proteins contains alternating glycine-rich and alanine block regions (Figure 1-1 D). These two residues comprise more than 60 percent of the whole sequence, indicating the importance of this composition. The alanine block may form crystalline structures to interact with different molecules, and the glycine-rich region is responsible for the elasticity of the protein (Hu et al., 2006; Rising et al., 2011). The non-repetitive C-terminal domain contains a signal peptide and can form intermolecular disulfide bond linkages due to the conserved cysteine residues, which will stabilize multimers involved in higher aggregate complex formation (Hu et al., 2006; Romer and Scheibel, 2008). In contrast, the importance of the non-repetitive N-terminal domain is still unclear (Hagn, 2012).

There is great interest in making use of the superlative properties of spider silk. However, unlike silkworm silk, which can be reeled from cocoons of silkworm pupae and is commercially available, a "spider silk farm" is impractical due to the territorial and cannibalistic natures of spiders and the low yield of silk from individual spiders (Hu et al., 2006; Romer and Scheibel, 2008). Therefore, researchers in biotechnology and bioengineering have focused on silk-derived synthetic materials. Many different hosts like bacteria, fungi, plants, insect and mammalian cells have been used to express cloned silks (Omenetto and Kaplan, 2010). Recombinant silk proteins can be used to make fibers, textiles, films, hydrogels, porous sponges and microcapsules (Kluge et al., 2008), but so far, no artificial spider silk is as perfect as the natural one (Hagn, 2012). The repetitive sequences may be one of the reasons that make the production of recombinant silk challenging, since it is hard to find a suitable expression host (Heim et al., 2009). Mimicking the spinning of spider silk is another challenge, because it is a very complicated and amazing process that involves different conditions and organs, including a liquid-liquid phase separation followed by a liquid-solid phase transition, converting the proteins from a concentrated solution to the final solid dry silk (Hu et al., 2006; Heim et al., 2009; Hagn et al., 2012).

Abductin was first found as a rubber-like protein in the internal triangular hinge ligament of *Pecten* (Kelly and Rice, 1967). The biological role of abductin is to keep the scallop shells open when the adductor muscle relaxes, using the elastic energy stored as the shell closes, thus this protein plays roles in swimming (Kelly and Rice, 1967; Rauscher and Pomes, 2012). Abductin is similar to elastin and resilin (discussed below) in physical properties, and they all

exhibit rubber-like elasticity when hydrated and become rigid and leather-like when dry (Kelly and Rice, 1967; Rauscher and Pomes, 2012). The amino acid composition of abductin is very different from elastin and resilin (Figure 1-1 C), and it has a high content of not only glycine but also methionine (Kelly and Rice, 1967). The amino acid sequences of abductin from *Argopecten* (the bay scallop) was reported by Cao et al. (1997) and they divided the sequences into two domains: the alanine-rich N-terminus and the remainder of the protein which is glycine/methionine-rich. Contained in this region is a highly repetitive sequence with a repeat motif of GGFGGMGGGX (Cao et al., 1997). The mechanical properties of abductin is mainly determined by its glycine content (Rauscher and Pomes, 2012). Disulfide cross-links, which are stable in the oxidative state of sea water, are suggested to be present in abductin (Bochicchio et al., 2008), but the residues involved are still unclear. Circular dichroism studies of abductin-derived peptides suggest the presence of polyproline Ⅱ structures and β-turns in the abductin protein (Bochicchio et al., 2008).

There are many other elastic proteins created by nature. For example, many people eat one group of elastic proteins everyday, which account for most of the dry weight of wheat grain, called gluten. Gluten is responsible for the viscoelasticity of bread dough, which is, however, a totally fortuitous property during evolution (Shewry et al., 2002). Some repeat motifs and conserved amino-acid sequences are also found in gluten. For example, the high molecular weight (HMW) subunit, which is the major determinant of gluten elasticity, is composed of three domains: a non-repetitive N-terminal domain, which is short but variable in length, the central domain, which contains a large number of short repeats, and a non-repetitive 42-residue C-terminal domain (Shewry et al., 2002). A high content of glutamine, proline and glycine residues is found in the repeat domains (Shewry et al., 2002; Anjum et al., 2007).

The resilience of abductin, elastin, major ampullate silk and resilin are 82-97%, 90%, 35% and 92%, respectively; the strength of elastin, major ampullate silk and resilin are 2 MPa, 1.1 GPa and 4 MPa, respectively (Rauscher and Pomes, 2012). One major characteristic of elastic proteins is their repeated amino acid sequences, and I show the repeat regions of the proteins discussed in this chapter in Figure 1-1.

Cuticular proteins

Arthropoda is the largest animal phylum, containing the Class Hexapoda, the insects and closely related organisms. Arthropod animals are found in almost every corner of the world, from river to ocean, from earth to air. To a great extent, this wide distribution profits from the exoskeleton or cuticle, which allows the animals to be able to adapt to various environments. Cuticles could have different mechanical properties and thus different functions, such as maintaining the body shape, locomotion, preventing dehydration and infection, protection from predators and some external stimuli like UV radiation, chemicals and temperature (Suderman et al., 2010; Nagasawa, 2012; Moussian, 2013). This versatile biomaterial is mainly composed of the polysaccharide chitin and a matrix of protein and catechols, together with some minor components such as lipids, wax esters and some inorganic materials (Nagasawa, 2012; Moussian, 2013). Crustaceans and a few species of Insecta use minerals such as calcium, zinc and manganese as another major component, resulting in very rigid cuticle compared to other arthropods (Morgan et al., 2003; Nagasawa, 2012), but the majority of insect cuticles lacks such minerals. After cellulose, chitin is the second most abundant polysaccharide in the world, and now is commercially available, mainly obtained from shellfish sources that are the waste products of the seafood industry (Gonil and Sajomsang, 2012). Chitin and its deacetylated derivative, chitosan, are biocompatible and biodegradable materials, which are well studied and used for many applications (Fernandez and Ingber, 2012). A good introduction to insect chitin is in the recent review by Doucet and Retnakaran (2012).

The outstanding mechanical properties of cuticle depend on both the individual protein chains and the interactions between proteins and chitin fibers (Andersen and Roepstorff, 2005; Fernandez and Ingber, 2012). The water content seems to be another important factor of the mechanical properties of insect cuticle (Klocke and Schmitz, 2011). The different cuticular proteins are a fundamental factor of cuticle properties, and they are even more important for the tick because the tick cuticle has rather poor chitin content (Andersen and Roepstorff, 2005).

Twelve cuticular protein families from arthropods have been described by Willis (2010). The largest is the arthropod-specific cuticular protein R&R (CPR) family, named because of a common motif of 35 amino acids recognized by Rebers and Riddiford (1988). The RR motif may be a chitin-binding domain. CPR proteins have two major subgroups: RR-1 and RR-2, and a third minor one RR-3 (Willis, 2010). The sequences of CPR proteins are very diverse even in the

R&R region, indicating their roles as structural proteins rather than enzymes (Moussian, 2013). Interestingly, the RR-1 proteins are found mainly in soft or flexible cuticles while the RR-2 proteins are associated with hard or rigid cuticles (Willis, 2010), suggesting that R&R domains maybe also play roles in regulation of protein-protein or protein-chitin structures. Most CPR proteins contain only one R&R motif, but a few exceptions found in some species have more than a dozen such regions (Willis, 2010).

Compared with CPRs, the cuticular proteins of the Tweedle group are less diverse, sharing a domain with an unknown function and lacking a known chitin-binding domain. Tweedle proteins are insect-specific and tissue and stage specifically expressed, suggesting that they play key roles in different types of cuticles but not as a cuticle organizer (Moussian, 2013). There also are some other protein families which seem order-specific. For instance, the family of "cuticular proteins glycine-rich" (CPG), a lepidopteran family, is named due to many GGYGG or GGxGG repeats; the family of alanine-rich cuticular proteins (CPLCA) has so far only been found in Diptera and contains 13%-26% alanine residues in the amino acid sequences (Willis, 2010; Moussian, 2013).

During cuticle development, cross-linking of cuticular proteins (CPs) with phenolic compounds results in the hardening of the cuticle, a process known as sclerotization (Moussian, 2013). The cross-linking of proteins with phenols is known as catecholation and is catalyzed by extracellular laccases (Suderman et al., 2006) An interesting feature of CPs is that despite some specific exceptions, most contain no cysteine residues in their secreted or mature form (Willis, 2010). Thus, in formation of cross-linked proteins in sclerotization , these proteins cannot use disulfide bonds or other sulfer adducts as the linkage. One alternate option for cross-linking is the dityrosine linkage between tyrosine residues of neighboring proteins (Moussian, 2013). This type of linkage occurs in the cuticle collagens and cuticlins of *C. elegans,* which are cross-linked by di- and tri-tyrosine cross linkages during cuticle synthesis (Page and Johnstone, 2007). Another type of cross-link in the cuticle may be catalyzed by extracellular transglutaminases, which may have a role in the pigmentation and stiffness of the cuticle (Moussian, 2013).

Resilin

Resilin is a structural cuticular protein first described in locusts and dragonflies by Weis-Fogh (1960) and called "a rubber-like" protein. Three elastic structures, the prealar arm and the

main wing-hinge ligament from desert locust, and the tendon of the pleuro-subalar muscle from a dragonfly, were tested in many aspects, such as water content, reactions with dyes and chemicals and mechanical properties, leading to the conclusion that it was the same protein present in all three samples. This protein was named "Resilin." Resilin will not dissolve in many solvents if the polypeptide is not broken up, but it can be easily digested by proteases. It is very stable upon heating and to pH changes, and provides the characteristic elasticity for some regions of insect cuticle (Weis-Fogh, 1960). In a wide pH range (from pH 3 to 9.5), resilin could be selectively stained by methylene blue and toluidine blue (Andersen and Weis-Fogh, 1964), which is a simple method to identify structures containing resilin. Resilin becomes rubber-like only when hydrated in water or other polar solvents and rigid when dry, and this process is totally reversible, suggesting that the protein is stabilized by some type of non-covalent interactions (Weis-Fogh, 1960). Andersen found that resilin fluoresces blue under ultraviolet light (excitation wavelength is around 300 nm, emission maximum is about 415 nm) due to the presence of dityrosine and trityrosine, which cross-link different molecules of resilin to form a network (Andersen, 1963, 1964). Nowadays, detecting the blue fluorescence of ditytosine has become a common method for detecting resilin, although Andersen and Weis-Fogh (1964) caution against using only one method for detecting resilin.

Resilin has been found in many arthropod animals, in some regions where resilience and/or energy storage are needed, and in most cases, the protein was identified based on its characteristic blue fluorescence. An important function of resilin in the pleuro-subalar tendon of the dragonfly is to store energy for flight (Andersen and Weis-Fogh, 1964). Elasticity is also needed for the vein twisting that occurs in dragonfly wings during flight (Gorb, 1999). In damselflies there are two types of vein joints in the wing: immobile and mobile; resilin was detected by fluorescence microscopy only in the mobile joints (Gorb, 1999). Earwigs have highly folded hind wings, and resilin is present there, mainly at the elastic joints of veins, which generate folding forces, and also along the radiating folds (Haas et al., 2000a). Resilin is also present in hind wings of two coleopteran beetles, *Pachnoda marginata* and *Coccinella septempunctata*, where extra mobility is needed (Haas et al., 2000b). These findings are all based on the blue fluorescence of resilin. Resilin is also used in jumping, for example, froghopper insects store energy for jumping using a composite structure of resilin and chitinous cuticle (Burrows et al., 2008). There is another well-known "resilin pad" at the base of flea hindleg used

to store energy in jumping (Sutton and Burrows, 2011). Wiesenborn made good use of the blue fluorescence under UV light and produced many beautiful pictures showing the presence of resilin in insects from 7 orders, 17 families and 18 genera (Wiesenborn, 2011), indicating a wide occurrence of reslin in insects. Wings and legs of insects have a relatively long fatigue lifetime, whereas other body parts have very different fatigue properties (Dirks et al., 2013). At least one of the reasons for this difference may be the different distribution of elastic resilin and the different degree of di- and trityrosine cross-linking of resilin in different regions (Andersen, 2004).

A milestone in resilin history was the identification of the resilin gene in *Drosophila melanogaster* by Ardell and Andersen (2001). Gene CG15920 was suggested as the *Drosophila* resilin precursor (Ardell and Andersen, 2001). One valuable product based on this gene was the generation of an antibody against a resilin-like protein (rec1-resilin) encoded by the first exon of the gene (Elvin et al., 2005); this antibody was used to detect resilin *in vivo*, a method beyond the classic blue fluorescence. Some regions of legs and wing bases of *Drosophila* pharate adults, which autofluoresce, also cross-react with the anti-rec1-resilin antibody in sections. Not only in *Drosophila*, but also in fleas and dragonflies, similar results were obtained using this antibody (Wong et al., 2012). This was the first time, to my knowledge, that an antibody was used to confirm the autofluorescence materials as resilin.

The *Drosophila* resilin protein (CG15920) sequence is characterized by three regions: the N-terminal region containing 18 repeats of a 15-residue sequence, the center region of 62 residues similar to the RR-2 consensus and the C-terminal region containing 11 repeats of a 13-residue sequence (Ardell and Andersen, 2001). Exons 1, 2 and 3 encode the N-terminal repeated region, the RR-2 consensus motif and the C-terminal repeated region, respectively. Based on this sequence, the N-terminal domain was expressed in *Escherichia coli* and the purified and concentrated protein solution was cast into a rubber-like rod by a rapid photochemical cross-linking method (Elvin et al., 2005). This system could be a useful tool in biomaterial and biomedical applications. The successfully used expression and purification methods (Kim et al., 2007; Lyons et al., 2007) for the rec1-resilin should be also helpful to other researchers. Several groups have further studied the *Drosophila* resilin. The chitin-binding function of the RR-2 domain was confirmed by an *in vitro* chitin-binding assay (Qin et al., 2009). A recombinant protein containing most of the repeat region from the first exon had similar properties to native

resilin (Lyons et al., 2009), and protein derived from exon 1 is more elastic than that from exon 3 (Qin et al., 2011), perhaps because the exon 1 protein would be more disordered in a random coil conformation, while exon 3 and full length resilin are predicted to form β-turns in a β-spiral conformation (Tamburro et al., 2010; Qin et al., 2011; Qin et al., 2012).

Tribolium castaneum (the red flour beetle) belongs to the order Coleoptera and is a "model insect" not only because its genome sequence is available, but also the RNA interference (RNAi) technique is a powerful and attractive experimental tool used in this beetle (Miller et al., 2012). The putative pro-resilin cDNA sequence (HQ110094) was submitted to NCBI by Dittmer and Kanost from our group in 2010. The sequence characteristic is similar to the *Drosophila* resilin gene, with a repeated sequences in the N-terminal and C-terminal regions flanking a RR-2 consensus motif (Figure 1-2). However, the *Tribolium* N-terminal repeat region is bigger, containing 9 repeats of 23 amino acid residues; the C-terminal repeat region contains 12 repeats of 12 amino acid residues. In both repeat regions, there is a conserved repeat of tyrosine (Y) residues, which may be responsible for the cross-linking of resilin. A high content of glycine (G) residues is also found in the two repeat regions. The sequence similarities of resilin from fruit fly, flea, horn fly, dragonfly and the red flour beetle are compared (Figure 1-3). All proteins have two repeat regions and conserved tyrosine residues. The N-terminal repeat region has higher similarity than the C-terminal repeat region. Two isoforms were found in some insects. Isoform A contains the RR motif, which is missing in isoform B. Only isoform A has been identified in *Tribolium* so far. However, no other information is available about resilin in *Tribolium*. Thus, my research focused on the protein resilin itself in the red flour beetle, *Tribolium castaneum,* with a long term goal of investigating its structure and function.

Specific aims

The first aim of this research was to express a recombinant resilin in *Bacillus subtilis*, and purify the protein for experiments to investigate the cross-linking reactions *in vitro*.

The second aim was to localize resilin in *Tribolium* hind wings by the classic autofluorescence property of resilin and immunohistochemistry using a purified antibody against a recombinant resilin expressed in *E. coli*.

The last aim was to characterize the phenotype(s) of adult beetles in which resilin expression had been depressed by RNA interference using double-stranded RNA.

(A) Human Elastin

VGVAPG
VGVAPG
VGVAPG
VGLAPG
VGVAPG
VGVAPG
VGVAPG

(B) Beetle Resilin

GQNGGGPSSTYGPPGQGGNGFGG
GQNGGRLSSTYGPPGQGGNGFGG
GQNGGRPSSTYGPPGQGGNGFGG
GQNGGRPSSTYGPPGQGGNGFGG
GQNGGRPSSTYGPPGQGGNGFGG
GQNGGRPSSTYGPPGQGGNGFGG
GQNGGRPSSTYGPPGQGGNGFGG
GQNGGKPSSTYGPPGQGGNGFGG
GQNGGRPSSTYGPPGQGGNGNGG

GGNGNGGYPSGG
PGGAGNGGYPSG
GPQGGNGGYPSG
GPQGGNGGYPSG
GPQGGNGGYPSG
GPQGGNGGYPSG
GPQGGNGGYPSG
GPQGGNGGYPSG
GPQGGNGGYPSG
GPQGGNGGYTSG
GPQGGNGGYPSG
GPQGGNGGSGPY

(C) Scallop Abductin

GGTGGMGGGMNA
GGFGGIGGGMGGGK
GGFGGMGGGP
GGFGGIGGGS
GGFGGM
GGFGGMGGGK
GGFGGMGSSM
GGFGGMGGGN
AGFGGMGGQS

(D) Spider major ampullate spidroin 1

---QGGYGQGAGSSAAAAAAAAAAAAAGGQG
GQGQGRYGQGAGSSAAAAAAAAAAAAAA
GQGQGGYGQGAGGNAAAAAAAAAAAAAAGQGGQGGQG
GLGQGGYGQGAGSSAAAAAAAAAAAAA
GRGQGGYGQGAGGNAAAAAAAAAAAAAAGQGGQGGYG
GLGQGGYGQGAGSSAAAAAAAAAAAAA
GRGQGGYGQGAGGNAAAAAAAAAAAAAAGQGGQGGYG
GLGQGGYGQGAGSSAAAAAAAAAAAAAGGQG
GQGQGRYGQGAGSSAAAAAAAAAAAAAA
GRGQGGYGQGSGGNAAAAAAAAAAAAASGQGSQGGQG
GQGQGGYGQGAGSSAAAAAAAAAAAAAS
GRGQGGYGQGAGGNAAAAAAAAAAAAAAGQGGQGGYG
GLGQGGYGQGAGSSAAAAAAAAAAAAGGQG
GQGQGGYGQGAGSSAAAAAAAAGSGQGGYG
GQGQGGYGQSSASASAAASAAS

(E) Wheat Glutenin

PGQGQQ
PGQGQQ
SGQGQQGYYPTSPQQ
PGQWQQ
PEQGQPGYYPTSPQQ
PGQLQQ
PAQGQQ
PGQGQQGRQ
PGQGQPGYYPTSSQLQ
PGQLQQ
PAQGQQGQQ
PGQGQQGQQ
PGQGQQ
PGQGQQGQQ
PGQGQQ
PGQGQQGQQ
LGQGQQGYYPTSLQQ
SGQGQPGYYPTSLQQ
LGQGQSGYYPTSPQQ
PGQGQQ
PGQLQQ
PAQGQQ
PEQGQQGQQ
PGQGQQGQQ
PGQGQQ
PGQGQPGYYPTSPQQ
SGQGQPGYYPTSSQQ
PTQSQQ
PGQGQQGQQ
VGQGQQAQQ
PGQGQQ
PGQGQPGYYPTSPLQ
SGQGQPGYYLTSPQQ
SGQGQQ
PGQLQQ
SAQGQKGQQ
PGQGQQ
PGQGQQGQQ
PGQGQQGQQ
PGQGQPGYYPTSPQQ
SGQGQQ
PGQWQQ
PGQGQPGYYPTSPLQ
PGQGQPGYDPTSPQQ
PGQGQQ
PGQLQQ
PAQGQQGQQ
LAQGQQGQQ
PAQVQQGQQ
PAQGQQGQQ
LGQGQQGQQ
PGQGQQ
PAQGQQGQQ
PGQGQQGQQ
PGQGQQ
PGQGQPWYYPTSPQE
SGQGQQ
PGQWQQ
PGQWQQ
PGQGQPGYYLTSPLQ
LGQGQQGYYPTSLQQ
PGQGQQ
PGQWQQ
SGQGQHGYYPTSPQL
SGQGQR
PGQWLQ
PGQGQQGYYPTSPQQ
SGQGQQ
LGQWLQ
PGQGQQGYYPTSLQQ
TGQGQQ
SGQGQQGYY

Figure 1-1 The repeat sequences of different elastic proteins.

The primary repeat sequences are highlighted in green and the extra residues are highlighted in light blue.

A) Human elastin (AAC98395).

B) *Tribolium* resilin (ADM26717). N-terminal repeats are highlinghted in green and C-terminal repeats are highlighted in yellow. The conserved tyrosine residues (Y) are highlighted in red.

C) Scallop abductin (AAB94680).

D) Spider major ampullate spidroin 1 (CAM32260). Only the repeats in C-terminal part are shown. The rest ones are similar to these.

E) Wheat glutenin (CAA27052).

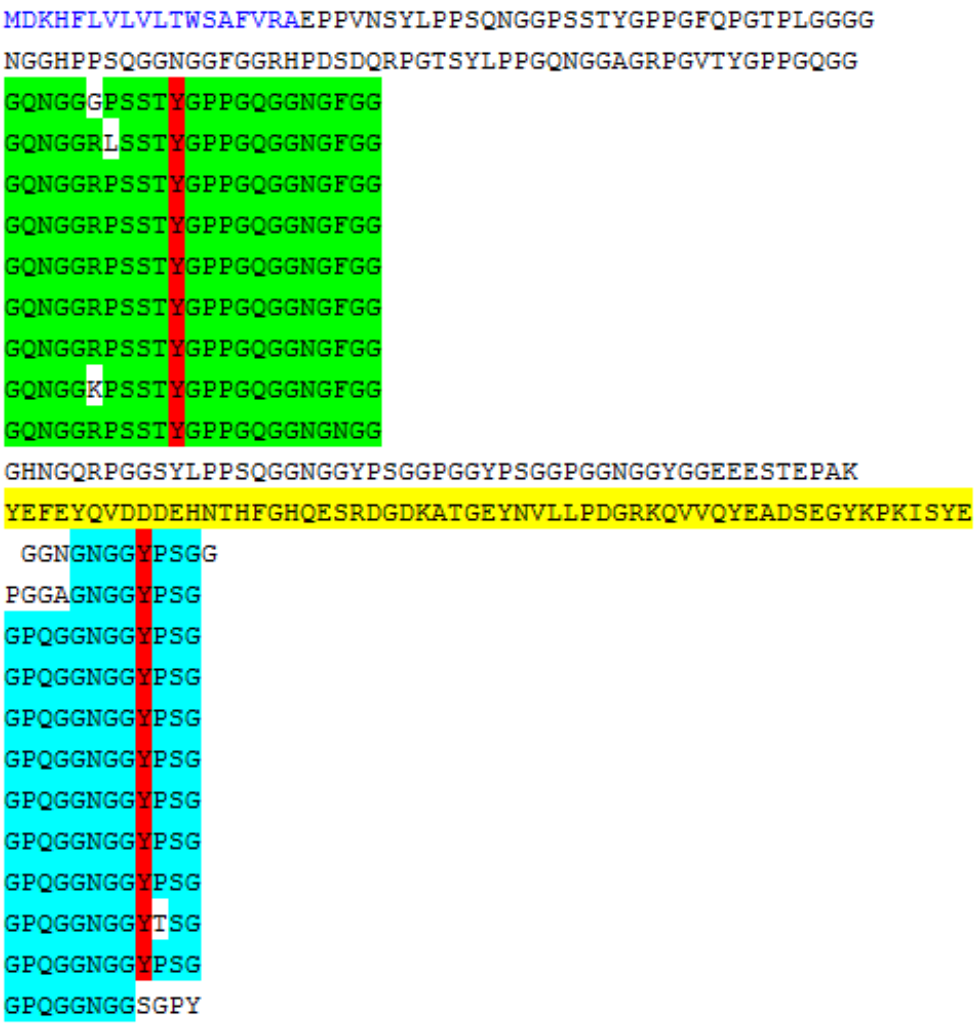
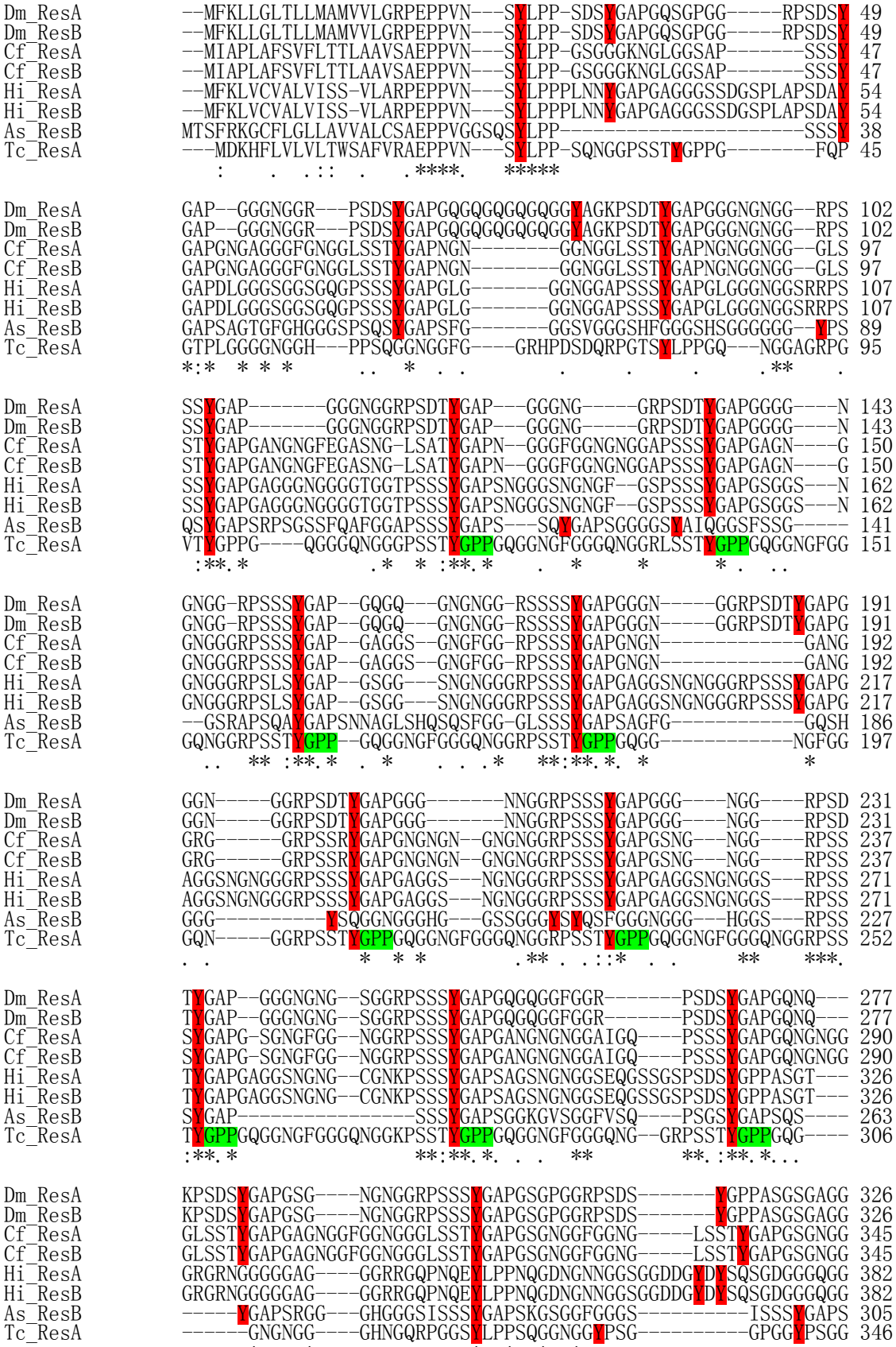


Figure 1-2. Features of the amino acid sequence of *Tribolium castaneum* resilin (revised from Neal Dittmer).

The beginning sequence in blue letters is the signal peptide. Two conserved large repeated regions in N-termini and C-termini are highlighted in green and light blue, respectively. The middle part, which is highlighted in yellow, is the RR-2 motif. Highly conserved tyrosine (Y) residues in the two repeated regions are highlighted in red.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Cf_ResB --MIAPLAFSVFLTTLAAVSAEPPVN---SYLPP-GSGGGKNGLGGSAP--------SSSY 47
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Hi_ResB --MFKLVCVALVISS-VLARPEPPVN---SYLPPPLNNYGAPGAGGGSSDGSPLAPSDAY 54
As_ResB MTSFRKGCFLGLLAVVALCSAEPPVGGSQSYLPP----------------------SSSY 38
Tc_ResA ---MDKHFLVLVLTWSAFVRAEPPVN---SYLPP-SQNGGPSSTYGPPG--------FQP 45
Dm_ResA GAP--GGGNGGR---PSDSYGAPGQGQGQGQGQGGYAGKPSDTYGAPGGGNGNGG--RPS 102
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Cf_ResA GAPGNGAGGGFGNGGLSSTYGAPNGN---------GGNGGLSSTYGAPNGNGGNGG--GLS 97
Cf_ResB GAPGNGAGGGFGNGGLSSTYGAPNGN---------GGNGGLSSTYGAPNGNGGNGG--GLS 97
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Hi_ResB GAPDLGGGSGGSGQGPSSSYGAPGLG--------GGNGGAPSSSYGAPGLGGGNGGSRRPS 107
As_ResB GAPSAGTGFGHGGGSPSQSYGAPSFG--------GGSVGGGSHFGGGSHSGGGGGG--YPS 89
Tc_ResA GTPLGGGGNGGH---PPSQGGNGGFG-----GRHPDSDQRPGTSYLPPGQ---NGGAGRPG 95
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As_ResB --GSRAPSQAYGAPSNNAGLSHQSQSFGG-GLSSSYGAPSAGFG-------------GQSH 186
Tc_ResA GQNGGRPSSTYGPP--GQGGNGFGGGQNGGRPSSTYGPPGQGG-------------NGFGG 197
Dm_ResA GGN-----GGRPSDTYGAPGGG-------NNGGRPSSSYGAPGGG-----NGG-----RPSD 231
Dm_ResB GGN-----GGRPSDTYGAPGGG-------NNGGRPSSSYGAPGGG-----NGG-----RPSD 231
Cf_ResA GRG------GRPSSRYGAPGNGNGN--GNGNGGRPSSSYGAPGSNG---NGG-----RPSS 237
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As_ResB GGG----------YSQGGNGGGHG---GSSGGGYSYQSFGGGNGGG---HGGS---RPSS 227
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As_ResB SYGAP----------------SSSYGAPSGGKGVSGGFVSQ----PSGSYGAPSQS---- 263
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As_ResB -----YGAPSRGG---GHGGGSISSSYGAPSKGSGGFGGGS----------ISSSYGAPS 305
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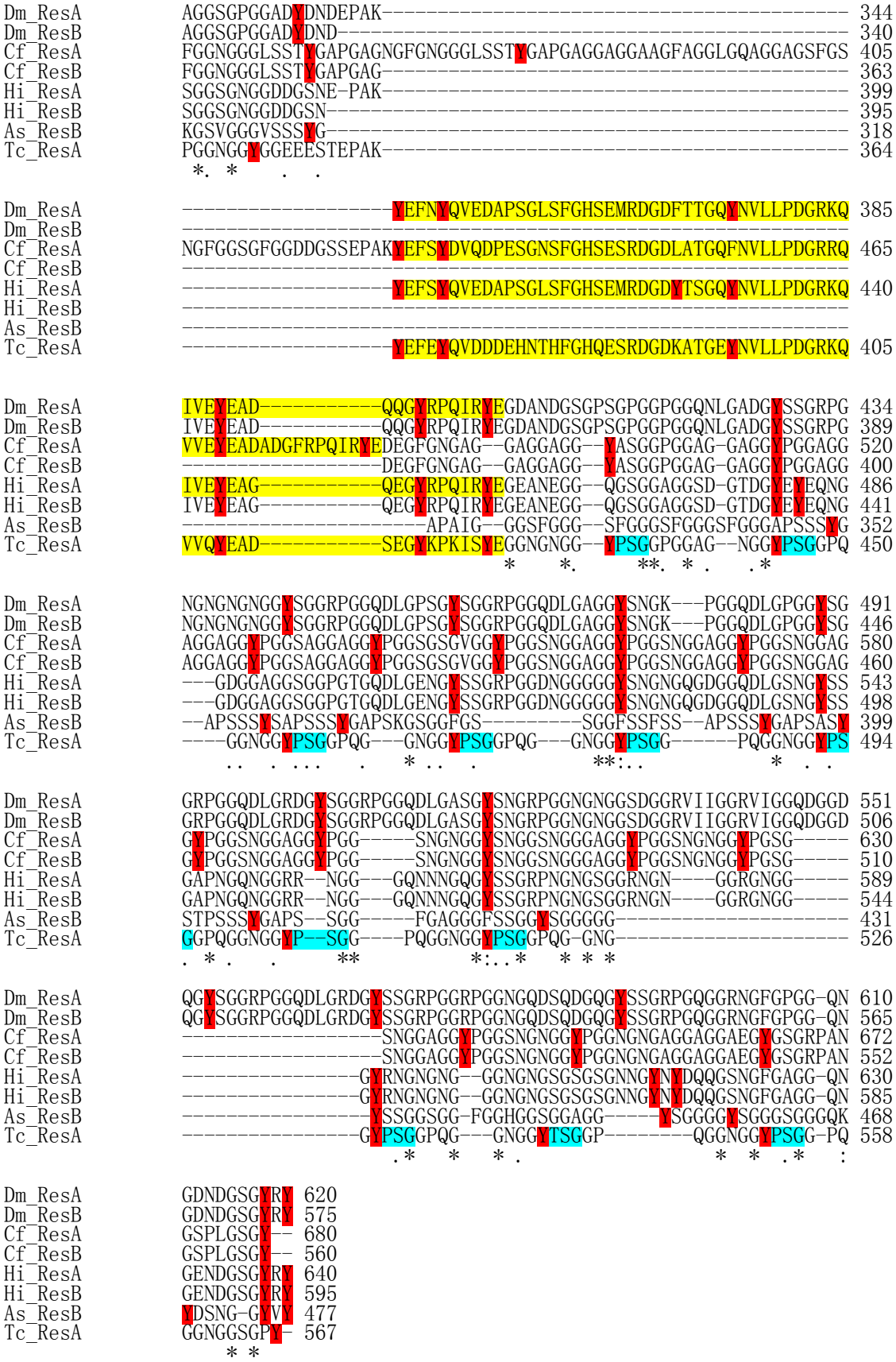
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Hi_ResA SGGSGNGGDDGSNE-PAK 399
Hi_ResB SGGSGNGGDDGSN 395
As_ResB KGSVGGGVSSSYG 318
Tc_ResA PGGNGGYGGEEESTEPAK 364
Dm_ResA YEFNYQVEDAPSGLSFGHSEMRDGDFTTGQYNVLLPDGRKQ 385
Dm_ResB
Cf_ResA NGFGGSGFGGDDGSSEPAKYEFSYDVQDPESGNSFGHSESRDGDLATGQFNVLLPDGRRQ 465
Cf_ResB
Hi_ResA YEFSYQVEDAPSGLSFGHSEMRDGDYTSGQYNVLLPDGRKQ 440
Hi_ResB
As_ResB
Tc_ResA YEFEYQVDDDEHNTHFGHQESRDGDKATGEYNVLLPDGRKQ 405
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Dm_ResB IVEYEAD----------QQGYRPQIRYEGDANDGSGPSGPGGPGGQNLGADGYSSGRPG 389
Cf_ResA VVEYEADADGFRPQIRYEDEGFGNGAG--GAGGAGG--YASGGPGGAG-GAGGYPGGAGG 520
Cf_ResB DEGFGNGAG--GAGGAGG--YASGGPGGAG-GAGGYPGGAGG 400
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Hi_ResB IVEYEAG----------QEGYRPQIRYEGEANEGG--QGSGGAGGSD-GTDGYEYEQNG 441
As_ResB APAIG--GGSFGGG--SFGGGSFGGGSFGGGAPSSSYG 352
Tc_ResA VVQYEAD----------SEGYKPKISYEGGNGNGG--YPSGGPGGAG--NGGYPSGGPQ 450
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Dm_ResB NGNGNGNGGYSGGRPGGQDLGPSGYSGGRPGGQDLGAGGYSNGK---PGGQDLGPGGYSG 446
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Tc_ResA GGPQGGNGGYP--SGG----PQGGNGGYPSGGPQG-GNG---------- 526
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Dm_ResB QGYSGGRPGGQDLGRDGYSSGRPGGRPGGNGQDSQDGQGYSSGRPGQGGRNGFGPGG-QN 565
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Cf_ResB ----------SNGGAGGYPGGSNGNGGYPGGNGNGAGGAGGAEGYGSGRPAN 552
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Hi_ResB ----------GYRNGNGNG--GGNGNGSGSGSGNNGYNYDQQGSNGFGAGG-QN 585
As_ResB ----------YSSGGSGG-FGGHGGSGGAGG-----YSGGGGYSGGGSGGGQK 468
Tc_ResA ----------GYPSGGPQG---GNGGYTSGGP--------QGGNGGYPSGG-PQ 558
Dm_ResA GDNDGSGYRY 620
Dm_ResB GDNDGSGYRY 575
Cf_ResA GSPLGSGY-- 680
Cf_ResB GSPLGSGY-- 560
Hi_ResA GENDGSGYRY 640
Hi_ResB GENDGSGYRY 595
As_ResB YDSNG-GYVY 477
Tc_ResA GGNGGSGPY- 567

Figure 1-3 Alignment of resilin protein sequence from five insects.

Drosophila melanogaster (fruit fly) resilin isoform A and B (NP_611157, NP_995860), *Ctenocephalides felis* (flea) resilin isoform A and B (AEQ49434, AEQ49435), *Haematobia irritans* (horn fly) resilin isoform A and B (AEQ49436, AEQ49437), *Aeshna sp.* (dragonfly) resilin isoform B (AEQ49438) and *Tribolium castaneum* (red flour beetle) resilin isform A (ADM26717) are included. Residues that are identical in all sequences are identified by an asterisk. A period (.) or colon (:) identify weak and strong conservative substitutions. *Tribolium* N-terminal YGPP repeat motifs are highlighted in green, and C-terminal YPSG repeat motifs are highlighted in light blue. The RR motifs are highlighted in yellow. All tyrosine residues are highlighted in red.

Chapter 2 - Materials and Methods

Insects

Tribolium castaneum adults (Ga-1 strain) were originally obtained from Dr. Richard Beeman at the Center for Grain and Animal Health Research, United States Department of Agriculture in Manhattan, Kansas and used to establish a laboratory colony. The colony was fed on whole wheat flour with 5% (by weight) brewer's yeast and kept in an incubator at 28℃.

Expression of recombinant resilin

Expression of recombinant resilin in Escherichia coli

Three different plasmids were constructed for expressing *T. castaneum* resilin in *Escherichia coli*. A glycerol stock of *E. coli* BL21 (DE3) strain containing the resilin sequence inserted into pET32a vector was obtained from Dr. Neal Dittmer (*E. coli* construct 1). Purified plasmid DNA of pProEX-HTc containing the resilin cDNA sequence (*E. coli* construct 2) was obtained from Dr. Neal Dittmer, and used to transform *E. coli* Rosetta strain (EMD Millipore) by heat shock in a 42℃ water bath for 30 s. The cells were shaken at 250 rpm, 37℃, for 1 h. Ampicillin (50 μg/mL) and chloramphenicol (34 μg/mL) were used in selection. Enzyme digested (*Nco*Ⅰ and *Xho*Ⅰ) pET28a vector and resilin cDNA were obtained from Dr. Neal Dittmer and ligated by T4 DNA ligase. The ligated plasmid DNA (*E.coli* construct 3) was used to transform Max Efficiency DH5α Competent *E. coli* (Life Technologies) by heat shock in a 42℃ water bath for 45 s. The cells were shaken at 225 rpm, 37℃, for 1 h and spread onto an LB agar plate containing 50 μg/mL kanamycin to grow overnight. One colony from the overnight plate was used to inoculate 3 mL of LB medium with 50 μg/mL kanamycin. The mixture was shaken at 225 rpm, 37℃ overnight. Plasmid DNA was purified from the culture using QIAprep Miniprep kit (Qiagen) and used to transform the *E. coli* strain, Rosetta (DE3) (EMD Millipore) by heat shock in a 42℃ water bath for 30 s. The cells were shaken at 250 rpm, 37℃, for 1 h. Kanamycin (50 μg/mL) and chloramphenicol (34 μg/mL) were used for the selection of recombinants.

The following expression process for different constructs and *E. coli* strains was similar. Cells were streaked out from a glycerol stock or freshly transformed mixture onto an LB agar

plate containing appropriate antibiotics and grown overnight at 37℃. One colony was used to inoculate 50 mL of LB medium with antibiotics in a 250 mL flask, and shaken overnight at 300 rpm at 37℃. Ten mL of the culture was added to 500 mL LB medium with antibiotics in a 500 mL flask, and shaken at 300 rpm at 37℃. IPTG was added to the culture to a concentration of 1 mM when the culture reached an OD_{600} of 0.5-1. After 4 h, the cells were harvested by centrifugation at 4000×g for 20 min at 4℃. The cell pellet was resuspended in cold lysis buffer (0.3 M sodium chloride, 50 mM sodium phosphate, pH 8; 4 mL buffer per gram of pellet), and then, sonicated using an Sonics VC505 sonicator in 6 cycles of 15 s pulse and 15 s interval. The soluble and insoluble fractions were separated by centrifugation at 10000×g for 30 min at 4℃, and analyzed by SDS-PAGE and immunoblot.

Expression of recombinant resilin in Bacillus subtilis

Resilin cDNA obtained as a PCR product with two restriction sites (*BamH* Ⅰ at the N-terminus and *Aat* Ⅱ at the C-terminus) was cloned into pCR4 vector using a TA TOPO Cloning kit (Invitrogen), and transformed into One Shot TOP 10 cells (Invitrogen) by heat shock at 42℃ for 30 s. The cells were shaken at 200 rpm, 37℃ for 1 h and spread on an LB agar plate containing 50 μg/mL of ampicillin, incubated at 300 rpm, 37℃ overnight. One colony from the plate was used to inoculate 3 mL LB medium containing 50 μg/mL ampicillin, and incubated at 300 rpm, 37℃ overnight. Plasmid DNA was purified from the culture using QIAprep Spin Miniprep kit (Qiagen). The pCR4/resilin plasmid DNA and pHT43 vector were digested by the two enzymes (*BamH* Ⅰ and *Aat* Ⅱ). The digested resilin fragment and pHT43 vector were purified following the QIA Quick Gel Extraction kit (Qiagen), and then ligated by T4 DNA ligase at 16℃ overnight. The ligated resilin/pHT43 was used to transform Max Efficiency DH5α Competent cells (Invitrogen) by heat shock at 42℃ for 45 s. The cells were shaken at 225 rpm, 37℃, for 1 h and spread onto an LB agar plate containing 100 μg/mL ampicillin to grow overnight at 37℃. One colony was used to inoculate 3 mL LB medium containing 50 μg/mL ampicillin and shaken at 300 rpm, 37℃ overnight. The recombinant plasmid DNA was purified from the culture following the QIAprep Miniprep kit (Qiagen) and used to transform JM101 Competent Cells (Fisher). The cells were spread on an LB agar plate containing 100 μg/mL of ampicillin and incubated at 37℃ overnight. One colony was used to inoculate 3 mL LB medium containing 100 μg/mL ampicillin and shaken at 300 rpm, 37℃ overnight. The plasmid DNA was

purified from the culture following the High-Speed Plasmid Mini Kit (IBI) and stored at -20℃ until the expression step.

Bacillus subtilis expression system was from MoBiTec (Germany). The stock cells (WB800N) were used to inoculate 5 mL of HS medium and shaken at 300 rpm, 37℃ overnight. A 0.5 mL aliquot of the overnight culture was used to inoculate 50 mL HS medium, and shaken which was at 300 rpm, 37℃ for about 9 h. Nine mL of the culture was mixed with 1 mL of 80% sterile glycerol and kept on ice for 15 min. The 10 mL mixture was fractionated into 1 mL aliquots and frozen in liquid nitrogen and stored at -80℃. For transformation experiments, one aliquot was thawed in a 37℃ water bath and used to inoculate 20 mL LS medium. The mixture was shaken at 70 rpm in a 30℃ water bath for 2 h. Ten μl of 0.1M EGTA was added to 1 mL of the cell culture and incubated for 5 minutes at room temperature. One μg of the pHT43/resilin plasmid DNA purified from *E. coli* strain JM101 was added to the 1 mL cell culture and shaken at 300 rpm, 37℃ for 2 h. The cells were collected by centrifugation at 4000×g for 2 min and plated on an LB agar plate containing 5 μg/mL chloramphenicol. The plate was incubated at 37℃ overnight. For recombinant protein expression, one colony was used to inoculate 50 mL of 2×YT medium containing 5 μg/mL chloramphenicol and shaken at 300 rpm, 37℃ overnight. Ten mL of the overnight culture was added to 500 mL of 2×YT medium containing 5 μg/mL chloramphenicol, and shaken at 300 rpm, 37℃ for 2 h. IPTG was added to a final concentration of 1 mM, and continued shaking for 4 h. The medium was separated from cells by centrifugation at 4000×g, 4℃ for 20 min and stored at 4℃ to be used later for purification of the recombinant resilin.

Purification of recombinant resilin

Purification of proteins expressed in E. coli

Recombinant resilins expressed in BL21 (DE3) or Rosetta cells were purified by NiNTA chromatography. The soluble proteins were mixed with NiNTA agarose (Qiagen) (1 mL for every 4 mL of cleared lysate) and rotated at 4℃ for 1.5 h. The mixture was poured into a 1.5 cm diameter column and washed twice with wash buffer (300 mM NaCl, 20 mM imidazole in 0.5 M sodium phosphate buffer, pH 8.0) at a volume equal to that of the clear lysate. The recombinant proteins were eluted 4 times with 10 mL of elution buffer (300 mM NaCl, 250 mM imidazole in

0.5 M sodium phosphate buffer, pH 8.0). Fractions were analyzed by SDS-PAGE and immunoblot.

A second expression of recombinant resilin in BL21 (DE3) was performed. The protein was first purified by NiNTA chromatography as described above. Elution fractions 1 and 2 were combined and dialyzed 3 times against dialysis buffer (50 mM NaCl, 20 mM Tris, pH 7.4) at 4℃. Fifteen mL of the dialyzed protein was filtered (0.45 μm) and loaded into an ion exchange column (HiPrep Q FF 16/10 column, GE Healthcare Life Sciences). Purification was performed using the BioLogic System using a gradient from 50 mM NaCl to 1 M NaCl in 20 mM Tris, 50 mM NaCl, pH 7.4 at rate of 5 mL/min. Fractions of 1 mL each were collected and analyzed by SDS-PAGE and immunoblot. The fractions 40 to 44 were combined and filtered (0.45 μm), and loaded into a gel filtration column (HiLoad 16/60 Superdex 200 prep grade, GE Healthcare Life Sciences). Proteins were eluted using a buffer of 20 mM Tris, 150 mM NaCl, pH 7.5 at rate of 1 mL/min. Fractions of 2 mL each were collected and analyzed by SDS-PAGE and immunoblot.

Purification of proteins expressed in B. subtilis

Proteins from the first expression in *B. subtilis* were purified in three steps: ammonium sulfate precipitation, ion exchange and gel filtration chromatography. Several trials were performed to determine the optimal concentration of ammonium sulfate for precipitation. To 20 mL of the medium after expression, 2.79 grams of ammonium sulfate was added and dissolved to obtain a 25% saturated solution. The mixture was incubated on ice and stirred frequently for 10 min. The precipitate was separated from the supernatant by centrifugation at 15000×g, 4℃ for 15 min. In a similar way, 3.09 grams or 3.6 grams more ammonium sulfate was used to obtain 50% and 75% saturated solutions, respectively. A second trial was performed to check more concentrations (0-25%, 25%-35%, 35%-45%, and 45%-55%) and a third trial was performed to compare 35% and 40% saturated ammonium sulfate, in a similar way as above. Different fractions were analyzed by SDS-PAGE and immunoblot. Forty per cent saturation was chosen as an optimal concentration of ammonium sulfate to precipitate proteins. Ammonium sulfate was added to 3 L medium from 4h expression to obtain 40% saturation. The mixture was stirred at 4℃ for 15 min and centrifuged at 15000×g, 4℃ for 30 min. The supernatant was removed, and the precipitate was dissolved and dialyzed 3 times against the buffer of 20 mM Tris, pH 7.5, at 4℃. The dialyzed solution was loaded into an ion exchange column (HiPrep Q FF 16/10 column,

GE Healthcare Life Sciences). The ion exchange chromatography was performed using the BioLogic System with a gradient from 0 to 1 M NaCl in 20 mM Tris, pH 7.4 at rate of 5 mL/min. Fractions of 1 mL each were collected and analyzed by SDS-PAGE and immunoblot. The fractions 38 to 54, except 50, were combined and concentrated to less than 1 mL, and loaded into a gel filtration column (HiLoad 16/60 Superdex 200 prep grade, GE Healthcare Life Sciences). Proteins were eluted using a buffer of 20 mM Tris, 150 mM NaCl, pH 7.4 at rate of 1 mL/min, with the BioLogic System. Fractions of 2 mL each were collected and analyzed by SDS-PAGE.

Proteins from the second expression in *B. subtilis* were purified in two steps: ammonium sulfate precipitation and gel filtration chromatography. The medium after expression was mixed with saturated ammonium sulfate solution to obtain 40% saturation. The mixture was centrifuged at 15000×g, 4℃ for 20 min. The pellet was resuspended into 10 mL of 20 mM Tris, 150 mM NaCl, pH 7.4, and 0.4 mL of Protease Inhibitor Cocktail (Sigma P8849) was added. The mixture was centrifuged at 4000 rpm, room temperature for 10 min, and the supernatant was concentrated to about 2.5 mL using a 30 kDa Millipore centrifugal filter. The solution was separated by a gel filtration as described above. Selected elution fractions were analyzed by SDS-PAGE and immunoblot.

Protein concentration assay

One method was to compare the resilin samples by SDS-PAGE together with several bovine serum albumin (BSA) samples of known amounts. The gel was stained with Coomassie blue staining solution and destained to visualize protein bands. Protein concentrations were estimated by comparing the band intensities of protein samples with the BSA standards.

A second method used the protein absorption at 280 nm and the concentration was calculated based on the Beer–Lambert law, with the calculated molar extinction coefficient of 52,150 $M^{-1}cm^{-1}$ for the recombinant resilin expressed in *B. subtilis*.

The concentration of the recombinant resilin was also measured using the Thermo Scientific Pierce BCA Protein Assay Kit. BSA was used as the standard and a standard curve of absorbance at 562 nm vs BSA concentrations was made to estimate the resilin concentration.

The last method utilized was the Thermo Scientific Pierce Coomassie Plus Protein Assay kit. Again, BSA was used as the standard protein and a standard curve of absorbance at 595 nm vs BSA concentrations was made to calculate the resilin concentration.

SDS-PAGE and Immunoblot Analysis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using NuPAGE 4-12% Bis-Tris gels (Invitrogen). Protein solutions were mixed with loading buffer containing SDS and β-mercaptoethanol. The mixture was heated at 95℃ for 5 min and centrifuged at 16000×g for 1 min. Samples were loaded onto the gel and separated in MOPS buffer by electrophoresis at a constant voltage (200 V) for about 45 min. The gel was then transferred into a staining solution of 0.2% (w/v) Coomassie Brilliant Blue R (Sigma) in 50% (v/v) methanol and 12% (v/v) glacial acetic acid and shaken gently for 30 min, followed by destaining in 30% (v/v) methanol, 10% (v/v) acetic acid. The destaining solution was changed after 30 min. The gel was rinsed with distilled water when blue bands of protein were visualized against a clear gel background and dried between two cellophane membranes.

For immunoblot analysis, a 0.45 μm nitrocellulose membrane was equilibrated in Western transfer buffer (48 mM Tris, pH 9.2, 39 mM glycine, 1.3 mM SDS, 20% methanol) for 15 minutes. The proteins were separated by electrophoresis as above and transferred from the gel to the membrane using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) for 70 min at a constant voltage (11V). The membrane was blocked for 1 hour with 3% dry milk dissolved in TTBS (25 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, pH 7.4, plus 0.05% Tween-20) and washed with TTBS for 5 min. Then, the membrane was incubated for 2 h at room temperature or overnight at 4℃ with an appropriate primary antibody diluted in 3% dry milk in TTBS. The membrane was washed three times with TTBS for a total of 15 min and incubated for 1 h with an appropriate secondary antibody (Goat Anti-Rabbit or Goat Anti Mouse IgG (H+L)-alkaline phosphatase conjugate, Bio-Rad) diluted 1:3000 in 3% dry milk dissolved in TTBS. The membrane was washed twice with TTBS and once with TBS (25 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, pH 7.4) for a total of 15 min, followed by development using an Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad) until purple bands of protein were visualized against a clear background. The membrane was rinsed with water and dried in air.

Affinity purification of the antibodies

The recombinant resilin of construct 1 was expressed in *E. coli* BL21 (DE3), and partially purified by NiNTA affinity chromatography. The partially purified protein sample was separated

by SDS-PAGE, and the band containing the resilin fusion protein was excised and sent to Cocalico Biologicals (Reamstown, PA) to generate a rabbit polyclonal antiserum.

AminoLink Plus Immobilization Kit was used to purify the resilin anbodies from rabbit antiserum, by removing antibodies against the fusion tag. The kit protocol was followed with minor changes. A new column was placed into a 15 mL plastic centrifuge tube to remove the storage buffer by centrifugation at 1000×g for 1 min. Two mL of Coupling Buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2) was added to the column (in the tube) and centrifuged again. About 4 mg TAG (the big N-terminal fusion tag from pET32a vector, purified by Dr. Maureen Gorman) and 40 μL of 5 M sodium cyanoborohydride solution were added to the column and mixed end-over-end for 4.5 h at room temperature. The column (in the tube) was centrifuged to remove non-bound proteins and washed twice with 2 mL of Quenching Buffer (1 M Tris-HCl, 0.05% sodium azide, pH 7.4). A mixture of 2 mL Quenching Buffer and 40 μL sodium cyanoborohydride solution was added to the column and mixed end-over-end for 30 min. Buffer was removed by centrifugation, followed by washing five times with 2 mL Wash Solution (1 M NaCl, 0.05% sodium azide). The column was equilibrated with 2 mL Coupling Buffer, five times. Two mL of antiserum was added to the column and dripped until the pink color reached the bottom of the column. One mL of Coupling Buffer was added to the column and mixed end-over-end for 2 h at room temperature. The flow through was collected, which was the partially purified antibody.

A second affinity purification step was used to separate resilin-binding antibodies from the partially purified mixture. Gel filtration fractions 34, 35 and 36 of recombinant resilin expressed in *E. coli* BL21 (DE3) were combined and dialyzed against Coupling Buffer. The protein solution was concentrated to about 2 mL and bound to a new AminoLink column in the same way as above. The column then was washed three times with 2 mL of Coupling Buffer. Two mL of the partially purified antibody was added to the column and dripped until the pink color reached the bottom of the column, and then 0.2 mL of Coupling Buffer was added and the sample was incubated at room temperature by mixing end-over-end for 1 h. The flow through was removed, followed by washing of the column with 2 mL Coupling Buffer, three times. Two mL of Elution Buffer (0.1 M Glycine-HCl, pH 2.5 was added to the column and collected into a 15 mL blue cap tube containing 200 μL Neutralization Buffer (1 M Tris, pH 8.0). Two mL more

of Elution Buffer was added to the column and collected into the same tube, which was the final preparation of purified antibody.

Cross-linking assays of recombinant resilin

Purified recombinant resilin from *B. subtilis* was used for *in vitro* cross-linking assays. A 30 μL reaction was set up, containing 10 μL of resilin solution (36.7 μg protein), 1 unit/μL of horseradish peroxidase (HRP, Sigma) and 0.01% H_2O_2 and incubated at room temperature. After 0.5, 1, 2, 4 and 18 h, 5 μL of the reaction mixture was stored at -20℃, and analyzed by SDS-PAGE later.

A second cross-linking experiment was set up to test how much HRP was needed for cross-linking. Four 10 μL reactions were set up in the following ways: (1) 2 μL of resilin, 0.01% H_2O_2; (2) 2 μL of resilin, 0.01% H_2O_2, 0.001 unit/μL HRP; (3) 2 μL of resilin, 0.01% H_2O_2, 0.01 unit/μL HRP; (4) 2 μL of resilin, 0.01% H_2O_2, 0.1 unit/μL HRP. All mixtures were incubated at room temperature for 30 minutes and analyzed by SDS-PAGE.

The final trial used a newly purified resilin solution, and ovalbumin (Sigma) as a control protein. Sixty μL reactions were set up as follows: (1) 2.7 μg resilin; (2) 2.7 μg resilin, 0.01% H_2O_2; (3) 2.7 μg resilin, 0.1 unit/μL HRP; (4) 0.01% H_2O_2, 0.1 unit/μL HRP; (5) 2.7 μg resilin, 0.01% H_2O_2, 0.01 unit/μL HRP; (6) 2.7 μg resilin, 0.01% H_2O_2, 0.1 unit/μL HRP. The control reactions utilized ovalbumin instead of resilin with all of the other conditions being the same. All reactions were incubated at room temperature for 30 min, and samples were saved for analysis by SDS-PAGE and immunoblot.

Isolation of total RNA and proteins from beetles

Hind wings and elytra dissected from *T. castaneum* pharate adults were used to isolate total RNA and proteins. Eggs, larvae from three stages (early, middle, late), pupae from six stages (day 0-5) and adults from three stages (day 0, 5, 10) were used for isolation of total RNA. Total RNA and proteins were isolated using the TRI Reagent (Sigma, T9424) following the protocol suggested by the manufacturer. The total RNA was used for the first-strand cDNA synthesis, and the proteins were analyzed by SDS-PAGE and immunoblot.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The first-strand cDNA was synthesized from the total RNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, 18080-051). The "First-Strand cDNA Synthesis" protocol was followed to complete the cDNA synthesis.

A sequence of 261 base pairs of the *T. castaneum* resilin nucleotide sequence was amplified from different cDNA samples using a forward primer (RTPCR-TcRes-F: 5'-CGG GCG GTT CGT ATC TC-3') and a reverse primer (RTPCR-TcRes-R: 5'-CTT GCT TCC TGC CAT CC-3'). The thermal cycling conditions were one cycle of 94℃ for 2 min, 30 cycles of 94℃ for 30 s, 55℃ for 30 s, 72℃ for 40 s followed by incubation at 72℃ for 10 min. *T. castaneum* ribosomal protein S6 (rpS6) was used as an internal control and amplified using the same set of cDNA. These primers were obtained from Dr. Subbaratnam Muthukrishnan's laboratory. The forward primer was rpS6-F (5'-AGA TAT ATG GAA GCA TCA TGA AGC-3') and the reverse primer was rpS6-R (5'-CGT CGT CTT CTT TGC TCA AAT TG-3'). The thermal cycling conditions were one cycle of 94℃ for 2 min, 24 or 26 cycles of 94℃ for 30 s, 54 ℃ for 30 s, 72℃ for 60 s followed by incubation at 72℃ for 10 min. The PCR products were analyzed by DNA electrophoresis.

Double-stranded RNA synthesis and injection

The DNA templates used for dsRNA synthesis were produced by PCR. For resilin template, the N-terminal part of resilin cDNA was amplified using the forward primer TcResilin(RNAi)-F2 (5'-TAA TAC GAC TCA CTA TAG GGG TCC ACA CTG CTT TG-3'), and the reverse primer TcResilin(RNAi)-R2 (5'-TAA TAC GAC TCA CTA TAG GGA CCG TAT GTT ACT CC-3'). The thermal cycling conditions were one cycle of 94℃ for 2 min, five cycles of 94℃ for 30 s, 48℃ for 30 s, 72℃ for 30 s followed by 30 cycles of 94℃ for 30 s, 64℃ for 30 s, 72℃ for 30 s. For the control vermillion template, the forward primer was TcVer(RNAi)-F (5'-TAA TAC GAC TCA CTA TAG GGG TCT TGG TGG A-3'), and the reverse primer was TcVer(RNAi)-R (5'-TAA TAC GAC TCA CTA TAG GGC CGC CAT TTC-3'). The thermal cycling conditions were one cycle of 94℃ for 2 min, five cycles of 94℃ for 30 s, 36℃ for 30 s, 72℃ for 40 s followed by 30 cycles of 94℃ for 30 s, 63℃ for 30 s, 72℃ for 40 s. The PCR products were electrophoresed on agarose gels followed by extraction from

the gels using the QIAquick Gel Extraction Kit (Qiagen). The extracted DNA was use as template for dsRNA synthesis using the MEGAscript RNAi Kit (Ambion). The dsRNA was finally eluted with distilled deionized water into a 1.5 mL RNase-free microcentrifugal tube and concentrated for injection.

dsRNA was injected into pre-pupae of *T. castaneum* using the Nanoliter 2000 and Micro4 Injection Controller (World Precision Instruments). Insects were injected with either 200 ng of dsRNA or 400 ng of dsRNA in 200 nL.

Visualization of cross-linked resilin by UV fluorescence

Hind wings from *Triblolium castaneum* adults were dissected and mounted in 100% glycerol on a glass slide and covered with a coverslip. Auto-fluorescence of the hind wings was observed using an LSM 5 Pascal microscope (Zeiss). The excitation wavelength was 360 nm and the emission wavelength was equal to or greater than 420 nm.

Immunohistochemistry

Hind wings of *T. castaneum* adult were dissected and placed in a small drop of phosphate buffered saline (PBS, 10 mM sodium phosphate, 2.7 mM KCl, 137 mM NaCl, pH 7.4) on a glass slide and covered with coverslip. Paraformadehyde (4%) was added to one edge of the coverslip to be absorbed into the space between the coverslip and the glass slide, thus to fix the wings for 30 min at room temperature, followed by rinsing with PBS containing 0.1% Tween 20 for 15 min at room temperature. In early trials, wings were then incubated with 0.1% Triton X-100 in TBS for 15 min at room temperature; in later trials, wings were incubated with either 1% Triton X-100 in TBS or 100% methanol for 1 h at room temperature. Wings were incubated in Blocking Solution (PBS plus 0.1% Tween 20 containing 10% Normal Goat Serum) for 1.5 h at room temperature. In early trials, wings were incubated with rabbit pre-immune serum or purified resilin antibody diluted 1:50 in Blocking Solution overnight at 4℃; in later trials, wings were incubated with crude (unpurified) antiserum diluted 1:100 or purified resilin antibody diluted 1:5 in Blocking Solution, or, as controls, pre-immune serum diluted 1:100 in Blocking Solution or Blocking Solution without primary antibody, overnight at 4℃. Wings were washed with PBS plus 0.1% Tween 20 for 15 min and incubated with secondary antibody (Goat-anti-Rabbit, AP-conjugate, Bio-Rad) diluted 1:500 in Blocking Solution for 1.5 h at room temperature or

overnight at 4℃. Wings were rinsed with PBS plus 0.1% Tween 20 for 15 min followed by NTMT (100 mM NaCl, 100mM Tris-HCl, 50 mM $MgCl_2$, 0.1% Tween 20, pH 9.5) for 15 min. Wings were incubated in developing solution (5 mL NTMT plus 2 drops NBT plus 2 drops BCIP) for 2-3 h at room temperature and rinsed with distilled deionized water to stop the development.

LB medium (1 L)	10 g tryptone, 5 g yeast extract, 10 g sodium chloride in distilled water, pH 7.0
2×YT medium (1 L)	16 g tryptone, 10 g yeast extract, 5 g sodium chloride in distilled water
10× S-base (100 mL)	2 g $(NH_4)_2SO_4$, 14 g K_2HPO_4, 6 g KH_2PO_4, 1 g sodium citrate, 0.1 ml 1M $MgSO_4$ in distilled water
HS medium (100 mL)	66.5 mL distilled water, 10 mL 10× S-base, 2.5 mL 20% (w/v) glucose, 5 mL 0.1% (w/v) L-tryptophan, 1 mL 2% (w/v) casein, 5 mL 10% (w/v) yeast extract (Difco), 10 mL 8% (w/v) arginine, 0.4% histidine
LS medium (100 mL)	80 mL distilled water, 10 mL 10× S-base, 2.5 mL 20% (w/v) glucose, 0.5 mL 0.1% (w/v) L-tryptophan, 0.5 mL 2% (w/v) casein, 5 mL 2% (w/v) yeast extract (Difco), 0.25 mL 1 M $MgCl_2$, 0.05 mL 1 M $CaCl_2$

Table 2-1 Formulations of media used for protein expression.

Chapter 3 - Results

Preparation of antibodies to recombinant resilin

T. castaneum resilin cDNA was cloned into pET32a vector (Resilin construct 1, Figure 3-1 A), which was used to transform *E. coli* strain BL21 (DE3) (by Neal Dittmer) for expression. The resilin expression was successfully induced by IPTG (Figure 3-2), and the soluble fraction of the cell lysate contained the resilin fusion protein, which was partially purified by NiNTA chromatography. The elution fraction 2 contained most of the resilin. This fraction was used for preparatory SDS-PAGE to isolate the resilin band (data not shown). The resilin band was excised and used as antigen to make resilin antibody (rabbit antiserum).

The resilin antiserum detected both the recombinant resilin and the N-terminal fusion tag (TAG) (Figure 3-3 A). The crude antibody could detect 4 ng of recombinant resilin but not the same molar equivalent of TAG (1 ng). This indicated that some of the antibodies in the serum were specific to resilin. However, the crude antibody still detected a small amount of TAG (such as 10 ng) and some other materials which may be *E. coli* proteins. To improve the specificity of the antibody, it was purified in two steps using affinity chromatography.

To remove antibodies to the TAG, the purified TAG protein (obtained from Dr. Maureen Gorman) was bound to an AminoLink Plus column and the crude resilin antibody was applied to the TAG column. Antibodies to the TAG should bind to the column while all other antibodies should be in the flow through. Antibodies that did not bind to this column were tested by immunoblot to detect binding to recombinant resilin or TAG (Figure 3-3 B). This partially purified antibody could detect recombinant resilin but did not detect the TAG, showing that this purification step was successful.

A further affinity purification of antibodies that bind to recombinant resilin was desired to improve the specificity for biological experiments. Resilin constructs 2 and 3 (Figure 3-1 B and C) were tried for expressing different recombinant resilins for further purification of the antibody. However, these expressions were not successful. For construct 2 expressed in *E. coli* Rosetta strain, the recombinant resilin was expected to be 54 kDa, but there was no obvious induction of protein of this size (Figure 3-4 A). The same situation was observed in the construct

3 expressed in *E. coli* Rosetta (DE3) strain (Figure 3-4 B). The predicted size of the recombinant resilin was 51 kDa, but still, no obvious induction of expression was observed.

Therefore construct 1 was used again to express the recombinant resilin, followed by NiNTA chromatography purification (Figure 3-5 A). The elution fractions 2 and 3 were combined and used in further purification by ion exchange chromatography, using a HiPrep Q FF 16/10 column (Figure 3-5 B). The ion exchange chromatography did not separate very well the recombinant resilin (red arrow) from the major contaminant proteins (Figure 3-5 C, D). Fractions 40 to 44 were combined and applied to a gel filtration column (HiLoad 16/60 Superdex 200 prep grade) (Figure 3-5 E). The peak fractions were analyzed by SDS-PAGE (Figure 3-5 F), showing a good separation of the recombinant resilin from the major contaminating proteins. Fractions 34, 35 and 36 contained very pure recombinant resilin and were combined and used for the second step of the antibody purification.

Recombinant resilin isolated by gel filtration was used to bind to an AminoLink Plus column. The partially purified antibody was then applied to the resilin-bound column for purification. After washing, antibodies were eluted at low pH and then rapidly neutralized. The purified antibody was used in an immunoblot analysis to test detection of both the recombinant resilin and the TAG (Figure 3-3 C). The purified antibody could detect 10 ng of the recombinant resilin (red arrow) but did not detect the TAG (4 ng, 1.5 times molar equivalent). The recognition of the recombinant resilin appeared to be relatively specific (a single band).

TAG removal by enterokinase and purification of the cleaved resilin

After purification by NiNTA chromatography, part of the construct 1 recombinant resilin was used for a trial of TAG removal. The recombinant resilin was incubated with enterokinase for 13 h and samples were analyzed by SDS-PAGE and immunoblot (Figure 3-6 A and B). The recombinant resilin (fusion protein) should be cut into two parts: resilin (51 kDa) and the TAG (17 kDa). The TAG was at the expected mobility (about 17 kDa, blue arrow). However, the cleaved resilin was much smaller (~40 kDa) than the expected size based on the SDS-PAGE analysis (Figure 3-6 A, black arrow). After NiNTA chromatography, the TAG, which contains a 6×His sequence, was mostly removed, but a major degradation product also appeared (Figure 3-6 C).

Expression and purification of recombinant resilin from *B. subtilis*

The fourth plasmid construct was made (Figure 3-1 D) and used to express a recombinant resilin in *B. subtilis*. The expressed protein was expected to be secreted into the medium of the expression system. This secreted form of resilin is the full length resilin without its own signal peptide and predicted to be 51 kDa.

The recombinant resilin expression was successfully induced by IPTG (Figure 3-7 A, red arrow). Three liters of medium was separated from the bacterial cells by centrifugation. Small aliquots were used for three trials to determine the best concentration of ammonium sulfate to precipitate resilin. In the first trial, 0-25%, 25%-50%, and 50%-75% saturated ammonium sulfate were tested (Figure 3-7 B, C). Most of the recombinant resilin was precipitated by 50% saturated ammonium sulfate (Figure 3-7 C, red arrow). The second trial was using 0-25%, 25%-35%,35%-45%, and 45%-55% saturated ammonium sulfate (Figure 3-7 D, E). Most of the recombinant resilin was precipitated by 45% saturated ammonium sulfate (Figure 3-7 E, red arrow). In the final trial was comparing 35% and 40% saturated ammonium sulfate (Figure 3-7 F), and a little more recombinant resilin was precipitated by 40% saturated ammonium sulfate. This concentration was used to precipitate proteins from nearly three liters of medium.

The proteins precipitated by 40% ammonium sulfate were further purified by ion exchange chromatography (Figure 3-8 A). The peak fractions were analyzed by SDS-PAGE and Immunoblot (Figure 3-8 B, C). Most of the recombinant resilin was contained in the main A_{280} peak fractions (red arrow), but still there were some contaminating proteins.

The ion exchange chromatography fractions 38-54, except 50, were combined and further purified by gel filtration chromatography (Figure 3-9 A). The peak fractions were analyzed by SDS-PAGE (Figure 3-9 B), showing that the recombinant resilin (red arrow) was separated well from other proteins. From 3 liters of culture, about 730 μg of pure recombinant resilin (combined fractions 34-37 after gel filtration chromatography) was obtained after three steps of purification.

Another 1 liter expression of recombinant resilin in *B. subtilis* was performed, and proteins were precipitated by 40% saturated ammonium sulfate from the medium (Figure 3-10 A, red arrow). The precipitated proteins were further purified by gel filtration chromatography (Figure 3-10 B) and the peak fractions were analyzed by SDS-PAGE (Figure 3-10 C). This simplified purification strategy worked well and is summarized in Figure 3-10 D. The presence of recombinant resilin was confirmed by immunoblot analysis (Figure 3-10 E, red arrow). From

one liter culture, about 200 μg of pure recombinant resilin (fraction 34 of gel filtration chromatography) was obtained after two steps of purification.

Recombinant resilin cross-linked by peroxidase *in vitro*

Resilin *in vivo* is thought to be cross-linked to itself and other proteins by dityrosine bonds (Andersen, 1964). The purified recombinant resilin from the first preparation from *B. subtilis* expression was used for trials of cross-linking reactions to examine whether the recombinant resilin could be cross-linked *in vitro*. Reactants were recombinant resilin, horseradish peroxidase (HRP) and hydrogen peroxide. A time course analysis was performed using 36 μg recombinant resilin, 1 u/μL HRP and 0.01% H_2O_2 per reaction (Figure 3-11 A). Within 0.5 h, all recombinant resilin was apparently cross-linked (red arrow), appearing as a band at the top of the sample well. Another trial was performed using different concentrations of HRP, and all of the reaction mixtures were incubated at room temperature for 0.5 h (Figure 3-11 B). All of the recombinant resilin could be cross-linked (red arrow) by 0.1 u/μL HRP, but the recombinant resilin became only partially cross-linked by 0.01 u/μL HRP. From the SDS-PAGE analysis (Figure 3-11 B), a degradation of the recombinant resilin was observed, so more recombinant resilin was expressed and purified for additional cross-linking assays.

The purified recombinant resilin from the second *B. subtilis* expression was used for more cross-linking reactions. The recombinant resilin could be cross-linked only in presence of both HRP and H_2O_2, and different HRP concentrations led to similar results as before (Figure 3-12 A). Both the cross-linked resilin (red arrow) and the uncross-linked resilin (blue arrow) could be detected by the purified resilin antibody in the immunoblot analysis (Figure 3-12 B). Also, the cross-linked resilin (red arrow) could be detected by an anti-dityrosine antibody (Figure 3-12 C), indicating the recombinant resilin was cross-linked via dityrosine. This dityrosine cross-link was also indicated by the blue fluorescence under ultraviolet light visible at the sample well, as very high molecular weight material in the same position detected by both the resilin and dityrosine antibodies (Figure 3-12 D, red arrow). Control reactions were performed using ovalbumin instead of the recombinant resilin (Figure 3-12 E), revealing no cross-linked proteins in the presence of HRP and H_2O_2, and no dityrosine was detected by the anti-dityrosine antibody (Figure 3-12 F). These results support the hypothesis that the recombinant resilin is specifically cross-linked by horseradish peroxidase in presence of hydrogen peroxide *in vitro*.

Resilin mRNA levels during development

A developmental mRNA profile of *T. castaneum* resilin was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR). The total mRNA of *T. castaneum* from thirteen different stages was extracted and used to make the first strand cDNA, which was used as the template of the RT-PCR. The *T. castaneum* resilin mRNA was abundant in the late pupal stage (day 4 and 5) (Figure 3-13). The new adult (day 0) had a very low level of mRNA and almost no resilin mRNA was found in any other stages. *T. castaneum* ribosomal protein S6 (rpS6) was used as an internal control, and its mRNA levels were approximately equal in all stages.

Knockdown of resilin expression by RNA interference

Double stranded RNA (dsRNA), which targeted the *T. castaneum* resilin gene or the vermillion gene (control) encoding tryptophan oxygenase, was injected into pre-pupae, and the mRNA and protein extracted from pharate adult was used to check for the extent of knockdown of mRNA or protein levels by RT-PCR or immunoblot, respectively. Injection of 200 ng of dsRNA partially knocked down the resilin transcript, and 400 ng of dsRNA almost completely knocked down the transcript (Figure 3-14 A). The resilin transcript was unaffected by vermillion knockdown. The rpS6 transcript level was unaffected by resilin or vermillion knockdown. This experiment also demonstrated that the resilin transcript level was high in hind wings, but it was undetectable in elytra.

The purified resilin antibody was used in an immunoblot analysis to detect resilin in the protein samples extracted from hind wings or elytra of wild type (WT) or dsResilin-treated (RNAi) pharate adults (Figure 3-14 B). The resilin expression was successfully knocked down by 400 ng of dsRNA. Also, the resilin protein was detected in hind wings but not in elytra.

No phenotype detected after resilin RNAi

The dsResilin-injected prepupae survived to the adult stage, and no obvious morphological phenotype was observed (Figure 3-15). A few beetles with abnormal hind wings were observed, but the abnormal hind wings were not apparently due to RNAi because some of the wild type (WT) beetles also exhibited similar defective hind wings, and some of the control dsVermillion-treated beetles also had abnormal hind wings (15 out of 90 treated beetles), which was similar to dsResilin-treated beetles (13 out of 92). The vermillion gene expression was

successfully knocked down in control beetles since the white-eyed phenotype was observed (data not shown).

The resilin RNAi did not affect the life span of *T. castaneum* adults (Figure 3-16). The resilin knocked-down beetles survived as long as the vermillion knocked-down beetles.

Auto-fluorescence of hind wings

Hind wings of *T. castaneum* adults were dissected and examined under ultraviolet light (wavelength of 360 nm) to observe its auto-fluorescence. Wild type, dsVermillion-treated and dsResilin-treated samples were all used. In wings from 0 to 1 day old beetles (Figure 3-17 A), there was no obvious difference in the distribution of the blue fluorescence between control and dsResilin insects. Wings from 2 to 3 day old beetles (Figure 3-17 B) also showed little difference in the distribution of the blue fluorescence. The blue color was found mainly in the tip area of the wing, which is highly folded in the normal resting state, or at the base of the wing where the wing hinge is located. This result suggested that the blue fluorescence may be due to some materials other than resilin.

Immunohistochemistry of hind wings

Both of the crude resilin antibody and purified antibody were used in the immunohistochemistry of wild type *T. castaneum* hind wings. In the early trial (Figure 3-18 A), 0.1% Triton X-100 was used to permeabilize the hind wings. No obvious signal (expected to be purple) was observed. The faint purple color in the hinge part (right column) was also found in all of the control insects, suggesting that it was not an authentic signal for resilin.

In the later trial, a higher concentration of Triton X-100 (1%) or 100% methanol was used to permeabilize the hind wings (Figure 3-18 B, C). Some purple color was observed on the edges of the wings, but that color appeared to be due to handling damage rather than a resilin signal, and the pre-immune group had the same pattern as in wings treated with resilin antibody, suggesting that the purple color was not due to resilin.

These results suggested that using the whole hind wing was not a good way to do immunohistochemistry, probably due to a lack of permiablization of the cuticle. Some alternatives are needed to localize resilin by immunohistochemistry in *T. castaneum* hind wings, such as using younger pharate adult hind wings or cross sections of the wing to overcome the intractable nature of mature hind wings.

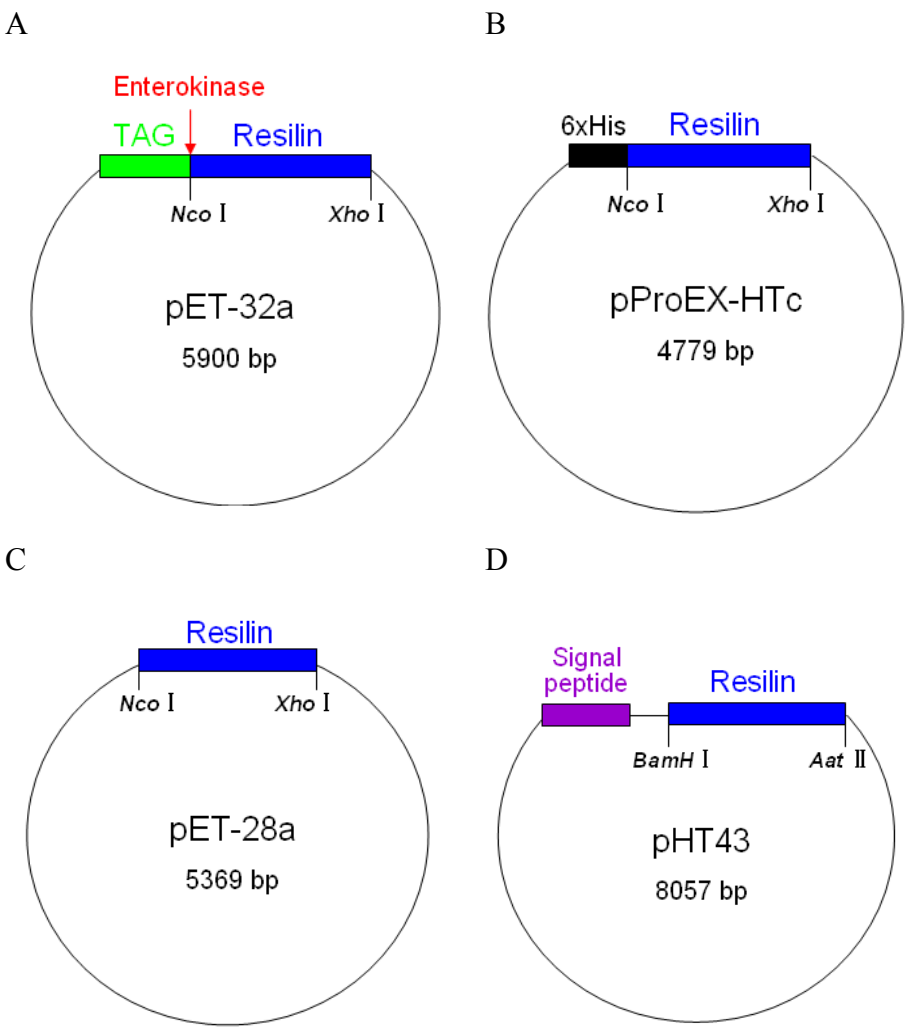


Figure 3-1. Four different plasmids were constructed for expressing *T. castaneum* resilin.

A) Construct 1 was used in *E. coli* BL21 (DE3) strain. The recombinant resilin contained an N-terminal fusion tag (TAG) and *T. castaneum* resilin sequence. The TAG could be cleaved by Enterokinase.

B) Construct 2 was used in *E. coli* Rosetta strain. The recombinant resilin contained an N-terminal fusion 6×His tag and *T. castaneum* resilin sequence.

C) Construct 3 was used in *E. coli* Rosetta (DE3) strain. The recombinant resilin contained only the *T. castaneum* resilin sequence.

D) Construct 4 was used in *Bacillus subtilis*. The recombinant resilin contained an N-terminal signal peptide derived from the vector and *T. castaneum* resilin sequence. The signal peptide would be cleaved by the bacteria so the secreted form of the recombinant resilin contained only *T. castaneum* resilin sequence.

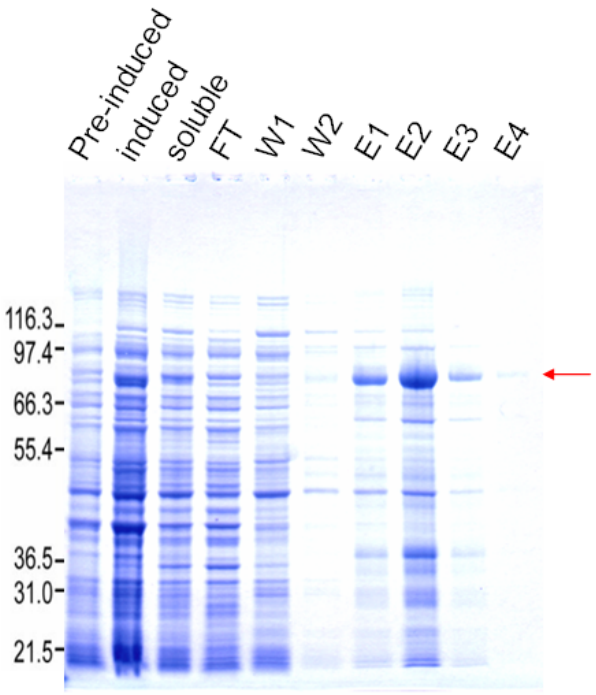


Figure 3-2. Recombinant resilin expression in *E. coli* strain BL21 (DE3) and purification by NiNTA chromatography.

Samples of pre-induced, induced, soluble fraction, flow through (FT), wash fractions (W1, W2) and elution fractions (E1-4) from expression and purification were treated with sample buffer containing β-mercaptoethanol and separated by SDS-PAGE (4-12% Bis-Tris gel) followed by Coomassie Blue staining. Ten µL of each sample was loaded. The red arrow shows the position of the recombinant resilin (~68 kDa).

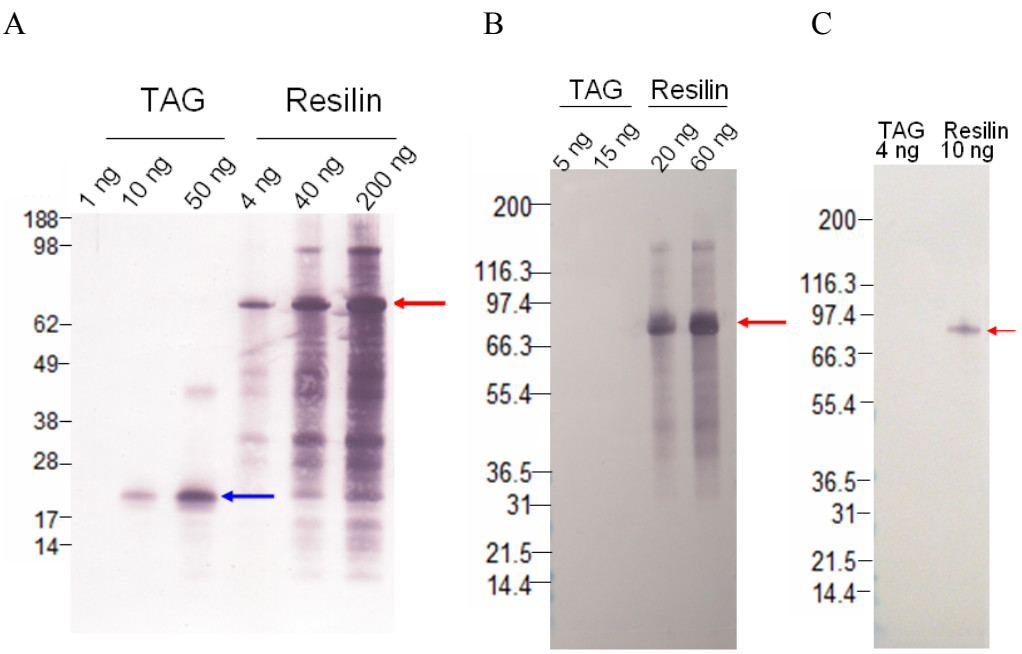


Figure 3-3. Characterization and purification of antibodies to recombinant resilin.

All protein samples were treated with sample buffer containing β-mercaptoethanol and separated by SDS-PAGE (4-12% Bis-Tris gel) followed by immunoblotting. The red arrows show the position of the recombinant resilin (construct 1) and the blue one shows TAG.

A) Crude antiserum was used to detect samples of the purified TAG or partially purified recombinant resilin.

B) Antibodies that did not bind to a TAG-affinity column were tested for removal of antibodies against the TAG protein.

C) Analysis of antibodies after affinity purification by binding to recombinant resilin.

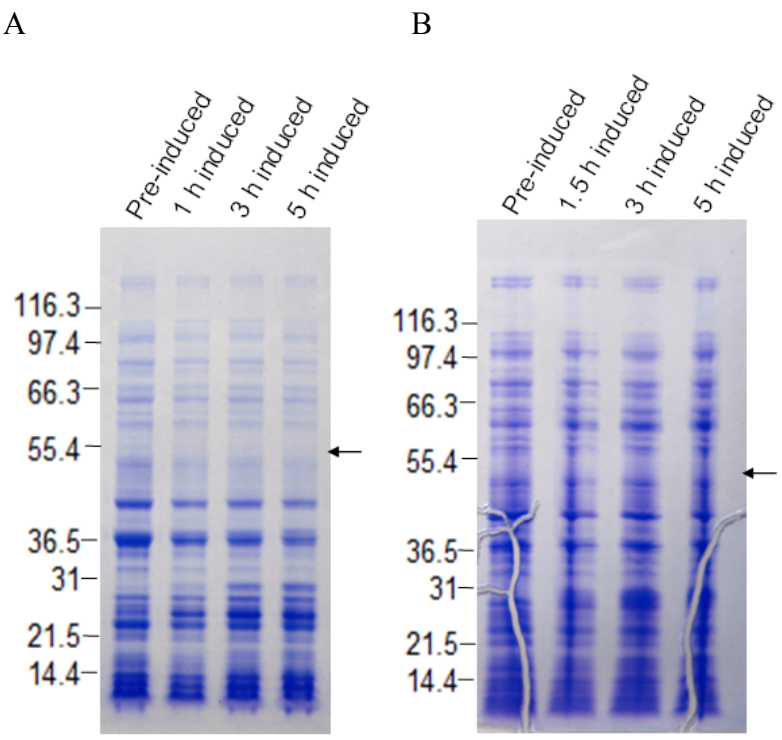


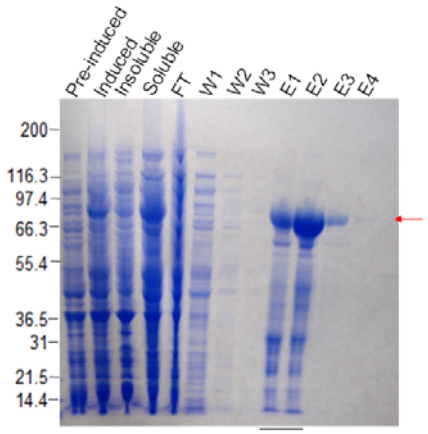
Figure 3-4. Trial expressions of two different recombinant resilins.

Pre-induced and induced protein samples were treated with sample buffer containing β-mercaptoethanol and separated by SDS-PAGE (4-12% Bis-Tris gel) followed by Coomassie Blue staining. Ten µL of each sample was loaded.

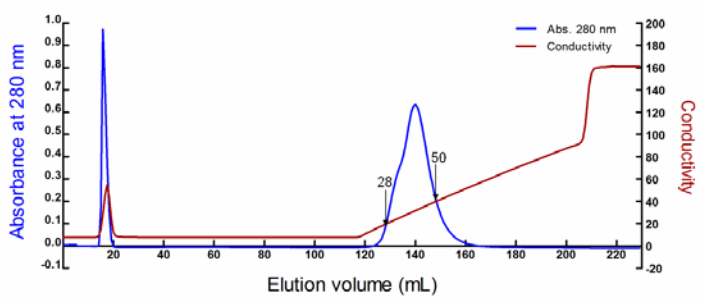
A) The construct 2 was expressed in *E. coli* Rosetta strain. The arrow shows the expected position of the recombinant resilin (54 kDa).

B) The construct 3 was expressed in *E. coli* Rosetta (DE3) strain. The arrow shows the expected position of the recombinant resilin (51 kDa).

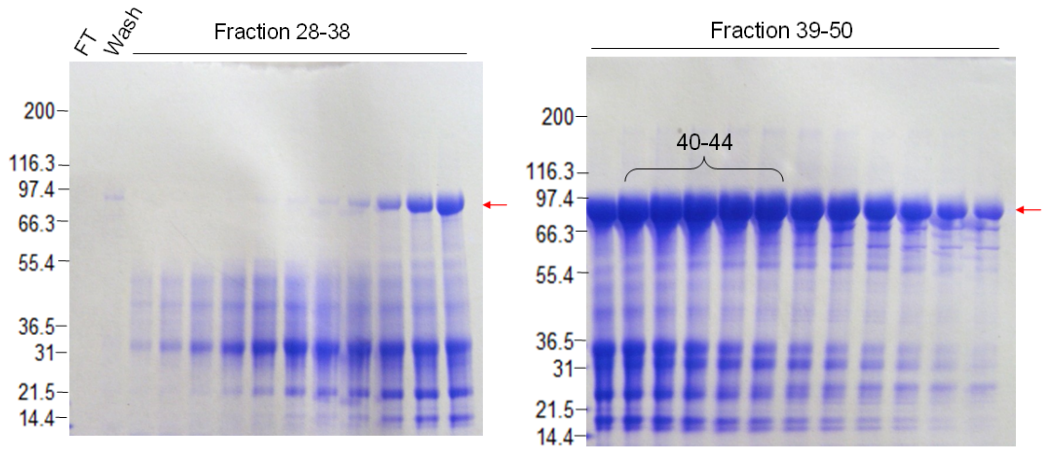
A


Pre-induced
Induced
Insoluble
Soluble
FT
W1
W2
W3
E1
E2
E3
E4
200
116.3
97.4
66.3
55.4
36.5
31
21.5
14.4

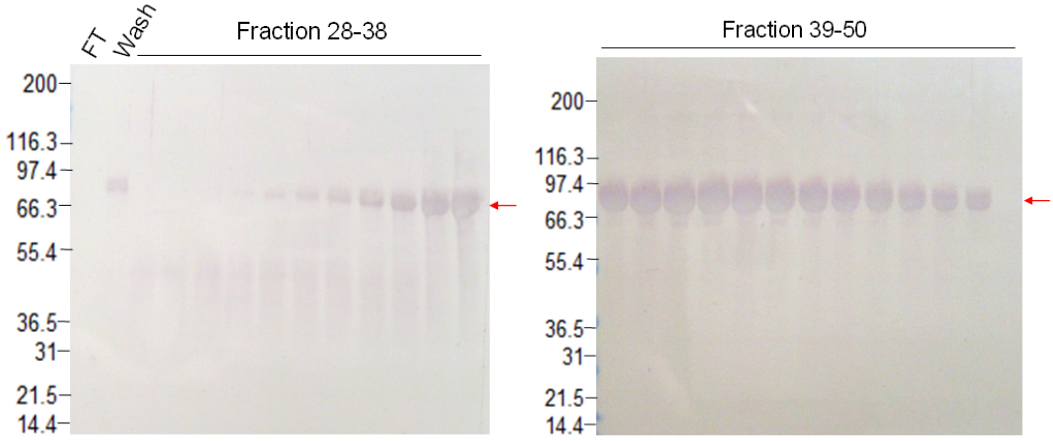
B


Abs. 280 nm
Conductivity
Absorbance at 280 nm
Conductivity
Elution volume (mL)
28
50

C


FT
Wash
Fraction 28-38
Fraction 39-50
40-44
200
116.3
97.4
66.3
55.4
36.5
31
21.5
14.4

D


FT
Wash
Fraction 28-38
Fraction 39-50
200
116.3
97.4
66.3
55.4
36.5
31
21.5
14.4

E F

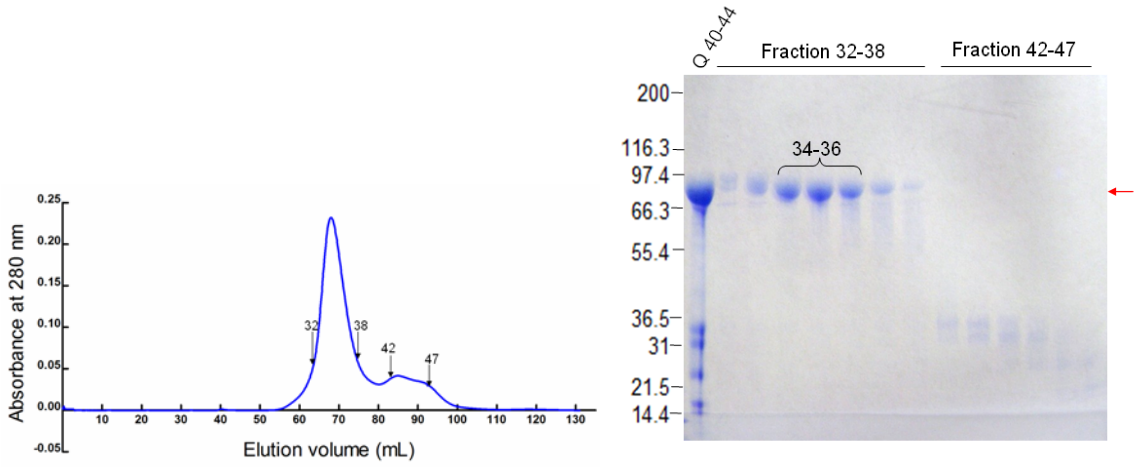


Figure 3-5. Expression and purification of recombinant resilin from *E. coli* construct 1. Protein samples from expression and purification were treated with sample buffer containing β-mercaptoethanol and separated by SDS-PAGE (4-12% Bis-Tris gel) followed by Coomassie Blue staining or immunoblotting with the partially purified resilin antibody. Ten µL of each sample was loaded. Red arrows show the position of the recombinant resilin (construct 1).

A) Analysis of protein samples from expression and NiNTA chromatography by SDS-PAGE.

B) Elution fractions 1 and 2 from NiNTA chromatography were combined and separated by ion exchange chromatography. Arrows show elution fractions in the peak area.

C) Analysis of fractions from ion exchange chromatography by SDS-PAGE.

D) Immunoblot analysis of the same samples as in (C).

E) Fractions 40-44 from ion exchange chromatography were combined and separated by gel filtration chromatography. Arrows show elution fractions in peak areas.

F) SDS-PAGE analysis of fractions from gel filtration chromatography.

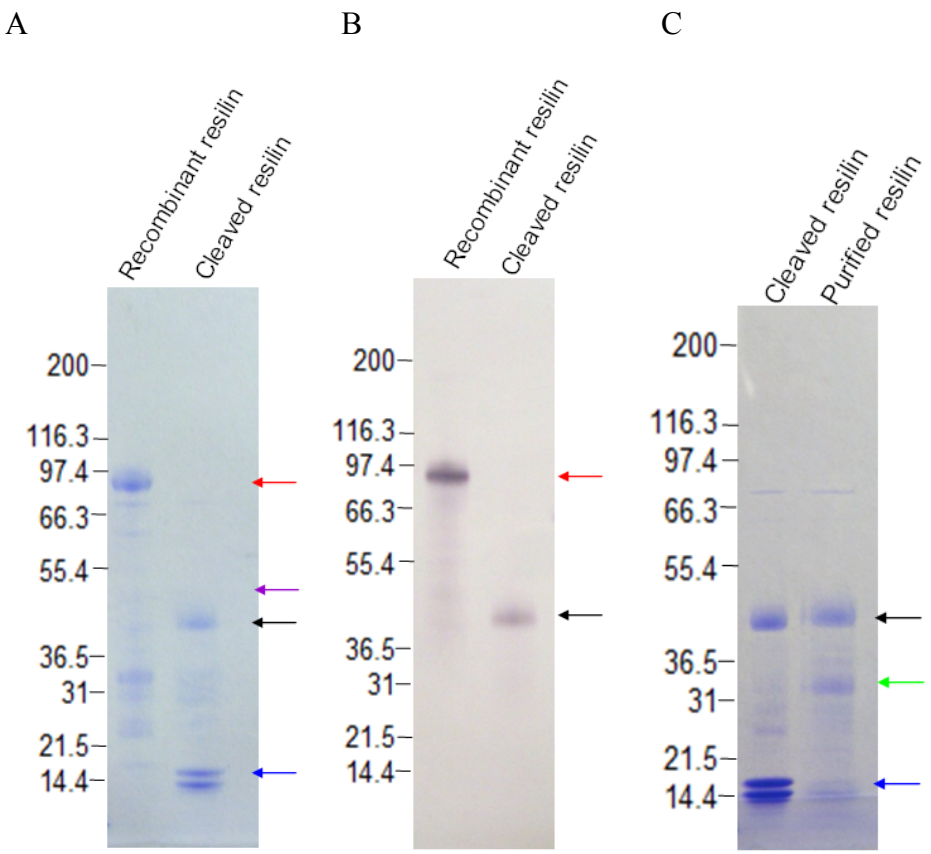


Figure 3-6. Removal of the TAG from the recombinant resilin and purification of cleaved resilin by NiNTA chromatography.

Resilin fusion protein was treated with enterokinase to cleave and release the TAG. An untreated sample and a cleaved sample were analyzed by SDS-PAGE followed by Coomassie Blue staining (A) or immunoblot using partially purified antibody (B). (C) the resilin sample after treatment with enterokinase was separated by NiNTA chromatography to remove the TAG, and sample before and after chromatography were analyzed by SDS-PAGE followed by Coomassie Blue staining. The red arrows show the position of the recombinant resilin fusion; the black arrows show the cleaved resilin; the blue arrows show the TAG; the purple arrow shows the expected position of cleaved resilin. A major degradation band appeared (green arrow) after NiNTA chromatography.

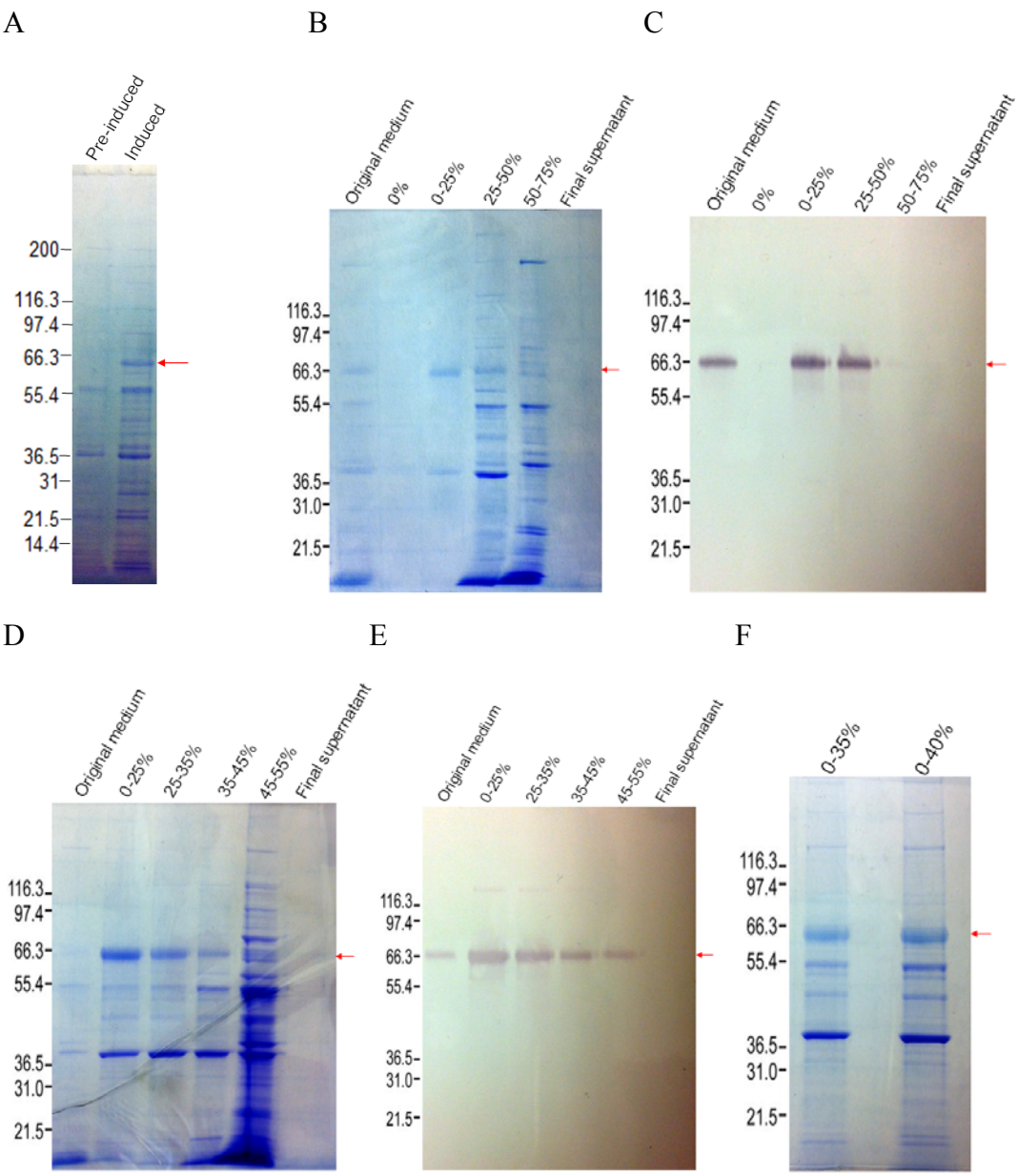


Figure 3-7. Expression and purification of recombinant resilin from *B. subtilis*.

Protein samples from expression and purification were treated with sample buffer containing β-mercaptoethanol and separated by SDS-PAGE (4-12% Bis-Tris gel) followed by Coomassie Blue staining or immunoblotting with the purified resilin antibody. Red arrows show the position of the recombinant resilin.

A) SDS-PAGE analysis of TCA-precipitated protein from culture medium before and after induction by IPTG.

B) SDS-PAGE analysis of resilin after precipitation from medium by different concentrations of ammonium sulfate.

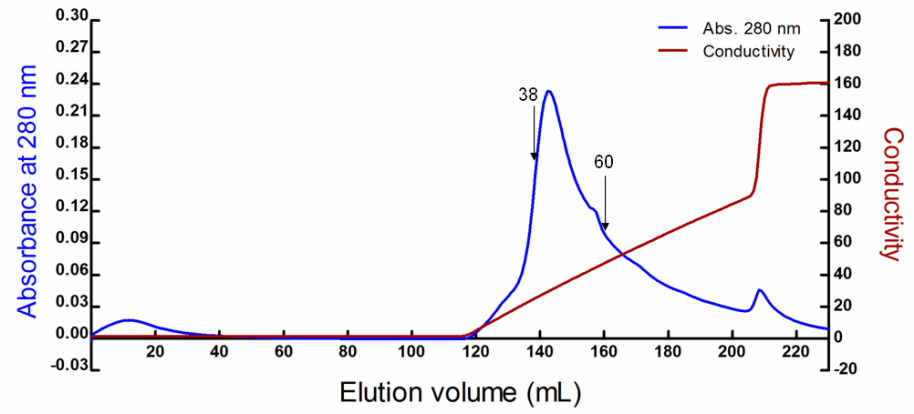
C) Immunoblot analysis of the same samples as in (B).

D) SDS-PAGE analysis of resilin after precipitation from medium by different concentrations of ammonium sulfate.

E) Immunoblot analysis of the same samples as in (D).

F) SDS-PAGE analysis of a comparison of resilin after precipitation from medium by two different concentrations of ammonium sulfate.

A



B C

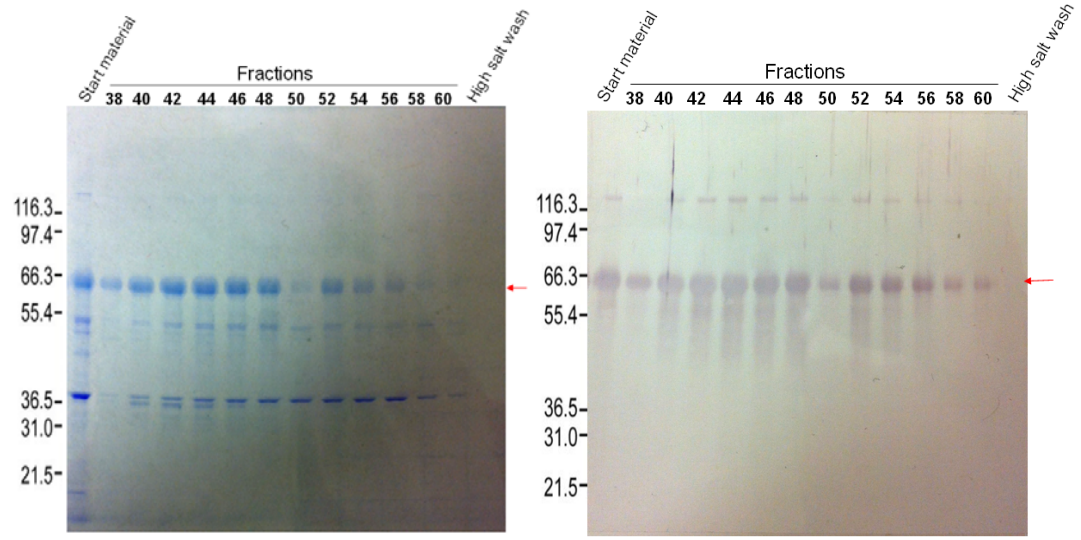


Figure 3-8. Purification of recombinant resilin from *B. subtilis* by ion exchange chromatography

A) Proteins precipitated by 40% saturated ammonium sulfate from the culture medium were separated by ion exchange chromatography. Arrows show elution factions in the peak area.

B) Protein samples after ion exchange chromatography were treated with sample buffer containing β-mercaptoethanol and separated by SDS-PAGE (4-12% Bis-Tris gel) followed by Coomassie Blue staining. Red arrow shows the position of the recombinant resilin.

C) Immunoblot analysis of the same samples as in (B) with the purified resilin antibody. Red arrow shows the position of the recombinant resilin.

A B

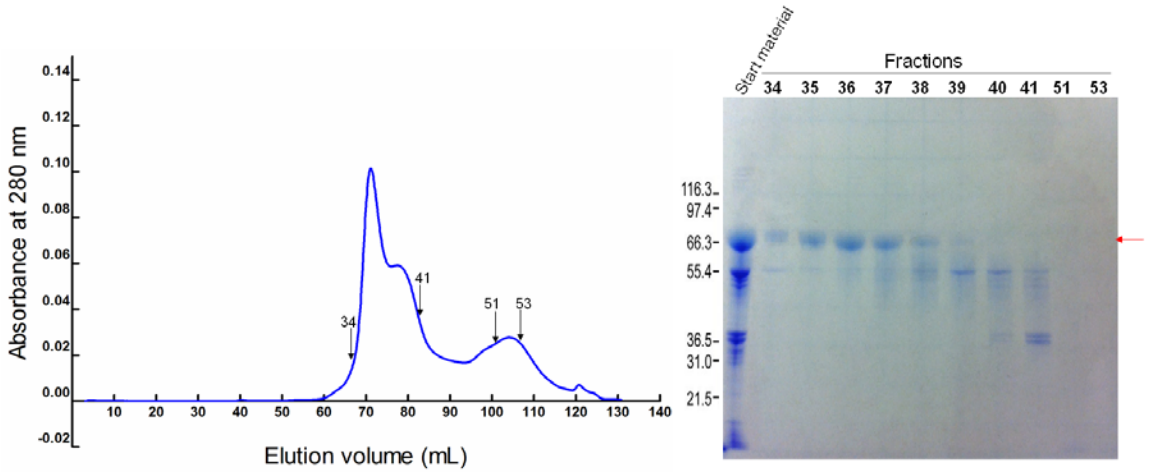


Figure 3-9. Purification of recombinant resilin from *B. subtilis* by gel filtration.

A) Elution fractions 38-54, except 50, after ion exchange chromatography were combined and separated by gel filtration. Arrows show elution fractions in peak areas.

B) Protein samples of elution fractions after gel filtration were treated with sample buffer containing β-mercaptoethanol and separated by SDS-PAGE (4-12% Bis-Tris gel) followed by Coomassie Blue staining. Red arrow shows the position of the recombinant resilin.

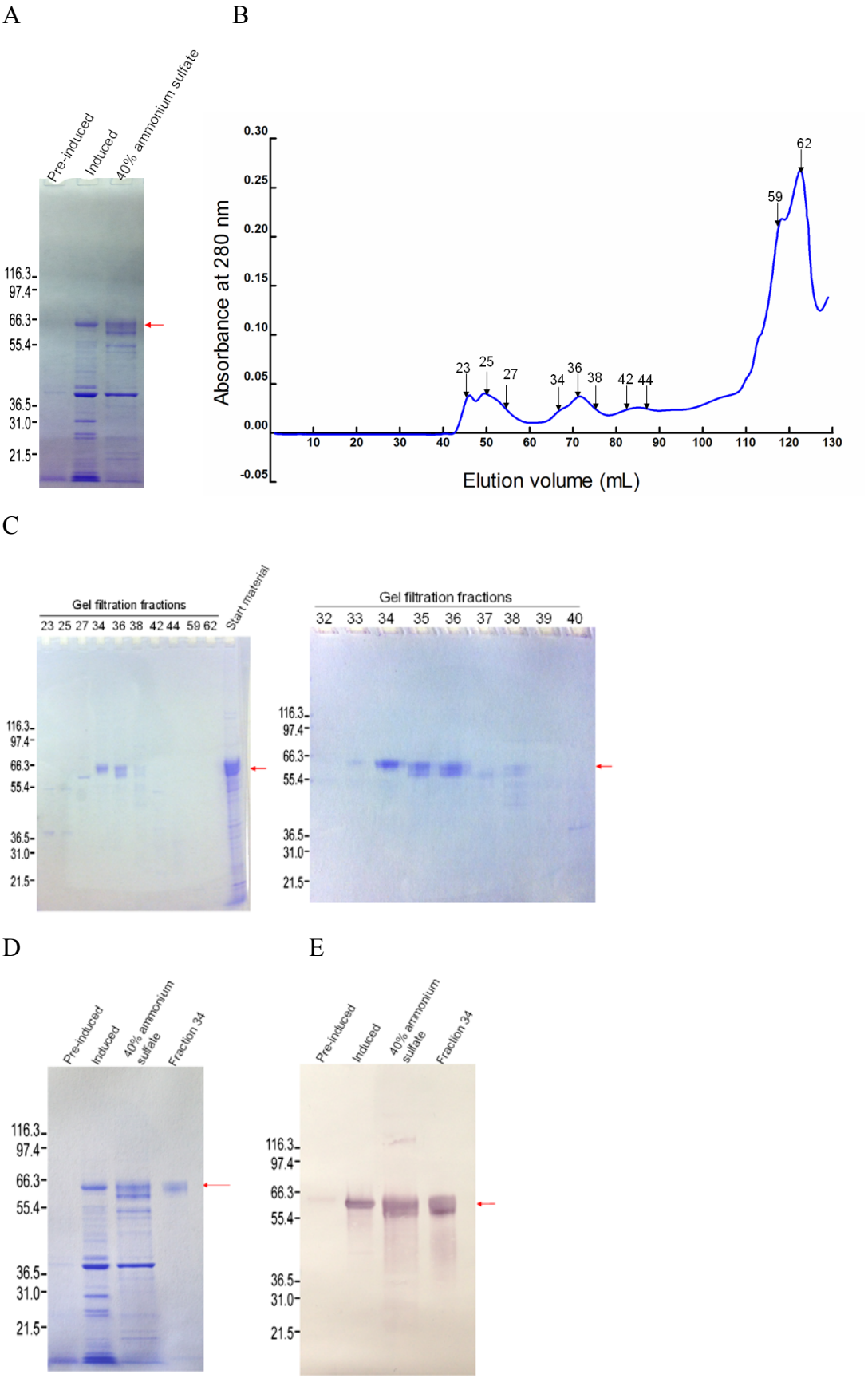

A
B
Pre-induced
Induced
40% ammonium sulfate
116.3
97.4
66.3
55.4
36.5
31.0
21.5
Absorbance at 280 nm
Elution volume (mL)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
10 20 30 40 50 60 70 80 90 100 110 120 130
23 25 27 34 36 38 42 44 59 62
C
Gel filtration fractions
23 25 27 34 36 38 42 44 59 62
Start material
Gel filtration fractions
32 33 34 35 36 37 38 39 40
D
E
Pre-induced
Induced
40% ammonium sulfate
Fraction 34

Figure 3-10. Additional expression and purification of recombinant resilin from *B. subtilis*. Protein samples from expression and purification were treated with sample buffer containing β-mercaptoethanol and separated by SDS-PAGE (4-12% Bis-Tris gel) followed by Coomassie Blue staining or immunobloting with the purified resilin antibody. Red arrows show the position of the recombinant resilin

A) SDS-PAGE analysis of TCA-precipitated protein from culture medium before and after induction by IPYG and 40% saturated ammonium sulfate-precipitated protein from culture medium after induction.
B) Proteins precipitated by 40% saturated ammonium sulfate were separated by gel filtration chromatography. Arrows show elution fractions in peak areas.
C) SDS-PAGE analysis of elution fractions of gel filtration chromatography.
D) SDS-PAGE analysis of proteins before and after induction (TCA precipitated), precipitated by 40% saturated ammonium sulfate and elution fraction 34 of gel filtration chromatography.
E) Immunoblot analysis of the same samples as in (D).

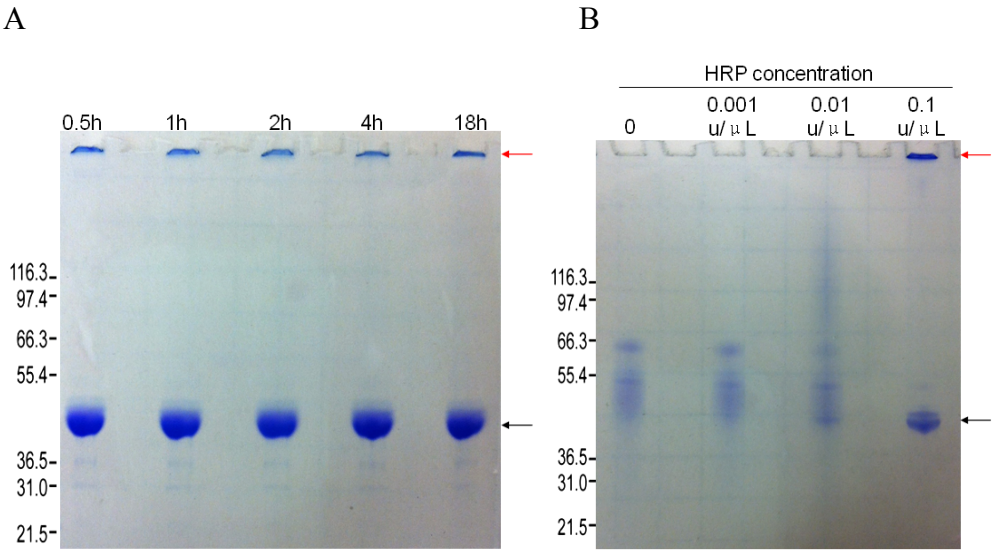


Figure 3-11. The purified recombinant resilin was cross-linked by peroxidase *in vitro*.

Protein samples of each reaction were treated with sample buffer containing β-mercaptoethanol and separated by SDS-PAGE (4-12% Bis-Tris gel) followed by Coomassie Blue staining. Red arrows show the position of cross-linked resilin and black arrows show HRP.

A) SDS-PAGE analysis of cross-linking reactions for different times.

B) SDS-PAGE analysis of cross-linking reactions using different concentrations of HRP.

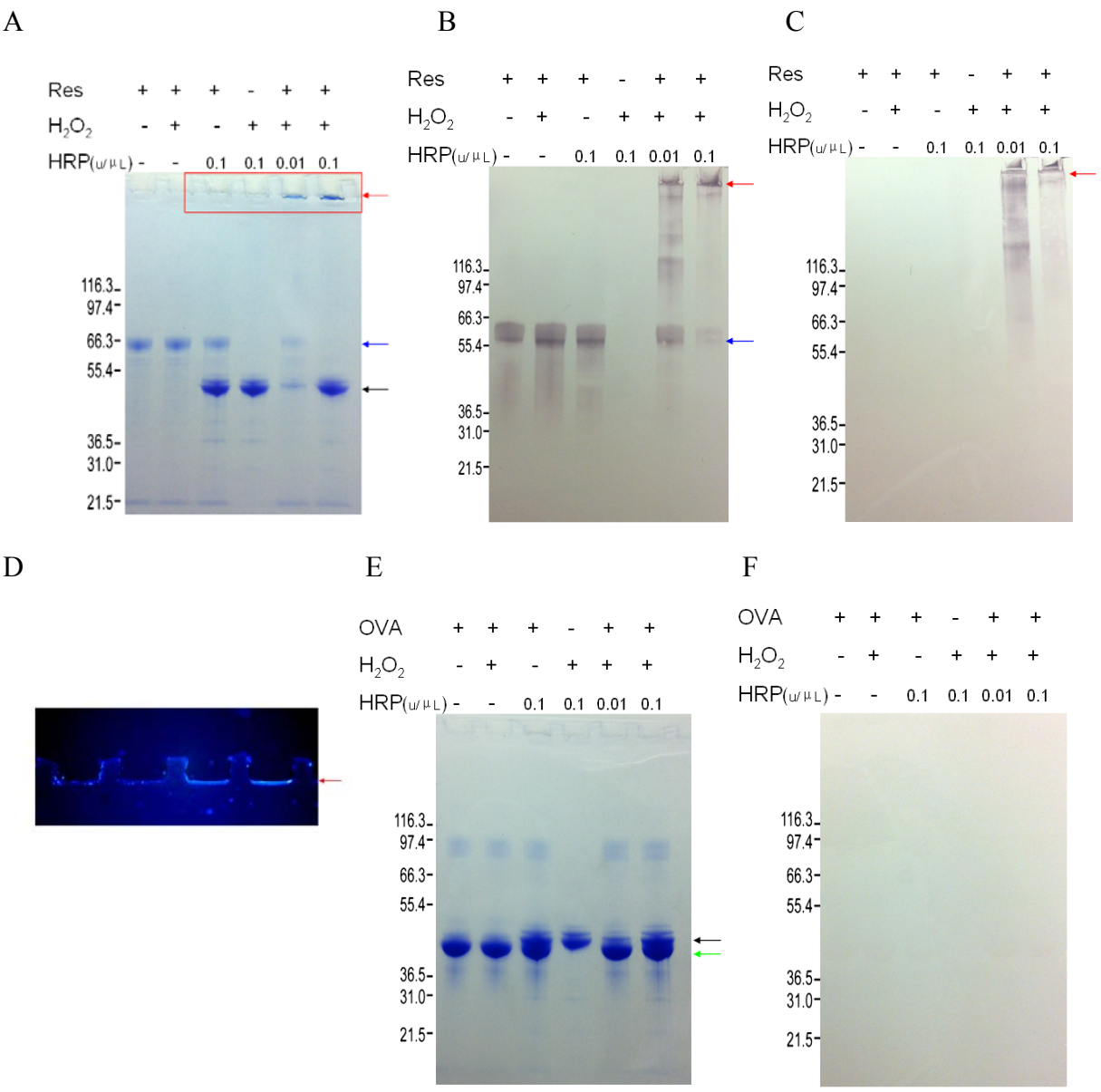


Figure 3-12 *In vitro* cross-linking of the recombinant resilin by horseradish peroxidase. Protein samples of each reaction were treated with sample buffer containing β-mercaptoethanol and separated by SDS-PAGE (4-12% Bis-Tris gel) followed by Coomassie Blue staining or immunoblotting. Red arrows show the position of cross-linked resilin.

A) SDS-PAGE analysis of cross-linking reactions containing different contents. The blue arrow shows uncross-linked recombinant resilin and the black arrow shows HRP.

B) Immunoblot analysis of the same samples as in (A) with purified resilin antibody. The blue arrow shows uncross-linked recombinant resilin.

C) Immunoblot analysis of the same samples as in (A) with the dityrosine antibody 1C3 (Kato et al., 2000) graciously supplied by Dr. Yoji Kato, University of Hyogo, Japan.

D) The area in the red rectangle in (A) was visualised under ultraviolet light before Coomassie Blue staining.

E) SDS-PAGE analysis of cross-linking reactions containing different contents. The green arrow shows ovalbumin (OVA) and the black arrow shows HRP.

F) Immunoblot analysis of the same samples as in (E) with dityrosine antibody.

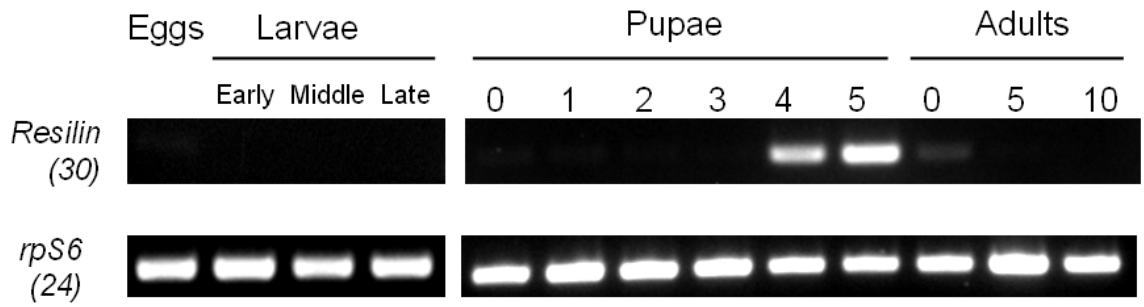


Figure 3-13. *T. castaneum* resilin mRNA level is the highest in late pupal stage.

PCR products were analyzed in agarose gels by DNA electrophoresis. 30 cycles was used for resilin PCR and 24 cycles was used for rpS6 PCR. The numbers are the day of different developmental stages.

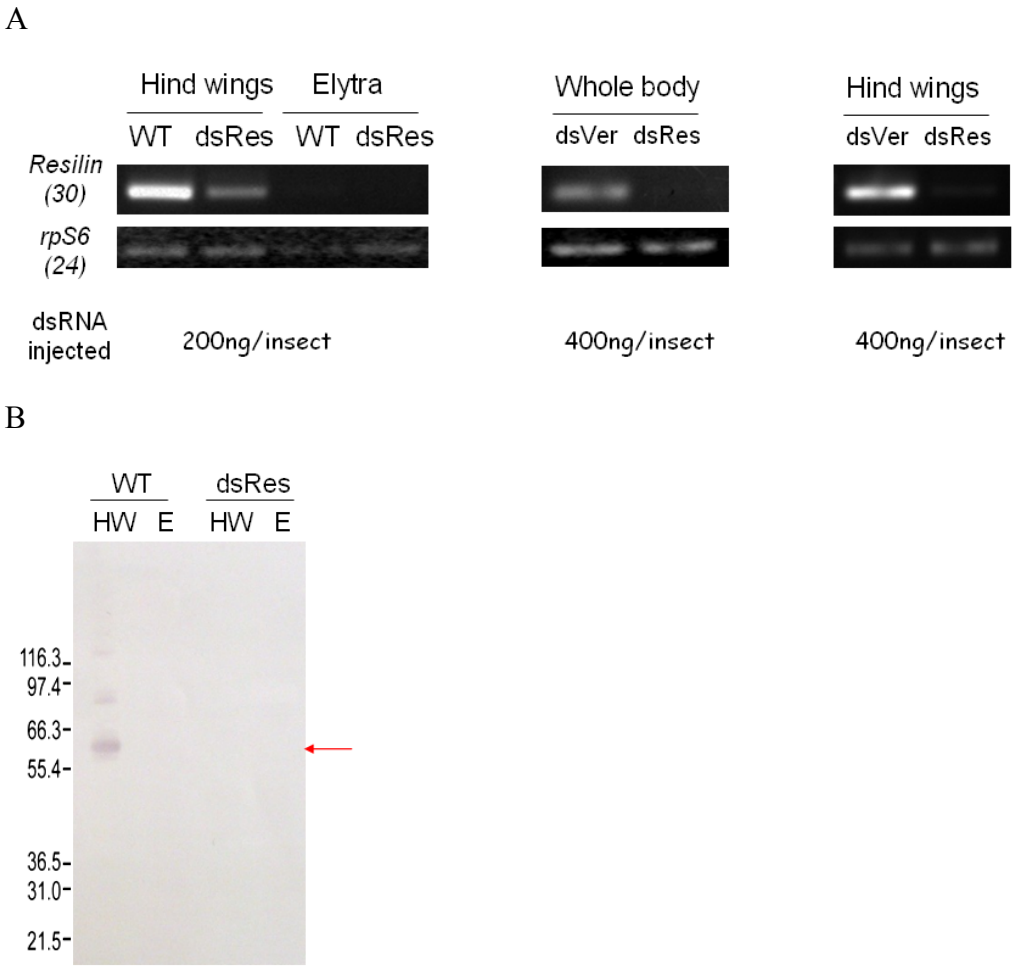


Figure 3-14. *T. castaneum* resilin was knocked down by RNAi.

The total mRNA and proteins were extracted from hind wings or elytra at the pharate adult stage of insects injected with dsRNA for resilin or vermillion, and used for RT-PCR or immunoblot analysis.

A) PCR products were analyzed in agarose gels by DNA electrophoresis. Thirty cycles was used for resilin PCR and 24 cycles was used for rpS6 PCR. WT: wild type; dsRes: resilin knocked down; dsVer: vermillion knocked down.

B) Immunoblot analysis with the purified resilin antibody. HW: hind wings; E: elytra. The red arrow shows the position of resilin.

Figure 3-15. RNAi did not affect the morphology of *T. castaneum* adults.

dsRNAs were injected in the pre-pupal stage, and these all survived to become adults. WT: wild type; dsVer: vermillion knocked down beetles; dsRes: resilin knocked down beetles.

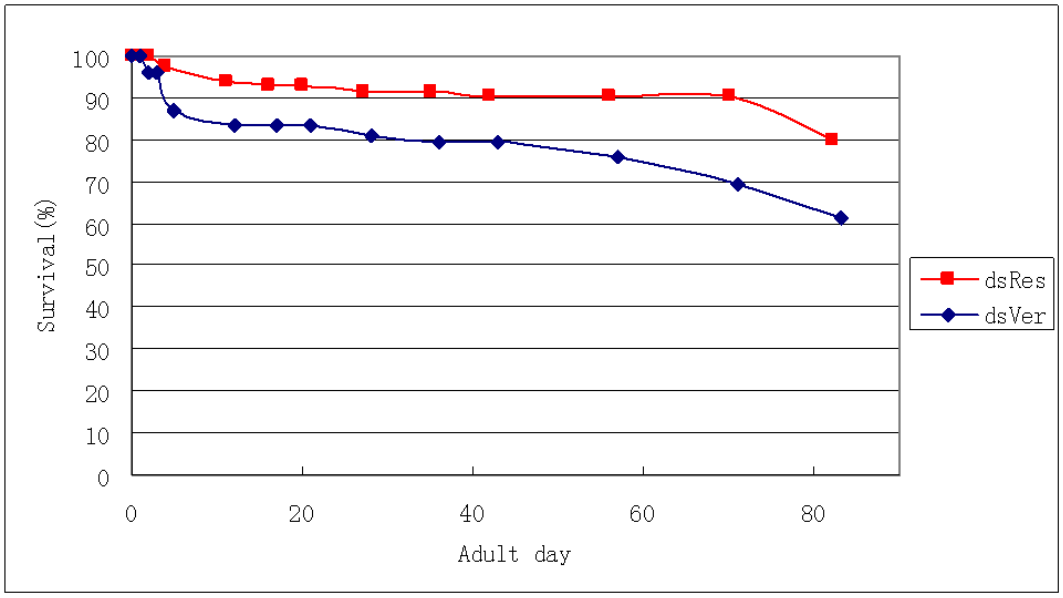


Figure 3-16. Resilin RNAi did not affect the lifespan of *T. castaneum* adults.

The red line shows beetles injected with dsRes and the dark blue line indicates beetles injected with dsVer. For dsRes, n=84; for dsVer, n=78.

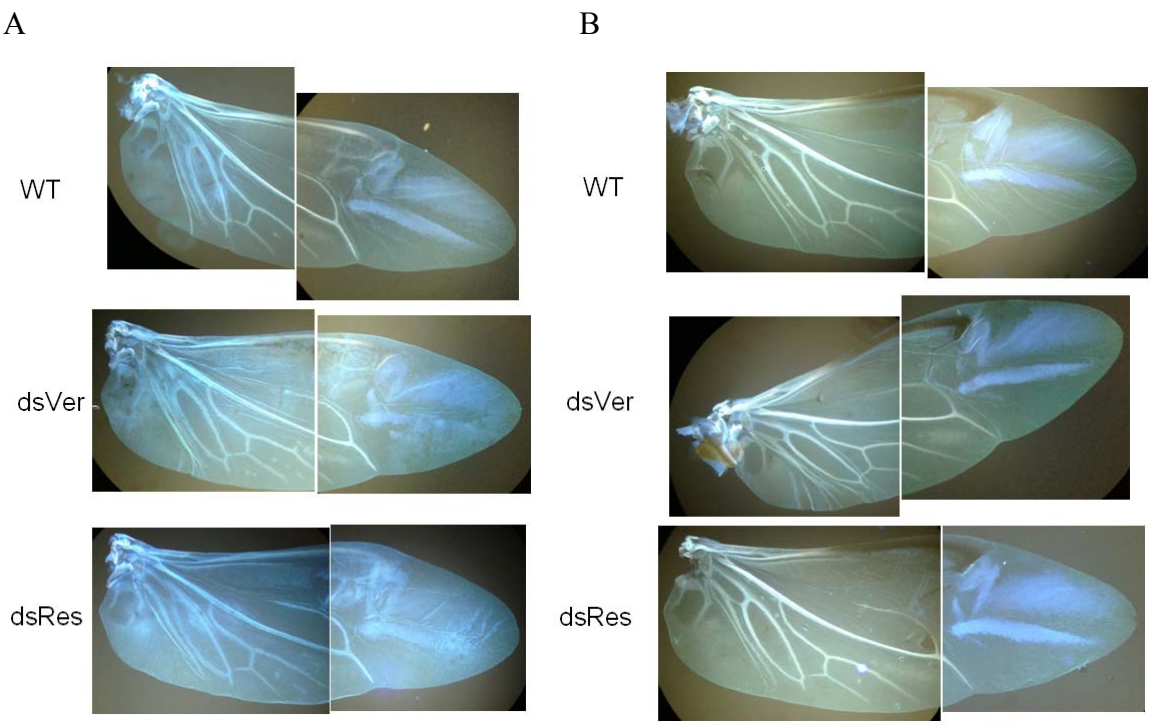
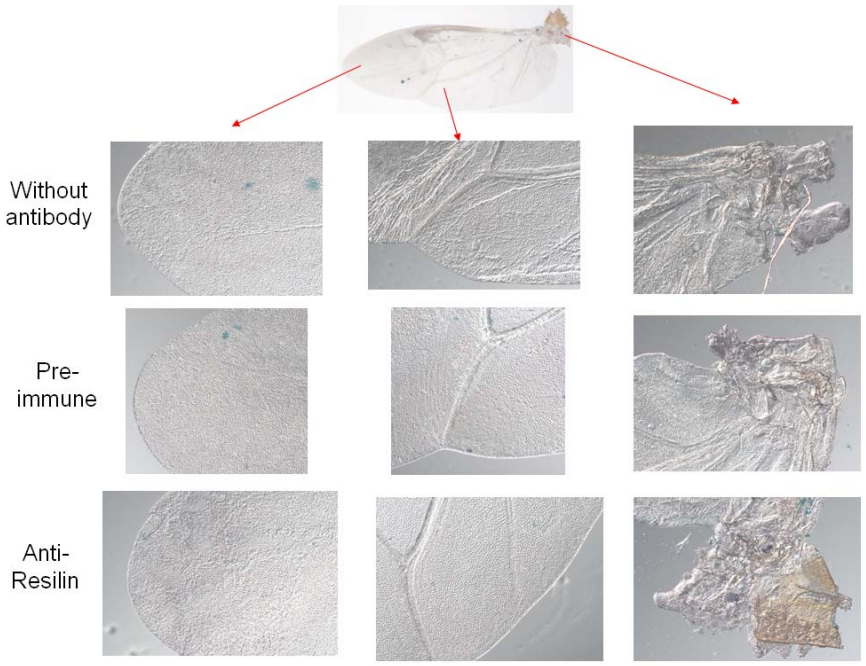


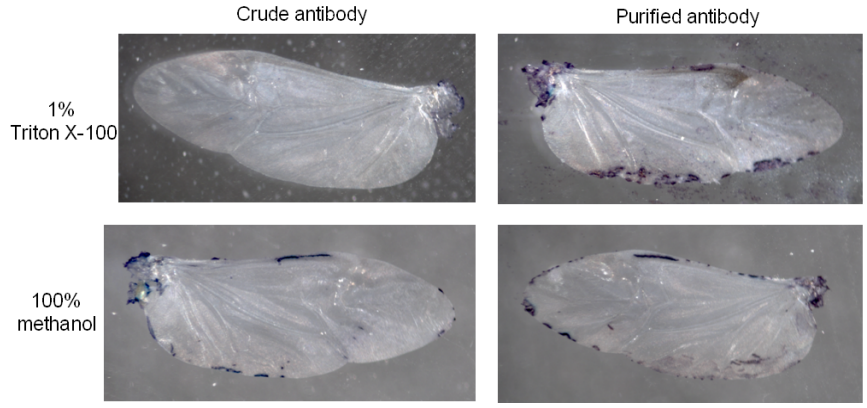
Figure 3-17. Blue fluorescence of *T. castaneum* adult hind wings.

Adult hind wings were dissected and mounted in 100% glycerol. The excitation wavelength was 360 nm and the emission wavelength was ≧ 420 nm. The upper panel was wild type (WT) samples; the middle panel was dsVer injected samples; the lower panel was dsRes injected samples. (A) Samples from 0-1 day old adults. (B) Samples from 2-3 day old adults.

A


Without antibody
Pre-immune
Anti-Resilin

B


Crude antibody
Purified antibody
1% Triton X-100
100% methanol

C

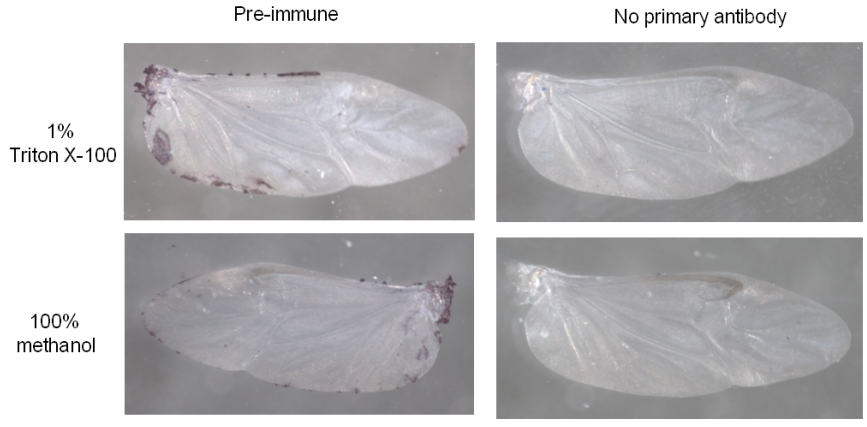

Pre-immune
No primary antibody
1% Triton X-100
100% methanol

Figure 3-18. Immunohistochemistry of resilin in *T. castaneum* adult hind wings.

T. castaneum adult hind wings were dissected and used in two trials with different permeabilizaion steps.

A) The first trial of immunohistochemistry using 0.1% Triton X-100. The upper panel was not treated with primary antibody; the middle panel was treated with pre-immune antibody; the lower panel was treated with purified resilin antibody.

B) The second trial using stronger permeabilization. The upper panel was treated with 1% Triton X-100; the lower panel was treated with 100% methanol. The left column was using the crude antibody while the right column was using the purified antibody.

C) The control group of the second trial. The upper panel was treated with 1% Triton X-100; the lower panel was treated with 100% methanol. The left column was using the pre-immune antibody while the right column was not using the primary antibody.

Chapter 4 - Discussion

Since the discovery of resilin in insects (Wei-Fogh, 1960), one goal has been to localize this protein in different body parts (Andersen and Weis-Fogh, 1964; Gorb, 1999; Haas et al., 2000a, 2000b; Elvin et al., 2005; Burrows et al., 2008; Sutton and Burrows. 2011; Lyons et al., 2011; Wiesenborn, 2011; Wong et al., 2012). In most previous studies, resilin was identified by its autofluorescence under ultraviolet light, which is due to the dityrosine cross-links in the protein (Andersen, 1964). Only researchers in Elvin's group used a *Drosophila* resilin antibody (anti-Rec1 antibody) to localize resilin in some insect tissues (Lyons et al., 2011; Wong et al., 2012). No resilin knockdown or mutation has been reported. Except the putative resilin cDNA sequence, no other information is available about resilin in the red flour beelte, *T. castaneum*. Thus, in this research I focused on the resilin protein in *T. castaneum* to investigate its structure and function.

Recombinant resilin proteins were expressed in *E. coli* or *B. subtilis*, and used to investigate the cross-linking of resilin *in vitro* and to generate antibodies against the protein. I also knocked down resilin expression in *T. castaneum* by RNAi to investigate its functions *in vivo*. I also attempted to localize resilin in *T. castaneum* adult hind wings by both autofluorescence and immunohistochemistry using the *Tribolium* resilin antibody.

Expression and purification of the recombinant resilin

The first identified resilin gene was the *D. melanogaster* CG15920 gene (Ardell and Andersen, 2001). Because the resilin protein exhibits very special properties, such as high resilience and energy storage, many researchers were interested in synthesizing "resilin-like" proteins or peptides to further study their material properties. Several different portions as well as the full length of the *Drosophila* resilin have been successfully expressed and purified (Elvin et al., 2005; Kim et al., 2007; Lyons et al., 2007, 2009; Qin et al., 2009, 2011, 2012; Tamburro et al., 2010). In this research, I attempted to express the full length *T. castaneum* resilin using four different constructs (Figure 3-1) in *E. coli* or *B. subtilis.* The most successful expressions were using vector pET32a expressed in BL21(DE3) *E. coli* strain (Figure 3-2) and pHT43 expressed in *B. subtilis* (Figure 3-7 A). The expression level was very low using pProEX-HTc or pET28a

expressed in the Rosetta or Rosetta (DE3) *E. coli* strain, respectively. No apparent induction was observed in SDS-PAGE analysis (Figure 3-4), but the more sensitive immunoblot analysis detected low levels of resilin expression (data not shown) with these constructs. A major difference between the four constructs was the N-terminal fusion tag. Construct 1 has a larger fusion tag (about 17 kDa); construct 2 has a smaller one (about 3 kDa); construct 3 and 4 have no tag. A possible explanation for the low expression level is that the synthesized resilin may be "toxic" to the *E. coli* cells due to the special repeated amino acid sequence of resilin. The larger fusion tag may somehow eliminate or suppress this toxicity, while the smaller one had little or no effect on the toxicity. Construct 4 had no tag, but it was expressed by *B. subtilis*, which secreted resilin into the culture medium. Thus, only a very small amount of resilin would be present inside the cells, which may not affect the cells. A second possibility is that *T. castaneum* resilin is not as toxic to *B. subtilis* cells as it is to *E. coli* cells.

For purification of the recombinant resilin, NiNTA chromatography, ammonium sulfate precipitation, ion exchange and gel filtration chromatography were all used. The NiNTA column worked well for His-tagged recombinant resilin (Figure 3-2; 3-5 A). Ion exchange chromatography did not improve the purity very much (Figure 3-5 C, D; 3-7 H). Gel filtration chromatography worked quite well in all cases (Figure 3-5 H; 3-7 K; 3-8 C, D). Based on this research, a conclusion is that NiNTA column chromatography and gel filtration chromatography can be used to purify His-tagged recombinant resilin, and ammonium sulfate precipitation together with gel filtration chromatography can be used to purify non-His-tagged secreted recombinant resilin.

The recombinant resilin expressed by *B. subtilis* was predicted to be about 51 kDa, but it did not migrate at the expected position in SDS-PAGE (such as in Figure 3-7 D). This is also possibly because of the unique repeated amino acid sequence of resilin, which may adopt a structure that results in abnormal migration in the gel.

A similar explanation is also for the case of protein amount determination (Table 4-1). BCA Protein Assay and Coomassie Plus Protein Assay were both used, but results between different times of the same assay or between the two assays were inconsistent. Before concentrating the recombinant resilin solution, the total protein was about 720 μg measured by the A_{280} method, and about 150 μg by the BCA assay. After concentrating the solution, the total protein was about 730 μg measured by the A_{280} method, and about 400 μg by the BCA assay.

Coomassie assay was also used and the result was about 160 μg, which was different from any of the other results. The hypothesis is that the sequence of the repeating structure of resilin made it difficult to react with the reagents to produce the color. Tyrosine residues of resilin protein may affect the colored complex in the BCA assay, and a small portion of basic amino acids of resilin may influence the color development in Coomassie assay. In contrast, results from the absorbance at 280 nm were consistent because no reagents/reactions was involved. So, the concentrations of recombinant resilin solutions were measured by this method.

Cross-linking reactions of the recombinant resilin

The mature resilin *in vivo* is believed to be cross-linked by dityrosine or tritytosine linkages (Andersen, 1964). In this research, the full length *T. castaneum* resilin was expressed in *B. subtilis*, and the purified resilin was used to conduct cross-linking reactions *in vitro*. The reactants were horseradish peroxidase, hydrogen peroxide and the purified resilin. The cross-linking reactions were successful (Figure 3-9). Horseradish peroxidase could cross-link resilin within 30 min into a large species that could hardly migrate into the gel during electrophoresis. The cross-linkages, at least in part, were apparently dityrosine, which was supported by immunoblot analysis using the anti-dityrosine antibody (Figure 3-9 E) and the blue fluorescence under ultraviolet light (Figure 3-9 F). Presumably, the horseradish peroxidase was randomly cross-linking the resilin molecules, since in the immunoblot analysis (Figure 3-9 D, E), a continuous smear was present between the resilin monomer and the large cross-linked polymer, indicating the presence of a set of molecules with continuously increased sizes. This cross-linking reaction only occurred in the presence of both horseradish peroxidase and hydrogen peroxide. Lacking either led to a failure of the cross-linking (Figure 3-9 C, D, E). The exact mechanism of resilin cross-linking in *T. castaneum* is still unclear. Another possible enzyme in the insect cuticle that could catalyze the cross-linking reaction is laccase. Knockdown of the possible enzyme(s) by RNAi is a way to investigate the mechanism of resilin cross-linking in *T. castaneum*.

Expression pattern of resilin in *T. castaneum*

In this research, I examined the transcript level of resilin in thirteen developmental stages of *T. castaneum* by RT-PCR (Figure 3-13). The results showed that resilin was highly expressed in the late pupal stage (pupae day 4 and 5), and a very low level was detected in newly emerged

adults (adults day 0). In any other stages, the resilin transcript was undetectable. This result suggested that the resilin protein may be not critical for eggs, larvae or pupae (at least early and middle pupae), but it apparently plays some functional roles in adults. The synthesis of the resilin protein likely begins at the late pupal stage and is completed soon after the adult emerges. Thus, new adults could have enough resilin for modifications (like cross-linking) and to build structures where resilin is needed. *D. melanogaster* has a similar expression pattern of resilin when peak expression is observed in the late puparial stage, but little or no expression occurs in any larval or adult stage (Marygold et al., 2013).

Resilin transcript was highly expressed in hind wings but not detectable in elytra of *T. castaneum* pharate adults (Figure 3-14 A), which was further confirmed by immunoblot analysis of proteins extracted from pharate adult hind wings or elytra (Figure 3-14 B). For pharate adult hind wings, two larger bands were detected , one of which had a size close to resilin dimer, and the other between monomer and dimer, suggesting that resilin may be cross-linked with itself as well as some other proteins. These results suggested that resilin plays roles in hind wing rather than elytra development, maybe in flying behavior or wing folding.

Resilin RNAi in *T. castaneum*

To my knowledge, no report of resilin knockdown or mutation in insects has been published previously. In this research, I used RNA interference to knock down expression of the *T. castaneum* resilin to investigate whether resilin has some biological roles in this beetle, together with a comparison with control beetles to localize resilin in adult hind wings. About 400 ng of double stranded RNA was injected into each prepupa of *T. castaneum*. This stage was chosen because it is just before expression of resilin begins during development. Both immunoblot analysis using the purified resilin antibody and RT-PCR results indicated a successful knockdown of *T. castaneum* resilin (Figure 3-11).

However, no apparent developmental or behavioral differences were observed between the knockdown adults and the controls. The external morphology of the adults in which the resilin expression was decreased by RNAi was similar to those without resilin RNAi (Figure 3-12), and the life span of the adult was unaffected (Figure 3-13). Observation of the walking behavior of *T. castaneum* adults also showed no significant difference between dsResilin- and dsVermillion-treated beetles (data not shown). I hypothesize that *T. castaneum* resilin is

redundant and there may be some other proteins or substances that can function similarly to resilin and rescue the resilin knockdown. Another possibility is that resilin may play a role in flying behavior, but it is difficult to conduct experiments on beetle flying in our laboratory conditions. Thus, in this research, no obvious knockdown phenotype was observed.

Immunohistochemistry and autofluorescence of *T. castaneum* resilin

Very little evidence using resilin antibody to localize the protein in animals has been published except for that utilizing the *Drosophila* anti-Rec1 antibody (Elvin et al., 2005). This antibody was generated in rabbits against a recombinant protein composed of the first exon of the *Drosophila* resilin gene, CG15920. Using this antibody, resilin was detected in the epidermis of *D. melanogaster* and *B. tryoni* embryos in repeated segmental patterns, at the base of sensilla of legs, and wing bases of *D. melanogaster* pharate adults, as well as in the well-known dragonfly wing tendons and pleural arch in the thorax of fleas (Wong et al., 2012). In this research, I tried to localize resilin in *T. castaneum* adult hind wings by immunohistochemistry, since resilin transcription and expression were observed in pharate adult hind wings by RT-PCR and immunoblot analyses (Figure 3-11). The whole adult hind wing was used in this research. The early trials using 0.1% Triton X-100 for permeabilization yielded no real signals for resilin (Figure 3-14 A). The later trials using either 1% Triton X-100 or 100% methanol for increased permeabilization of the tissue also did not show evidence for resilin signals but instead only the damaged wing edges were immuno-positive (Figure 3-14 B, C). The most plausible reason for this failure was that the mature hind wings were too "hard" for the antibody to penetrate, due to the components of the outermost layer of insect cuticle, lipids and wax esters (Moussian, 2013). Sectioned wings or pharate adult wings may be better choices for samples to subject to resilin immunohistochemistry in *T. castaneum*.

Mature resilin can fluoresce blue under ultraviolet light due to dityrosine cross-links (Andersen, 1963), and this autofluorescence is the classical method used to detect resilin in animals. In this research, I also tried to detect the autofluorescence of resilin in *T. castaneum* adult hind wings. The blue color similar to many photographs in the literature was observed in hind wings of wild type adults. However, this fluorescence was not decreased in hind wings of beetles in which resilin expression was knocked down by RNAi. The fluorescence had a similar distribution as that in wings of control insects (Figure 3-15). I hypothesize that the observed blue

fluorescence in *T. castaneum* adult hind wings is derived not only from cross-linked resilin but also from components other than resilin, perhaps other cuticular proteins that contain dityrosine cross-links. This result, to some extent, is consistent with that of a phenotype from resilin konckdown, namely one in which some other cuticular components present at least in *T. castaneum* adult hind wings also fluoresce blue under ultraviolet light and function similarly to resilin.

Here I list some of the earlier studies that detected resilin, and the results from this research (Table 4-2). In most cases, resilin was detected by its autofluorsecence. Some of the data was obtained using the anti-Rec1 antibody from one laboratory. No previous study has used RNAi. The only two beetles (other than *Tribolium*) are highlighted in green. Resilin was found in mobile joints of veins and locations with extra mobility in the hind wings of these two beetles, based on the autofluorescence (Haas et al., 2000b). No other evidence was shown to confirm that the blue areas contained resilin. In this research, I also observed blue fluorescence in hind wings of the red flour beetle, and the presence of resilin was confirmed by RT-PCR and immunoblot analysis with a resilin antibody. I used RNAi and successfully knocked down resilin expression in *T. castaneum* for the first time, but the lack of any phenotype and no decreased blue fluorescence in hind wings after RNAi was a surprise.

Conclusions and future directions

I believe that the work in this thesis furthered the understanding of the elastic cuticular protein resilin, and for the first time, resilin in the red flour beetle, *T. castaneum*, was studied. Resilin was highly expressed only in late pupal stages, as well as in the pharate adult hind wings but not in the elytra. Resilin expression was successfully knocked down by RNAi, but no obvious phenotype was observed, and no significant difference of the autofluorescence of adult hind wings between knockdown and control insects was observed, suggesting that some other cuticular components, which also fluoresce blue under ultraviolet light, may be present in hind wings and function similarly to resilin. The whole adult hind wing was not a good choice for immunohistochemistry, since no resilin signal was obtained in several trials. The full length resilin was successfully expressed and purified from both *E. coli* and *B. sutilis*, and the purified resilin from *B. subtilis* was cross-linked *in vitro* by horseradish peroxidase in the presence of

hydrogen peroxide. Based on this research, some future work could be done to further study *T. castaneum* resilin:

1. Immunohistochemistry of the whole or sectioned pharate adult hind wings and methylene blue and toluidine blue staining for localization of reslin.
2. RT-PCR and immunoblotting of different body parts, such as legs, thorax and abdomen, for expression pattern of resilin.
3. Observe flying behavior of the beetle to characterize the knockdown phenotype.
4. Cross-linking reactions *in vitro* using laccase instead of peroxidase.
5. Improve expression of the recombinant resilin to obtain enough protein and investigate whether they can be cross-linked into a solid material and determine its mechanical properties (Su et al., 2013).
6. Identify the substance(s) responsible for the blue fluorescence in resilin-knockdown beetles.
7. Identify the enzyme responsible for catalyzing the cross-linking of resilin *in vivo*.

	A_{280}	BCA assay	Coomassie assay
Before concentration	720 μg	150 μg	
After concentration	730 μg	400 μg	160 μg

Table 4-1 Protein amount determination using different methods.

Fraction 35 of the gel filtration chromatography was used to estimate the protein amount before concentration. Fractions 34, 35, 36 and 37 were combined and concentrated, and used to estimate the protein amount.

Species	Body parts	Methods	Reference
Schistocerca gregaria	Prealar arm; forewing hinge	Methylene blue or toluidine blue staining	Weis-Fogh, 1960
Aeshna cyanea	Tendon for pleuro-subalr muscle	Methylene blue or toluidine blue staining	Weis-Fogh, 1960
Schistocerca gregaria	Ligaments in wing system	Auto-fluorescence	Andersen and Weis-Fogh, 1964
Enallagma cyathigerum	Vein joints in the wings	Auto-fluorescence	Gorb, 1999
Forficula auricularia	Hind wings	Auto-fluorescence	Haas et al., 2000a
Pachnoda marginata; Coccinella septempunctata	Hind wings	Auto-fluorescence	Haas et al., 2000b
Zyxomma sp.	Wing tendon	Auto-fluorescence	Elvin et al., 2005
Aphrophora; Philaenus	Metathorax	Auto-fluorescence	Burrows et al., 2008
Archaeopsyllus erinacei	Joint of hind leg and thorax	Auto-fluorescence	Sutton and Burrows, 2011
Ctenocephalides felis	Pleural arch at top of hind leg	Auto-fluorescence and immunostaining by anti-Rec1 antibody	Lyons et al., 2011
Insects in 7 orders, 17 families, 18 genera	External of the whole body	Auto-fluorescence	Wiesenborn, 2011
Drosophila melanogaster; Bactrocera tryoni	Embryo	Immunostaining by anti-Rec1 antibody	Wong et al., 2012
Drosophila melanogaster	Pupal leg; pharate adult wing base	Auto-fluorescence and immunostaining by anti-Rec1 antibody	Wong et al., 2012
Aeshna cyanea	Wing tendon	Auto-fluorescence and immunostaining by anti-Rec1 antibody	Wong et al., 2012
Tribolium castaneum	Hind wing	RT-PCR; immunoblot by resilin antibody; auto-fluorescence; RNAi; protein extraction	This research

Table 4-2 Some previous studies which detected resilin by different methods.

Two beetles are highlighted in green.

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