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# Peroxinectin catalyzed dityrosine crosslinking in the adhesive underwater silk of a casemaker caddisfly larvae, *Hysperophylax occidentalis*

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ABSTRACT

Aquatic caddisfly larvae use sticky silk fibers as an adhesive tape to construct protective composite structures under water. Three new silk fiber components were identified by transcriptome and proteome analysis of the silk gland: a heme-peroxidase in the peroxinectin (Pxt) sub-family, a superoxide dismutase 3 (SOD3) that generates the $H_2O_2$ substrate of the silk fiber Pxt from environmental reactive oxygen species (eROS), and a novel structural component with sequence similarity to the elastic PEVK region of the muscle protein, titin. All three proteins are co-drawn with fibroins to form silk fibers. The Pxt and SOD3 enzymes retain activity in drawn fibers. In native fibers, Pxt activity and dityrosine crosslinks are co-localized at the boundary of a peripheral layer and the silk fiber core. To our knowledge, dityrosine crosslinks, heme peroxidase, and SOD3 activities have not been previously reported in an insect silk. The PEVK-like protein is homogeneously distributed throughout the fiber core. The results are consolidated into a model in which caddisfly silk Pxt-catalyzed dityrosine crosslinking occurs post-draw using $H_2O_2$ generated within the silk fibers by SOD3. The ROS substrate of caddisfly silk SOD3 occurs naturally in aquatic environments, from biotic and abiotic sources. The radially inhomogeneous dityrosine crosslinking and a potential titin-like PEVK protein network have important implications for the mechanical properties of caddisfly silk fibers.



## 1. Introduction

Aquatic caddisfly larvae fashion composite shelters under water with adhesive silk fibers and materials gathered adventitiously from their environment (Fig. 1). The underwater silk comprises a distinct peripheral adhesive layer on a viscoelastic, energy-dissipating, self-recovering fiber (Ashton et al., 2013a; Engster, 1976a). Caddisflies have been divided into three suborders (Integripalpia, Annulipalpia, and Spicipalpia) that deploy their larval silks in distinct fashions (LaFontaine, 1981; Wiggins, 2004). The Integripalpia case makers are mobile foragers that build portable protective cases, like body armor, around their soft larval bodies in species specific styles using species specific materials: stones, sticks, or leaves. The Annulipalpia web spinners build stationary retreats with stones or leaves, and suspend silk capture nets from the retreat to gather food from the water stream. Spicipalpia use their silk only to spin underwater cocoons just before pupation.

Caddisflies (Trichoptera) are a sister order of silk spinning terrestrial moths and butterflies (Lepidoptera), having diverged from a common silk-spinning ancestor about 250 million years ago (Wiggins, 2004). The common ancestry is reflected in physiological and structural homologies in the silk spinning machinery and silk fibers. In both orders, the fibers are drawn through labial spinnerets from a pair of large silk storage glands (Sehnal and Sutherland, 2008). The major structural components of the silk fibers are H- and L-fibroins (Yonemura et al., 2009). Although there is little primary sequence homology between the H-fibroins of aquatic

*Abbreviations:* Pxt, peroxinectin; SOD3, superoxide dismutase 3; eROS, environmental reactive oxygen species; H-fibroin, heavy chain fibroin; L-fibroin, light chain fibroin; $H_2O_2$, hydrogen peroxide; DAB, 3,3′-Diaminobenzidine; PTFE, polytetrafluoroethylene; FT-ICR, Fourier transform ion cyclotron resonance; MS/MS, tandem mass spectrometry; HCl, hydrochloric acid; HPLC, high performance liquid phase chromatography; UV, ultra violet; PCR, polymerase chain reaction; RNA, ribonucleic acid; cDNA, complementary deoxyribonucleic acid; NCBI, national center for biotechnology information; RADAR, rapid automatic detection and alignment of repeats in protein sequences; SEM, scanning electron microscopy; TEM, transmission electron microscopy; BSA, bovine serum albumin; PBS, phosphate saline buffer; Cu/Z, copper/zinc.

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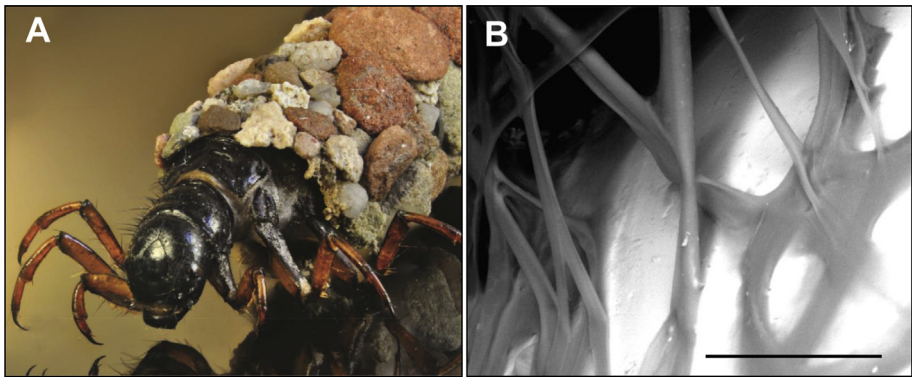


**Fig. 1.** A.) Casemaker *Hesperophylax occidentalis* fifth instar larvae within its natural stone case. B.) SEM of silk fiber ties adhered to the inside surface of a case particle. Scale bar = 50 μm.

caddisflies and terrestrial moths, there are several structural homologies. Both are large proteins with short non-repetitive termini flanking long and highly repetitive central core regions. Terrestrial silkworm silk H-fibroin has a hierarchical structure of nanocrystalline H-bonded β-domains, which provide strength, embedded in an extensible matrix (Keten et al., 2010; Marsh et al., 1955; Omenetto and Kaplan, 2010). In caddisfly H-fibroin, repeating $(pSX)_n$ motifs, where pS is phosphoserine, X is usually aliphatic, and $n = 2–6$, may form analogous H-bonded β-domains with similar structural and mechanical roles (Ashton et al., 2011; Stewart and Wang, 2010). The $(pSX)_n$ motifs comprise about 30% of the H-fibroin sequence. Ionic $Ca^{2+}$/phosphate interactions stabilize the H-bonded $(pSX)_n$ β-domains in the aqueous niche, and are responsible for the stiffness, tensile strength, and energy-dissipating qualities of the fibers (Ashton et al., 2013a; Strzelecki et al., 2011). Likewise, the primary sequence homology between silkworm and caddisfly L-fibroins is low, <25% overall identity (Yonemura et al., 2009). However, Yonemura and colleagues pointed out that the number and position of cysteine residues responsible for disulfide crossing of H- and L-fibroins in silkworm silk are conserved in caddisfly H- and L-fibroin, which suggested caddisfly H- and L-fibroin is also likely crosslinked through disulfides (Yonemura et al., 2009).

Caddisfly silk fibers are extremely resistant to solubilization with detergents, protein denaturants, reductants, metal chelators, high temperature, proteolytic enzymes, and combinations thereof (unpublished observations; Engster, 1976a; Wang et al., 2010). The extreme insolubility, as well as reddening of the fibers over time, suggested caddisfly silk may be stabilized by covalent crosslinks in addition to fibroin disulfide bonds. To identify enzymes that may be responsible for post-draw covalent crosslinking of caddisfly silk fibers, as well as other unidentified fiber components, the previously reported *Hysperophylax occidentalis* silk gland *de novo* transcriptome (Ashton et al., 2013) was searched with tryptic peptide sequences identified by tandem mass spectrometry of silk gland proteins. The presence and distribution of newly identified silk gland protein components in drawn silk fibers were confirmed by *in situ* enzyme activity or immunolocalization with synthetic peptide antibodies.

## 2. Material and methods

### 2.1. Animal preparation

Fifth instar larvae of the casemaker caddisfly *H. occidentalis* were collected locally from Red Butte Creek (Salt Lake County, Utah, USA) and maintained in an aerated lab aquarium in de-chlorinated tap water at 11 °C. Captive larvae were fed apples, potatoes, or fish food pellets.

### 2.2. Silk harvesting

Native portable caddisfly cases were carefully removed from larvae with fine forceps. Each larva was kept separately in a clean glass vial containing ~1 $mm^3$ polytetrafluoroethylene (PTFE) blocks cut from a 1 mm thick PTFE sheet. After 12 h, cases rebuilt with PTFE were collected, silk fibers were isolated and cleaned of debris with a fine forceps, then lyophilized.

### 2.3. Silk tandem mass spectrometry

#### 2.3.1. Silk gland proteins

Dissected intact silk glands were cut open and stored silk proteins were drained into an eppendorf containing 25 mM ammonium bicarbonate on ice. The proteins were denatured at 95 °C for 10 min, then immediately chilled on ice for 5 min. The protein was digested with a 1:25 ratio of trypsin to silk protein for 2 h at 37 °C. Peptides were introduced into the spectrometer by nanoLC (Eksigent, Inc.) using a C18 nanobore column and nanoelectrospray ionization (ThermoElectron Corp). Peptides were eluted with a 50 min linear gradient of 5–60% acetonitrile with 0.1% formic acid. Primary peptide molecular masses were determined by FT-ICR and peptide sequences by collision-induced dissociation in the linear ion trap of the LTQ-FT hybrid mass spectrometer.

#### 2.3.2. Silk fibers

Ten mg of silk fibers were hydrolyzed in 6 N HCl at 95 °C for 16 h. Hydrolyzed sample was filtered with 0.22 μm filter before HPLC. The samples were fractionated on a C18 reverse phase column. Fractions that absorbed at 280 nm, expected to contain dityrosine, were collected and analyzed with LC/MS/MS using a LTQ-FT hybrid mass spectrometer (ThermoElectron Corp). A peak with the expected mass of 3,3 dityrosine was identified with Xcalibur (Thermo Scientific).

### 2.4. Identification of silk protein transcripts

The *H. occidentalis* silk gland transcriptome *de novo* assembly and partial assembly of the H-fibroin transcript was described previously (Ashton et al., 2013). In further analysis, homologs of previously reported caddisfly genes from other species were identified in the translated version of the transcriptome database