Peroxidase-catalysed interfacial adhesion of aquatic caddisworm silk

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Casemaker caddisfly (Hesperophylax occidentalis) larvae use adhesive silk fibres to construct protective shelters under water. The silk comprises a distinct peripheral coating on a viscoelastic fibre core. Caddisworm silk peroxinectin (csPxt), a haem-peroxidase, was shown to be glycosylated by lectin affinity chromatography and tandem mass spectrometry. Using high-resolution H₂O₂ and peroxidase-dependent silver ion reduction and nanoparticle deposition, imaged by electron microscopy, csPxt activity was shown to be localized in the peripheral layer of drawn silk fibres. CsPxt catalyses dityrosine cross-linking within the adhesive peripheral layer post-draw, initiated perhaps by H₂O₂ generated by a silk gland-specific superoxide dismutase 3 (csSOD3) from environmental reactive oxygen species present in natural water. CsSOD3 was also shown to be a glycoprotein and is likely localized in the peripheral layer. Using a synthetic fluorescent phenolic copolymer and confocal microscopy, it was shown that csPxt catalyses oxidative cross-linking to external polyphenolic compounds capable of diffusive interpenetration into the fuzzy peripheral coating, including humic acid, a natural surface-active polyphenol. The results provide evidence of enzyme-mediated covalent cross-linking of a natural bioadhesive to polyphenol conditioned interfaces as a mechanism of permanent adhesion underwater.

1. Introduction

Silk is a generic term used to describe protein fibres produced by animals within several arthropod clades, terrestrial and aquatic. Silks originated independently numerous times within Arthropoda, in different gland types, but converged on several common features that functionally define silk [1]. Silks are tough and lightweight structural fibres spun externally into fabrics to create protective shelters, nest linings, egg cases, larval cocoons or prey capturing nets. The silk fibre precursors are stored as concentrated, structured, aqueous fluids in silk glands. The major structural silk proteins by molecular mass and total mass, H-fibroins, have repetitive low complexity primary sequences that form repeating secondary structural motifs that give the fibres strength and extensibility. As the protein fluid is drawn through narrow ‘spigots’ or ‘spinnerets’, shear-induced molecular reconfiguration of fibroins may assist in converting the structured fluids into insoluble fibres [2,3]. Another common feature of silks is adhesive coatings. The silk produced by domesticated silkworms, as a familiar example, is coated by a heterogeneous mixture of glue-like sericin glycoproteins that adhere paired filaments into single fibres, nascent cocoons to branches and silk fibres to one another in the cocoon [4,5]. Orb weavers spiders produce adhesive piriform silk to secure fibre junctions and to anchor the web framework to surfaces [6]. Capture spiral silk is coated with sticky viscoelastic micro-beads, comprising hygroscopic salts and glycoproteins that retain captured prey [7–9]. The silk fibres and adhesive coatings of aquatic silk-spinning arthropods have many of the same characteristics and are used for similar purposes as terrestrial silks, but must be tough and adhesive while fully submerged in water. Biochemical, structural and mechanical comparison of terrestrial and aquatic silks, diverged from a common ancestral silk, provides an opportunity to identify molecular
features resulting from the evolutionary adaptation of silk to underwater environments.

As a case in point, aquatic caddisflies (Trichoptera) are a sister-order of terrestrial moths and butterflies (Lepidoptera) that diverged from a common silk-spinning ancestor approximately 250 Ma. The majority of their life cycle is spent in larval stages feeding under water. Larvae (caddisworms) in the casemaker sub-order Integripalpia are mobile foragers that meticulously armour and camouflage their soft abdomens using adhesive silk to tape together carefully selected stones, cut-to-size sticks or leaves [10]. Caddisworms of the retreat building sub-order Annullipalpia construct stationary composite shelters with found materials, and suspend silk nets in the stream flow to capture food. The nano-fibrillar core of caddisworm silk is coated with a fuzzy adhesive layer that fuses paired fibres, emanating from paired silk glands, into a single ribbon and bonds the silk to the case or retreat construct [11,12]. The tough fibre backing displays energy-dissipating, nonlinear viscoelasticity and spontaneous complete recovery from tensile deformation [13]. Part of the molecular adaptation of caddisworm silk to underwater environments, the fibre toughening mechanism, was extensive phosphorylation of repeating serine-rich motifs in H-fibroin, the major structural protein of the silk core [14–16]. Calcium ion-cross-linked phosphoserine β-domains may sequentially and reversibly unfold during strain, manifesting as yield behaviour and a force plateau. The force plateau limits the stress on the adhesive interface between fibre and substrate [13–15,17]. Spontaneous recovery of the fibre’s initial stiffness and dimensions allows repeated dissipation of strain energy to protect the adhesive bonds in the high-energy caddisworm environment.

As glue that adheres fibres to substrate and to one another, the adhesive coating on caddisworm silk is functionally homologous to the sericin coating on silkworm silk. Similar to sericins, which are added to the silk dope in the middle silkworm gland [4], the constituents of the fluid caddisworm silk dope are spatially segregated to the peripheral layer and fibre core within the silk gland; a peripheral layer is apparent along the entire length of the silk gland lumen [18]. The silkworm sericin coating and the caddisworm peripheral coating are not molecularly homologous, however. Whereas the sericin coating of silkworm silk can be removed with hot slightly alkaline water, the peripheral coating on caddisworm silk is difficult to separate from the fibre core with even harsh chemical treatments [11]. Furthermore, homologues of silkworm sericins were not identified in a deep RNA-seq de novo transcriptome of caddisworm silk glands [19]. On the other hand, the caddisworm peripheral coating contains negatively charged glycoproteins that likely contribute to underwater adhesion [18,20]. The peripheral layer also contains a peroxidase enzyme in the peroxinectin (Pxt) family that catalyses peripheral layer localized dittryrosine cross-linking after the silk is drawn out of the anterior conducting channel [19]. A silk gland-specific superoxide dismutase 3 (SOD3) with a secretion signal peptide may generate the hydrogen peroxide (H2O2) substrate of peroxinectin within the peripheral layer from reactive oxygen species (ROS) present in natural waters [19]. The dittryrosine cross-links may covalently link the coating to fibre core, and stabilize both against the solubilizing power of liquid water.

Here, we present evidence that caddisworm silk peroxinectin (csPxt) in the peripheral layer can catalyse covalent cross-linking of silk to external polyphenolic compounds, including humic acid, an abundant, surface-active polymer in natural waters. Peroxidase-catalysed covalent cross-linking to conditioned surfaces may be another molecular adaptation of caddisworm silk to underwater adhesion.

2. Material and methods

### 2.1. Animal collection and maintenance

Fifth instar caddisfly larvae were collected from Red Butte Creek, Salt Lake county, UT, USA. Larvae were maintained in the laboratory in aerated, dechlorinated tap water at 13–15 °C, and fed their natural diet of vegetative detritus gathered from the creek.

### 2.2. Silk collection

Individual caddisfly larvae were removed from their natural case and placed in glass containers with 0.3–0.5 mm3 polytetrafluoroethylene (PTFE) blocks at the bottom of an aquarium. The larvae, in a frustrated effort to reconstruct their case, apply silk onto the PTFE blocks. The silk fibres adhere weakly to PTFE and can be recovered with a fine forceps. The harvested fibres were used immediately or stored in deionized (DI) water at 4 °C.

### 2.3. Gel electrophoresis

To collect silk gland proteins, dissected intact caddisworm silk glands were cut and the contents were drained into distilled water. Gland proteins were transferred into a clean vial containing sodium dodecylsulfate sample buffer and boiled for 7 min to denature silk gland proteins. Protein samples were separated by 4–20% gradient sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with coomassie blue for general proteins, with a periodic acid-Schiff (PAS) kit (Life Technologies, Inc.) for glycoproteins, and ProQ Diamond for phosphoproteins (Life Technologies, Inc.). To isolate glycoproteins, the silk gland contents were passed through a concanavalin A column according to the manufacturer’s instructions (Life Technologies, Inc.). Proteins in the eluted fractions were separated by SDS-PAGE, then stained with PAS.

### 2.4. Tandem mass spectrometry

Glycoprotein bands on SDS-PAGE were excised with clean razor blades and the gel blocks were rinsed several times with ultra-pure water. ConA purified protein fractions were precipitated with a commercial reagent (Calbiochem, Inc.), then vacuum-dried. Excised gel blocks were reconstituted in 25 mM (NH4)HCO3 in 50% acetonitrile, washed with 100% acetonitrile, then vacuum-dried. Protein reduction was carried out in 25 mM dithiothreitol 25 mM (NH4)HCO3 at 56 °C for 20 min. The proteins were then alkylated in 55 mM iodoacetamide 25 mM (NH4)HCO3 at room temperature for 20 min. Gels were washed with 100% acetonitrile and vacuum-dried. The gels were then digested at 1:25 ratio of trypsin to silk protein in 25 mM (NH4)HCO3, for 2 h at 37 °C. Peptides were introduced into the spectrometer by nanoLC (Eksigent, Inc.) using a C18 nanobore column and nanoelectrospray ionization. Peptides were eluted with a linear gradient of 5–60% acetonitrile and 0.1% formic acid. Primary peptide molecular masses were determined by Fourier transform ion cyclotron resonance and peptide sequences by collision-induced dissociation in the linear ion trap of an LTQ-FIT hybrid mass spectrometer (ThermoElectron, Inc.). Peptides were identified using the Mascot search engine (v. 2.2.1, Matrix Science) against the *Hyllisia consimilis* transcriptome database. Mascot thresholds were set as primary mass errors of less than 3 ppm, MS/MS ion scores greater than 20, and expect values less than 1.
2.5. Glycoprotein staining
Silk fibres were embedded in Immuno-bed resin (Polysciences, Inc.) for sectioning according to the manufacturer’s instructions. Briefly, fibres were dehydrated in serial alcohol solutions (50, 70 and 100%) for 2 h each, then infiltrated with Immuno-bed resin for 2 h, repeated three times. After polymerization the blocks were cut with a rotary microtome (Leica Biosystems, Inc.). Sectioned fibres were stained for glycoproteins with a PAS kit (Life Technologies, Inc.) according to manufacturer’s instructions. After washing with 7% acetic acid and rinsing with DI water, the stained sections were mounted on microscope slides and imaged by brightfield microscopy using a 100× objective (Zeiss, Inc.).

2.6. CsPxt localization by silver deposition
The commercial EnzMet™ HRP detection kit (Nanoprobes, Inc.), based on peroxidase enzyme-mediated Ag⁺ ion reduction to metallic silver and deposition of insoluble metallic silver nanoparticles, was used for high-resolution localization of csPxt in silk fibres following the manufacturer’s instructions. The kit components are proprietary. Silk fibres were treated with 60 μl of solution A for 2 min, then 20 μl of solution B was added to solution A and mixed for 2 min at room temperature, and then 20 μl of solution C containing H₂O₂ was added for Ag deposition. Reactions were stopped by washing five times with distilled water. To irreversibly inhibit peroxidase enzymes, fibres were treated with 0.015 wt% sodium azide for 15 min, then 20 μl of solution A, then 20 μl of solution B, then 20 μl of solution C containing H₂O₂, then 20 μl of solution D, and then 20 μl of solution E. After labelling, the sections were rinsed with DI water, lyophilized and analysed by transmission electron microscopy (SEM; 100 kV) using a backscattered secondary electron detector.

2.7. Scanning electron microscopy
After silver deposition, fibres were rinsed with DI water, lyophilized, mounted on carbon tape and examined with a FEI Quanta 200 SEM in low vacuum mode at the University of Utah Surface and Nanoimaging Facility using a secondary electron detector. To examine fibres in cross section, fibres were embedded in Immuno-bed (Polysciences, Inc.) and cut into 5 μm sections. The sections were rehydrated in DI water, and labelled with the EnzMet™ HRP detection kit. Some sections were pre-treated with 0.015% sodium azide for 15 min to inactivate peroxidases. After labelling, the sections were rinsed with DI water, lyophilized and analysed by scanning electron microscopy (SEM; 15 kV) using a backscattered secondary electron detector.

2.8. Transmission electron microscopy
Native silk fibres were incubated with the EnzMet™ HRP detection kit with and without H₂O₂ (solution C). The fibres were then fixed overnight in 2% OsO₄ in cacodylate buffer, then rinsed in DI H₂O. The samples were stained with 4% uranyl acetate, dehydrated with serial ethanol solutions, washed with acetone, then embedded in epoxy Embed-812 (Electron Microscopy Sciences, Inc.) following the manufacturer’s instructions. Polymerized blocks were cut into approximately 70 nm sections with a diamond Ultra 45° knife (Diatome, Inc.). Sections were mounted on copper grids and stained with saturated uranyl acetate, rinsed with water, then stained with Reynold’s lead citrate. The sections were imaged by transmission electron microscopy (TEM) in a Tecnai 12 (FEI, Inc.) microscope.

2.9. Phenolic polymer synthesis
A copolymer containing phenol sidechains, with a target molecular mass of 40 kg mol⁻¹, was synthesized by reversible addition fragmentation chain transfer copolymerization of hydroxyethyl methacrylamide (HEMA), methacrylic acid (MAA), methacryloyl-thiocarbamoyl-rhodamine (RhoMA), 35 mg of the transfer agent 2-(butylthiocarbonothioylthio) propanoic acid and 6.8 mg of azoisobutyronitrile in 35 ml of dimethylformamide. The polymerization reaction proceeded in the dark at 55°C for 15 h, after which the copolymer was dialysed extensively against DI H₂O, then lyophilized. The final sidechain molar ratios were determined by ³¹P NMR (figure 1). Phenolic sidechains were conjugated to 1.0 g of the HEMA-co-MAA-co-RhoMA copolymer by addition of 253 mg N-ethyl-N-(3-dimethylaminopropyl)carbodiimide (EDC) hydrochloride and 150 mg of tyrannine in DI water. The reaction proceeded for 15 h at 22°C. The phenol-conjugated polymer was isolated by dialysis against water for 3 days, then lyophilized. The final concentration of phenol sidechains was 5 mol% as determined by ³¹P NMR (figure 1).

2.10. CsPxt-catalysed polyphenol cross-linking
The rhodamine-labelled polymer with 5 mol% phenol sidechains was dissolved in phosphate buffered saline (PBS; pH 7.4) at 1 mg ml⁻¹. One mg of silk fibres were incubated in the phenolic polymer at room temperature for 1 h in the dark in each of the following conditions: with 10 μM H₂O₂, without H₂O₂, with 100 μM sodium azide plus 10 μM H₂O₂, and 100 μM L-tyrosine plus 10 μM H₂O₂. After incubation, silk fibres were washed six times for 5 min each with PBS (pH 7.4) to remove unbound polymer. Each treated batch of silk fibres was divided into two groups. The first group was mounted on microscope slides and the surface of whole fibres was imaged by laser scanning confocal microscopy (LSCM) using a 60× objective on an Olympus FX2000 microscope. The second group was embedded in Immuno-bed resin (Polysciences, Inc.) following the manufacturer’s instructions, then cut into 5 μm sections on a Leica rotary microtome (Leica Biosystems, Inc.). Cross-sectioned silk fibres were imaged by LSCM.

To examine diffusion of the rhodamine-labelled phenolic polymer into the peripheral layer, phenolic polymer was incubated with
silk fibres without 10 µm H₂O₂ for 1 h, then embedded without extensively washing (electronic supplementary material, figure S2). Polymerized silk fibres were sectioned to 5 µm and imaged by LSCM. The same microscope laser and detector settings were used for imaging all samples. Dityrosine fluorescence was imaged with 325 nm excitation and 340 to 500 nm emission. Rhodamine-labelled phenolic polymer was imaged with 540 nm excitation and 570 nm emission. Humic acid was imaged with 490 nm excitation and 518 nm emission.

3. Results

3.1. Components of the peripheral coating
Silk fibres were embedded, sectioned and treated with PAS carbohydrate stain to localize glycosylated components. Consistent with Engster’s earlier work [11], the peripheral coating on the silk fibres stained strongly compared with the fibre core (figure 2). The weak staining in the fibre core suggested there may be a low concentration of carbohydrate components in the core.

To identify silk glycoproteins, the contents of the silk gland were isolated by rupturing dissected silk glands and allowing the fluid contents to drain into a test tube. The contents were then separated by SDS-PAGE and labelled with PAS (figure 3). The two prominent glycoprotein bands running at approximately 95 and 110 kDa were separately excised from the gel and digested with trypsin in gel. The resulting tryptic peptides were analysed by tandem mass spectrometry. The top band was identified as a PEVK-like protein, the sequence and silk gland-specific expression of which was previously reported [19]. Eighteen unique tryptic peptides covering 37% of the protein sequences were identified in the MS/MS spectrum (electronic supplementary material, figure S1). The lower band was identified as the previously reported csPxt enzyme that catalyses tyrosine cross-linking in the peripheral layer [19]. Twenty-two unique peptides covering 32% of the protein sequences were identified in the gel band (electronic supplementary material, figure S1). Both proteins ran at substantially higher molecular mass on SDS-PAGE than predicted from their sequences, 56 kDa and 75 kDa, respectively.

As a second approach to identify silk glycoproteins, the fluid silk gland contents were fractionated on a column of immobilized concanavalin A lectin, which specifically binds α-D-mannosyl and α-D-glucosyl residues. Lectin bound proteins were eluted, trypsin digested and analysed by tandem mass spectrometry. Peptides corresponding to both the PEVK-like protein and csPxt were identified. In addition, 13 unique peptides covering 59% of the sequence of the previously reported caddisworm silk SOD3 (csSOD3) enzyme were identified (electronic supplementary material, figure S1). Staining with phosphate-specific ProQ Diamond showed that H-fibroin, which was too large to enter the running gel, is the only phosphorylated protein in caddisworm silk (figure 3, lane 3).

3.2. Localization of csPxt activity to the peripheral adhesive coating
Whole silk fibres treated with Ag⁺ ions in the presence of H₂O₂ using a commercial peroxidase localization kit had silver nanoparticles uniformly distributed on their surface when observed by SEM (figure 4a). Pre-treatment of the fibres with...
Figure 4. CsPxt localization by silver deposition. (a) SEM image of whole fibres with Ag⁺ and H₂O₂. (b) Whole fibres pre-treated with sodium azide. (c) SEM image of a thin silk fibre cross section treated with Ag⁺ and H₂O₂. (d) Thin cross section pre-treated with sodium azide before Ag⁺ and H₂O₂.

Figure 5. Fibre ultrastructure and csPxt localization in the fibre peripheral layer. (a) TEM image of native fibre cross section. (b) Boxed region in (a). (c) Longitudinal section of fibres treated with Ag⁺ in the presence of H₂O₂. Electron dense metallic Ag nanoparticles are indicated by arrowhead. (d) Longitudinal section without H₂O₂. PL, peripheral layer; CO, fibre core.
sodium azide, an irreversible inhibitor of haem-peroxidases, greatly reduced silver nanoparticle formation (figure 4b), consistent with csPxt-catalysed reduction of Ag$^{+}$ to elemental silver on the fibre coating. To investigate the distribution of csPxt between peripheral coating and fibre core, silk fibres were embedded, thin-sectioned, then treated with Ag$^{+}$ ions and H$_2$O$_2$, with and without sodium azide. A dense crust of silver particles formed only within the peripheral layer of the fibres, which suggested csPxt was located exclusively in the adhesive coating (figure 4c). Silver deposition was substantially reduced in silk fibres pre-treated with sodium azide (figure 4d).

The ultrastructure of the adhesive layer and high-resolution csPxt-catalysed silver particle deposition were examined by TEM. As previously reported by Engster [18], the fibres are coated with a distinct fuzzy layer of material ranging in thickness from 0.5 to 1 $\mu$m (figure 5a). The density of the coating was considerably higher near the electron dense interface with the fibre core and between the fibres (figure 5b). The density decreased toward the outer surface. In the presence of H$_2$O$_2$, silver nanoparticles accumulated within the peripheral layer (figure 5c), but not in its absence (figure 5d). The adhesive layer collapsed somewhat in the acidic conditions (0.1 M citrate buffer, pH 3.8) of the silver reduction assay. A matrix with grainy electron density similar to the peripheral layer was dispersed between the nanofibrils of the core region in all of the TEM images.

### 3.3. CsPxt catalyses covalent cross-linking of phenolic compounds to the peripheral layer

A water-soluble fluorescent polymethacrylate with phenolic and rhodamine sidechains was synthesized as shown in figure 1. To investigate whether csPxt embedded in the diffuse peripheral layer can cross-link external polyphenolic compounds, freshly gathered native silk fibres were incubated in 1 mg ml$^{-1}$ of the synthetic polyphenol for 1 h at 22 $^\circ$C with 10 $\mu$M H$_2$O$_2$, and as a control, without H$_2$O$_2$. After washing with water to remove unbound polymer, a portion of the fibres was embedded and thin-sectioned. Examination by LSCM revealed strong labelling with the fluorescent polyphenol in the presence, but not in the absence of H$_2$O$_2$ (figure 6a–d). Cross-sectioned fibres were labelled with the fluorescent polymer only in the peripheral layer in the presence of H$_2$O$_2$, but not in its absence (figure 6e–h). When the red fluorescence of the synthetic polyphenol (figure 6f) was overlaid on the blue fluorescence of dityrosine (figure 6i), it was apparent the synthetic polymer partially penetrated the peripheral layer; an innermost blue zone was separated from an outermost red zone by an interstitial magenta zone (figure 6j,k). The 40 kDa polymer also partially penetrated into the peripheral fibre layer. The extent of diffusion of the polyphenol into the peripheral fibre layer was shown by omitting the washing step to remove unbound polymer (electronic supplementary material, figure S2).
As further evidence that immobilization of the polyphenol in the peripheral layer was catalysed by csPxt, fibres were incubated with the synthetic polyphenol in the presence of H$_2$O$_2$ and an irreversible peroxidase inhibitor, sodium azide, or a competitive substrate, L-tyrosine (figure 7). In both cases, labelling of the fibre was greatly inhibited. Only a faint trace of rhodamine fluorescence is visible in the presence of L-tyrosine (figure 7).

In natural waters, submerged surfaces are fouled with surface-active polyphenolic macromolecules, such as humic acid, that originate from the breakdown of plant matter [21]. To investigate whether csPxt in the peripheral silk fibre layer can act on phenolic compounds present on surfaces in the natural environment of caddisfly larvae, silk fibres were incubated in a solution of humic acid with and without H$_2$O$_2$, the image by fluorescent microscopy as described for the synthetic polyphenol. The strong green fluorescence of humic acid was localized to the peripheral layer in the presence of H$_2$O$_2$, but in its absence (figure 8a–h). Although humic acid, with molecular mass ranging from 1.5 to 3 kg mol$^{-1}$, is much smaller than the synthetic polyphenol, it appeared to penetrate the peripheral layer less well; the cyan zone between the green humic acid and blue dityrosine zones appeared to be less extensive and the boundaries more distinct than the overlap zone of the phenolic polymer (figure 8j–l). The peroxidase inhibitors, sodium azide and L-tyrosine, dramatically reduced the amount of humic acid covalently bound into the peripheral layer (electronic supplementary material, figure S3).

4. Discussion

A model of caddisworm silk interfacial adhesion is presented in figure 9. Initial adhesion at the interface with substrate is contributed by the peripheral glycoprotein layer [18]. Silver ions (figures 4 and 5) and large polymers (figures 6 and 8) can penetrate well into the fuzzy coating, which suggests the loose peripheral layer can contribute to adhesion through polymer–polymer interpenetration, chain entanglement and physical cross-linking with surface adsorbed macromolecules. The acidic, negatively charged carbohydrates in the peripheral layer can contribute chemically to interfacial bonding through electrostatic interactions, H-bonding through sugar –OH groups, and ligand exchange with surface associated metal complexes [20]. In support of the adhesive role of glycoprotein layer, the suitability of polysaccharides as wet adhesion promoters and as adhesive structural components is apparent from their ubiquitous occurrence in natural underwater adhesives. Some comparatively well-described examples include bacterial biofilms [22], the surface attachment adhesives of red alga spores [23, 24], brown alga zygoles [25–27] and gliding diatoms [28, 29], the adhesive on cunvierian tubules of sea cucumbers [30], the temporary adhesives of sea stars [31, 32] and marine cephalopods [33], sulfated polysaccharides in sandcastle worm adhesive [34] and carbohydrates in gooseneck barnacle adhesive [35].

After initial adhesion of the silk through the glycoprotein layer, we propose that csPxt, itself a glycoprotein embedded in the peripheral layer (figure 3, S1), catalyses permanent interfacial adhesion by cross-linking tyrosine residues in the peripheral layer with phenolic groups adsorbed onto submerged surfaces, such as humic acid (figure 9). The csPxt also catalyses dityrosine cross-linking within the peripheral layer, which may strengthen and stabilize the fibres (figure 9, blue ring) [19]. The H$_2$O$_2$ substrate of csPxt may be generated by a proximate SOD3 from environmental ROS, which are
present at steady-state picomolar concentrations in natural waters [36,37]. CsSOD3 is specifically expressed in the silk glands [19] and is a constituent of the silk gland proteins as shown by tandem mass spectrometry (electronic supplementary material, figure S1), although its activity is yet to be directly demonstrated in silk fibres. Of the known constituents of the silk fibres, csPxt is itself the best candidate as its own phenolic substrate within the silk fibres. CsPxt contains

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\text{dityrosine cross-links} \quad \text{peripheral adhesive layer}
\]

Figure 8. CsPxt-catalysed cross-linking of humic acid into the silk peripheral layer. (a–d) Differential interference contrast (DIC) and fluorescent images of silk fibres incubated with humic acid with and without \( \text{H}_2\text{O}_2 \). (e–h) DIC and fluorescent images of cross-sectioned silk fibres incubated with humic acid with and without \( \text{H}_2\text{O}_2 \). (i) Humic acid autofluorescence of silk fibre in (e) and (f). (j) Overlay of humic acid fluorescence, (f), and dityrosine autofluorescence, (i). (k–l) Higher magnification of boxed regions in panel (j). All scale bars represent 5 \( \mu \text{m} \), except were indicated otherwise.

Figure 9. Schematic of csPxt-catalysed underwater interfacial adhesion. CsPxt embedded in the peripheral adhesive fibre coating catalyses covalent cross-linking between tyrosine sidechains in the peripheral coating and between tyrosine sidechains and natural polyphenolic primers on submerged surfaces. The csPxt \( \text{H}_2\text{O}_2 \) substrate may be generated, wholly or in part, by proximate csSOD3 acting on environmental ROS.

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\text{ROS} = \text{reactive oxygen species} \\
\text{Pxt} = \text{peroxinectin} \\
\text{SOD} = \text{superoxide dismutase} \\
\text{PXP} = \text{phenolic cross-link}
\]
24 tyrosine residues in total, five of which are in the approximately 150 amino acid N-terminal region outside of the peroxidase domain. Another candidate substrate of csPxt is H-fibroin, which contains repeating GGYGGL motifs. Hence, csPxt may self-cross-link to become an enzymatically active structural component of the fibres, exposed at the surface to catalyse interfacial adhesion, and also cross-link itself to H-fibroin to covalently attach the peripheral coating to the fibre core (figure 9).

Experimental evidence of enzyme-mediated covalent cross-linking in natural adhesives is rare in the literature. As one example, the separate parts of the multi-part polyampholytic adhesive of sandcastle worms, marine polychaetes in the Sabelarlidae family, are each encapsulated in secretory granules along with a high concentration of catechol oxidase that becomes an active structural component of the adhesive [34,38]. Copackaging, storage, and delivery of the pro-enzyme with the other adhesive components guarantees spatially homogeneous and rapid oxidative cross-linking of the glue through dopa sidechains, and requires minimal mixing of the pre-organized, pre-assembled adhesive packets. The robust sandcastle worm catechol oxidase, like csPxt in caddi worm silk in the peripheral layer, remains active in the fully cured adhesive. Although the sandcastle worm catechol oxidase is responsible for cohesive cross-linking, it could conceivably also catalyse cross-linking to surfaces primed with polyphenolics. Another example of adhesive enzymes are vanadium haloperoxidases, co-secreted with polyphenolic phloroglucinols and anionic polysaccharides by brown alga zygotes to adhere to surfaces after settlement [27,39]. Haloperoxidases catalyse two electron oxidation of halides (Cl–, Br–, I–) using H2O2 to produce hypohalites [27,39]. The paucity of evidence for enzyme-catalysed natural adhesive mechanisms is odd considering the myriad examples of enzyme-catalysed oxidative cross-linking of extracellular structural materials. Some examples are peroxidase-catalysed dityrosine cross-linking of sea urchin fertilization membranes of animals ranging from primitive metazoans to mammals [49–51], phenoloxidase catalysed cross-linking of arthropod cuticles [52,53], and transglutaminase catalysed cross-linking of fibrinogen to blood clotting [54]. The suggestion that adult barnacle adhesive may be cross-linked by a transglutaminase [55] was met with strong scepticism [35,56]. The few reported examples of enzyme-catalysed underwater adhesion or de-adhesion may be due to limited attention rather than to being rare in nature. The de-adhesive of the sea star duo-gland system may be an enzyme [57], but it has yet to be identified. Catechol oxidase activity was identified in mussel byssus [58] and may catalyse phenolic cross-linking through dopa residues, but its distribution and activity during byssus formation have not been investigated. Instead, the focus in the mussel byssus has been on non-enzymatic cross-linking mechanisms [59,60]. The lack of attention may be due in part to notions that large enzymes cannot diffuse through viscous adhesive agents and, therefore, would be inefficient cross-linking agents, or that a high concentration of enzymes would structurally weaken the adhesive as enzymes are not commonly thought of as structural components.

5. Conclusion

Casemaker caddi worm silk is a pressure-sensitive adhesive tape with an energy-dissipating viscoelastic core used to construct protective structures with building materials gathered in the caddi worm’s natural water environment. Without surface preparation, the silk adheres to wet organic and inorganic materials. The fuzzy adhesive coating contains an active peroxidase enzyme that catalyses dityrosine cross-linking at the boundary of the peripheral layer and fibre core to strengthen and stabilize the fibres, and to immobilize itself in the peripheral layer as a structural component of the silk. The robust peroxidase enzyme is disposed in the fibre coating in a way that allows covalent coupling between exterior polyphenolic compounds known to coat surfaces in natural water and the silk fibre. Enzyme-mediated covalent cross-linking may be an underappreciated interfacial adhesion mechanism in natural underwater adhesives. Further characterization of the biochemistry, assembly, structure, mechanical properties and adhesive mechanisms of the sticky underwater silk may provide unique insights to inspire the design of soft biphasic synthetic materials similar to the fully wet, biphasic materials that make up the tissues of our bodies [61].

Competing interests. The authors declare there are no conflicts of interest, financial or otherwise.

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