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Metals and the Integrity of a Biological Coating:

The Cuticle of Mussel Byssus

Niels Holten-Andersen^{†,‡}, Thomas E. Mates[§], Muhammet S. Toprak^{||}, Galen D. Stucky^{||}, Frank W. Zok[§], and J. Herbert Waite^{*,‡}

[‡]Biomolecular Science and Engineering Program, University of California, Santa Barbara, Santa Barbara, California 93106

[§]Materials Department, University of California, Santa Barbara, Santa Barbara, California 93106

^{||}Department of Chemistry and Biochemistry, University of California, Santa Barbara, Santa Barbara, California 93106

Abstract

The cuticle of mussel byssal threads is a robust natural coating that combines high extensibility with high stiffness and hardness. In this study, fluorescence microscopy and elemental analysis were exploited to show that the 3,4-dihydroxyphenyl-L-alanine (dopa) residues of mussel foot protein-1 colocalize with Fe and Ca distributions in the cuticle of *Mytilus galloprovincialis* mussel byssal threads. Chelated removal of Fe and Ca from the cuticle of intact threads resulted in a 50% reduction in cuticle hardness, and thin sections subjected to the same treatment showed a disruption of cuticle integrity. Dopa-metal complexes may provide significant interactions for the integrity of composite cuticles deformed under tension.

Introduction

Conventional hard coatings for protection against abrasion and wear are of little use on flexible substrates for electronics and medical applications.^{1,2} High substrate compliance leads to premature cracking of the coatings under even moderate bending and/or tensile loads.^{3–5} This deficiency is a consequence of the inherent tradeoff between the hardness and extensibility of most engineering materials. That is, an increase in hardness is offset by a reduction in the tensile breaking strain.^{6,7} We previously reported on a natural organic coating material—the protective cuticle of mussel holdfast threads—that evidently remains intact even when the threads are subjected to tensile strains up to 70%.^{8,9} Here we probe the cuticle composition with the goal of better understanding the operative strengthening mechanisms.

The mussel holdfast better known as the byssus consists of a bundle of threads evolved to enhance mussel survival in the wave-swept seashore.¹⁰ Each has a fibrous core covered by a thin protective cuticle and self-assembles from an ensemble of precursor proteins in the groove of the mussel foot in a process that resembles reaction injection molding.¹¹ The core is composed of three different collagen-like proteins with silk- and elastin-like blocks arranged in such a way as to resemble the compliance of nylon at the distal end and a rubbery elastomer at the proximal end of each thread.¹² In resisting mussel dislodgement by waves, the threads experience repeated high strain (e.g. 40% or more¹⁰). Consistent with its function to protect

the fibers, the cuticle covering the fibrous core exhibits hardness and stiffness that are about an order of magnitude greater than those of the thread interior yet remains surprisingly extensible, with breaking strains approaching 70%.⁹ Microstructural examinations revealed that cuticle extensibility is enabled in part by deformable microphase-separated granules. The granules serve to arrest the growth of matrix microcracks that form during tensile strain, thereby keeping the coating over underlying fibers of the thread core intact.⁹

Given the evident contribution of microarchitecture to the mechanical properties of the cuticle, it is also necessary to understand the interrelationship between mechanics and chemistry. We have previously discussed how the microcracks themselves could result from the breaking of reversible noncovalent bonds.⁹ The high content of 3,4-dihydroxyphenyl-L-alanine (dopa) in the cuticle protein, mussel foot protein 1 (mfp-1), coupled with its affinity for metal coordination led us to propose dopa-metal complexes as candidates of such reversible cross-links in the thread cuticle.^{8,13} In the present study, we demonstrate that dopa and metals are colocated within the cuticle of *M. galloprovincialis* mussel threads and that metal removal significantly reduces cuticle hardness and integrity.

Results

The autofluorescence of dopa ($\lambda_{\text{em}} = 400\text{--}500\text{ nm}$) has previously been used to verify its presence in biological materials.^{14–18} Consistent with the high dopa content in the cuticle protein, mfp-1 (10–20 mol%),¹⁹ we observed a strong intrinsic blue fluorescence from the cuticle of *M. galloprovincialis* threads in transverse sections (Figure 1a–c). The signal intensity was not differentiated by the characteristic composite structure of the cuticle (Figure 1d).

Elemental maps of transverse sections of the mussel threads were generated using secondary ion mass spectroscopy (SIMS) (Figure 2). They reveal that both Fe and Ca are concentrated within the cuticle whereas N appears to be more concentrated in the thread interior. Corroborating evidence was obtained from SIMS depth profiles taken on the external cuticle surface (Figure 3a) and using thermogravimetric analysis (TGA) of entire threads followed by electron desorption spectroscopy (EDS) (Figure 4 in Supporting Information). The total inorganic content of the byssal threads was found to be less than 1 wt %, with more than 90% present as Ca.

Both Ca and Fe were extracted from the thread cuticle by incubation in ethylene diamine tetraacetic acid (EDTA) (Figure 3a). The contribution of the metals to mechanical properties was investigated by comparing nanoindentational hardness values of cuticles in whole threads with and without the metals removed before sectioning. Metal chelation with EDTA from whole threads led to a 50% reduction in cuticle hardness (t-test $p < 0.001$, Figure 3b), yet the cuticle structure viewed by TEM appeared to be largely unchanged (Figure 3c). When metals were extracted from thin sections of cuticle, however, some structural disintegration was evident (Figure 3c).

Discussion

The actual dopa levels in cuticle cannot be directly determined because cuticle is not separable from the thread core. However, the fluorescence data indicate that dopa is uniformly distributed throughout the cuticle in that the signal intensity was not differentiated by the composite structure of the cuticle. On the basis of previous analyses, the distal portion of *M. galloprovincialis* threads contains an average dopa content of ~3 wt%.¹⁹ Because the cuticle represents about 10% of the total thread volume and the proteins of the thread core are reported to contain less than 0.5 wt % dopa,²⁰ the dopa content in the cuticle can be estimated to be

~25-30 wt % (assuming that cuticle and the thread core have similar densities). This estimate resembles the dopa content of pure mfp-1, which ranges from 15 to 30 wt %.¹⁹

The catecholic side chain of dopa exhibits a moderate affinity ($\log K_S \approx 7-10$) for many metal ions, but in some transition metals, the affinity is much higher (e.g., $\log K_S = 18$ for the monocatecholato-Fe^{III} complex).²¹ In this regard, Taylor et al. demonstrated that when mfp-1 or mfp-1-derived peptides were mixed with Fe^{III} at low iron to dopa ratios, tris-dopa-Fe^{III} complexes (as shown in Figure 1e) with cumulative log stabilities (i.e., $\log K_S$) of 37-40 formed.²² A conservative estimate would allow that at the pH of seawater (pH 8.2), about 80% of this affinity would be realized.²³ These complexes were later confirmed to be present in byssus by electron spin resonance.²⁴ Both EDS and the histochemical detection of Fe have established its localization to the cuticle.^{19,25} On the basis of elemental analysis and fluorescence microscopy, our results indicate that dopa has the same distribution as Fe and Ca in the cuticle of *M. galloprovincialis* mussel threads. The colocalization in situ falls short of proving a chemical interaction between dopa and the metals, but given the accumulated evidence, their interaction is beyond a reasonable doubt.

Recently, the strengths of dopa-metal complexes were measured by single-molecule tensile tests.²⁶ With a force to break of about 0.8 nN in water buffered at pH 8, these bonds are comparable to covalent bonds (~2 nN) yet are completely reversible. Noncovalent bonds have been demonstrated to increase extensibility in several types of biomimetic materials when engineered to replace covalent bonds as cross-links.^{27,28} During extension, these reversible bonds release stress buildup upon breaking and thereby prevent catastrophic material failure. Having tris-dopa-Fe³⁺ complexes (as shown in Figure 1e) as reversible cross-links for proteins in the *M. galloprovincialis* thread cuticle would be consistent with the high cuticle extensibility. Although the extent of tris-dopa-Fe³⁺ complexation in the *M. galloprovincialis* cuticle still needs to be determined, it is worth noting that compared to the cuticle of another mussel species (*Perna canaliculus*) known to contain a high density of cysteinyl-dopa cross-links the *M. galloprovincialis* thread cuticle exhibits a 300% greater extensibility with a compromise of only 30% in hardness.^{9,29}

With total inorganic content at less than 1 wt % of dry byssus (Figure 4 in Supporting Information) and all of the Fe and Ca sequestered within the cuticle (as judged by the elemental maps in Figure 2), we calculate a total metal content in the cuticle at 10 wt % or less using estimates of cuticle volume and density as above. Previous work, particularly by Taylor et al.,²² Wilker,^{24,30,31} and Messersmith,^{26,32} supports a significant albeit complex role for Fe^{III}-dopa chemistry within the cohesiveness of byssal cuticle. With Fe measured at less than 1/10 of the total inorganic content, we estimate an average molar ratio of between 3:1 and 4:1 of dopa/Fe for the cuticle after converting our weight percent measurements into molar equivalents. This estimate is stoichiometrically within the range of the tris-dopa-Fe³⁺ complex proposed by Taylor et al.²² However, given how variable Fe^{III} content is in individual byssal threads¹⁹ this conjecture, although plausible, may be premature and awaits further mechanical testing of byssal cuticle with and without Fe. Indeed, given the redox tendencies of both dopa and Fe^{III}, the outcome of mixing the two aerobically is still rather uncertain. For example, tris-dopa-Fe³⁺ complexes were reported to generate oxygen-free radicals that could lead to the formation of covalent didopa adducts.²⁴ Such cross-links were detected in byssal plaques by McDowell et al.,³³ but their existence in thread cuticle has not been shown.

Calcium, the other metal ion in cuticle, is consistently present and more abundant, but little thought has been given to its function. The catecholate portion of dopa binds Ca²⁺ with moderate stability (e.g., $\log K_S = 8$);²¹ however, in contrast to Fe^{III}, binding is usually through mono- to biscatecholato-Ca²⁺ complexation at pH 8. Moreover, Ca²⁺ significantly lowers the pK_a of the catecholic OH groups, and the dopa semiquinone is stabilized by Ca²⁺ ions.³⁴

Consistent with a cross-linking role, Ca^{2+} was observed to insolubilize films of tea tannins by catecholate bridging in the short term and by enhancing covalent cross-linking of tannins in the long term.³⁵ Alternatively, if all of the available dopa in the cuticle, as suggested above, is tied up with tris-dopa- Fe^{3+} complexes, then cuticular Ca may be largely unconnected with dopa chemistry. Fatty acids have been reported to be present in byssus,³⁶ and we have previously proposed that Ca^{2+} may interact with the polar head groups of such species.¹³ The observation that the nitrogen (N) content of the cuticle appears to be somewhat lower than the interior of the thread (Figure 2) supports the conjecture that macromolecules besides proteins are present in the cuticle.

The extraction data in conjunction with the homogeneous distribution of Fe and Ca within the cuticle supports the hypothesis that both metals are present as cross-linkers. Fe and Ca can be completely removed from the cuticle with a significant impact on its material properties as evinced by the 2-fold decrease in hardness (Figure 3a,b). Furthermore, metal removal from whole threads before sectioning has no apparent structural effect on the cuticular microstructure as viewed by TEM (Figure 3c). In contrast, treating thin thread sections with EDTA leads to detectable cuticle disintegration (Figure 3c) in agreement with the expected effect of breaking up intermolecular cross-links in a section that is only 80 nm thick. A better future assessment of the role of metal ions in the structural and mechanical integrity of thread cuticle would entail the testing of thread cuticles before and after metal removal and again following the restoration of Fe and Ca in the cuticles. Such studies were quite conclusive in the Zn- and histidine-rich proteins of *Nereis* jaws.³⁷

We have demonstrated that small amounts of metals lead to significant (2-fold) elevations in the hardness of cuticle from the mussel *M. galloprovincialis*. The results are consistent with the hypothesis that the metals form complexes with dopa that serve as effective noncovalent cross-linking agents. Lessons learned from such studies on natural coatings may guide the design and synthesis of future biomimetic coatings with desirable combinations of hardness and extensibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Scanning electron micrograph of an *M. galloprovincialis* thread sectioned perpendicular to the fiber axis to expose the fibrous core and the granular cuticle. The orientation of sections used throughout this study is indicated by the dashed line. (b) Bright-field image of the transverse cross-section of the thread. (c) Same section as in image b but viewed under 330-385 nm UV light. The image reveals blue autofluorescence of the cuticle, consistent with the presence of dopa. (d) Transmission electron microscopy image of cuticle corresponding to the area indicated in image b illustrating its granular composite nature. See Supporting Information for sample preparation. (e) Structure of the tris-dopa-Fe³⁺ complex proposed by Taylor et al.²² that may mediate cross-linking in the cuticle material.

Figure 2. Maps of Fe, Ca, N, and C distributions in a transverse cross-section of a mussel thread generated using SIMS. The maps illustrate the concentrations of Fe and Ca in the cuticle. N appears to be more concentrated in the thread interior. C is present in the thread as well as the embedding epoxy and was collected to demonstrate that the distributions of the other elements are not artifacts resulting from sample preparation. See Supporting Information for further details.

Figure 3.

(a) Depth profile of Ca and Fe generated using SIMS on threads with and without treatment with 0.2 M EDTA at pH 5.5. The inset shows a burn crater following analysis. The cuticle in the analyzed thread was $\sim 2.5 \mu\text{m}$ thick. Note the difference in the scales used for Ca and Fe signal intensities. (b) Hardness of the cuticle in whole threads as determined by the nanoindentation of native ($n = 12$) and EDTA-treated cuticles ($n = 10$). Error bars indicate the standard deviation. (c) Transmission electron micrographs of whole threads or thin sections treated with EDTA or deionized water (controls) as indicated. All are transverse cross-sections of threads with the core of the threads to the lower left, lower right, upper right, and right going

clockwise from the top left, respectively. The cuticle ultrastructure remains unchanged after EDTA treatment in whole threads. The thread cuticle in thin sections, however, falls apart upon exposure to EDTA. The inset in EDTA-treated thin section shows a sectioned granule that disconnected from the cuticle, displaying further disruption.