The Transition from Stiff to Compliant Materials in Squid Beaks

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Abstract

The beak of the Humboldt squid *Dosidicus gigas* represents one of the hardest and stiffest wholly organic materials known. As it is deeply embedded within the soft buccal envelope, the manner in which impact forces are transmitted between beak and envelope is a matter of considerable scientific interest. Here, we show that the hydrated beak exhibits a large stiffness gradient, spanning two orders of magnitude from the tip to the base. This gradient is correlated with a chemical gradient involving mixtures of chitin, water, and His-rich proteins that contain 3,4-dihydroxyphenyl-L-alanine (dopa) and undergo extensive stabilization by histidyl-dopa cross-link formation. These findings may serve as a foundation for identifying design principles for attaching mechanically mismatched materials in engineering and biological applications.

Living organisms are functional assemblages of different interconnected tissues. Not infrequently, tissues with highly disparate mechanical properties (e.g., bone and cartilage, shell and adductor muscle, nail and skin) are joined together (1). In practice, the joining of dissimilar materials can lead to high interfacial stresses and contact damage (2,3). In apparent contradiction to this, the contacts between mechanically mismatched biomolecular tissues are remarkably robust. Mechanical-property gradients are increasingly invoked as the principal reason for their mechanical performance. The dentino-enamel junction (4), arthropod exoskeleton (5), polychaete jaws, and mussel byssal threads (6) all exhibit such gradients. Optical properties in squid eyes have also been correlated to a protein-density gradient (7). Although much is known about the mechanical and biochemical properties of the separate tissues, surprisingly little has been done to explain how mixtures of macromolecules are adapted for incremental mechanical effects at interfaces.

The beak of the Humboldt squid *Dosidicus gigas* is an example of a system with grossly mismatched tissues. It is composed of slightly offset apposing upper and lower parts that make no hard pivotal contact with one another and are set into a muscular buccal mass that controls

their movement (8). The beak tip (or rostrum) is among the hardest and stiffest of wholly organic biomaterials (9). With a single notch through the thick dorsal integument of a captured fish, a squid beak can sever the nerve cord to paralyze prey for later leisurely dining (10). The predatory activity of a stiff razor-sharp beak embedded in a softer muscle mass might be compared to a person carving a roast with a bare metal blade. In both scenarios, there would be as much damage to self as to the intended objects. We found that the squid's task is facilitated by a beak design that incorporates large gradients in mechanical properties, intricately linked with local macromolecular composition, from the hard, stiff tip to the soft, compliant base.

When detached from the buccal mass, each Humboldt squid beak exhibits clearly visible gradients in pigmentation, ranging from translucent along the wing edge to black at the beak tip (Fig. 1, B and C). The pigmentation gradient appears to be closely coupled to a distribution of catechols that correspond to proteins containing 3,4-dihydroxyphenyl-L-alanine (dopa) (9). Catechol staining (red in Fig. 1D) is evident in the lightly tanned regions and more intensely (though largely obscured by black pigment) in the heavily tanned regions. Because the beaks are large enough to allow cutout specimens showing incremental differences in pigmentation (Fig. 1E), we performed coupled chemical and mechanical analyses to explore the consequences of pigmentation (figs. S1 and S2).

We carried out chemical analyses of consecutive cutouts after degradation by two treatments that preferentially attack different structural components: acid hydrolysis, which hydrolyzes protein and chitin but not the tanning pigment, and alkaline peroxidation, which solubilizes everything but chitin (see flowchart in fig. S1). The hydrolyzed untanned material is dominated by glucosamine, the basic structural unit of chitin [Fig. 2A(i)], whose presence was independently established by fiber x-ray diffraction (9). On the basis of glucosamine detection, chitin content in this region is estimated to be 90% of dry weight, compared to a low of 15 to 20% in the rostrum [Fig. 2A(ii)]. The amino acid composition of hydrolysates from all tanned regions is uniform and dominated by Gly, Ala, His and Asx (where Asx represents Asp and Asn combined) (27, 15, 11, and 7 mole percent, respectively) (Fig. 2, A and B). Only the untanned region differed in composition, with the Asx content being considerably higher and that of the other amino acids being somewhat lower than the corresponding values in the tanned regions. This disparity may be a result of the low protein content of the untanned material, as well as the difficulty of removing all adhering connective tissue derived from the buccal mass.

Alkaline peroxidation removes all proteins and pigments, leaving essentially pure chitin (95% of dry weight) [Fig. 2A(iii)]. The chitin is present in the form of a scaffold of fine (30-nm diameter) interconnected fibers that maintains beak shape (Fig. 2C). The scaffold structure appears to be the same from the base to the beak tip. The resistance of chitin to peroxide degradation was confirmed by treatment of a commercial chitin control. The protein liberated by alkaline peroxidation has essentially the same amino acid composition [Fig. 2A(iv)] as that detected in cutout specimens after direct beak hydrolysis [Fig. 2A(ii)], suggesting that protein is bound to and released with the tanning pigments during peroxidation.

The relative contributions of chitin, protein, and water in hydrated beak specimens were ascertained through gravimetric analysis, by weighing hydrated and freeze-dried samples before and after alkaline peroxidation. Tanning pigments were derivatized into soluble orange chromophores by alkaline peroxidation, and their concentrations were estimated by calculating the extinction of the orange chromophore with a known amount of nonhydrolyzable black pigment (fig. S4). The resulting compositions are summarized in Fig. 2D. The wing contains about 70 weight percent (wt %) water, 25 wt % chitin, and 5 wt % protein. The water content decreases with increasing concentrations of both protein and tanned pigment; water content reaches a minimum of 15 to 20 wt % in the rostrum, where the protein and tanning pigment levels are 60 and 20%, respectively. In contrast to protein and tanned pigment, chitin content

follows the opposite trend, decreasing to less than 15 wt % in the rostrum, but with a strong positive correlation to water content.

Because catechols are often coupled to pigmentation in living organisms, the relation between dopa-proteins and tanning pigment in squid beaks merited further scrutiny. Both dopa and several cross-link derivatives were captured from beak cutouts by phenylboronate chromatography after protein hydrolysis. The captured compounds were characterized by ultraviolet/visible spectroscopy (fig. S2), amino acid analysis (fig. S3), electrospray ionization mass spectrometry (ESI-MS), and tandem mass spectrometry (MS/MS). The major MS peaks detected after a 24 hour hydrolysis had mass/charge ratios (m/z) of 278 and 351; a less stable compound at m/z 335 was captured after a shorter (2 hour) hydrolysis. Structures deduced for each are consistent with a His-dopa peptide (m/z 335) and a His-dopa cross-link (m/z 351) plus a related derivative (m/z 278) (Fig. 3A and fig. S5). The tendency of these to undergo oxidation to black pigments in vitro at pH 7.0 suggests that the cross-links are soluble antecedents of the acid-insoluble black pigment. This hypothesis was tested by subjecting the acid-insoluble black pigment to direct laser desorption mass spectrometry with the pigment acting as its own matrix (Fig. 3B). Only a few prominent fragments, particularly at m/z 198 and 156, were observed. These resemble the dopa$\rceil^{H+}$ and His$\rceil^{H+}$ ions released during MS/MS of the dopa-His cross-links and are distinct from those in authentic eumelanin (m/z of 552, 365, and 208) (11). It is thus reasonable to assume that insoluble black pigments from squid beaks represent multimers of dopa-His cross-links.

Both tensile and nanoindentation tests show that when the coupons are hydrated, the Young's modulus E increases dramatically with the degree of pigmentation, from about 0.05 to 5 GPa (Fig. 4, A and B). However, after the peroxide treatment, the values drop drastically, to a mere 0.03 GPa, regardless of pigmentation. Evidently the chitin scaffold on its own has minimal structural integrity. In the freeze-dried state, E is uniformly high, diminishing by only a factor of 2 (from about 10 to 5 GPa) between the rostrum and the untanned wing (Fig. 4B). Essentially the same reduction was obtained in the rostrum after peroxide treatment, suggesting that the proteins play a minimal role in beak reinforcement in the absence of water.

To glean additional insights into compositional effects, E was replotted against both chitin content and combined chitin/protein content (Fig. 4C). In the freeze-dried state, the modulus depends only weakly on protein or chitin content. In the hydrated states, however, it exhibits a strong dependence on composition. Most notably, it appears to follow an inverse relation with chitin content—an unexpected result because chitin fibers are among the stiffest polysaccharides ($E = 40$ GPa in the dry state) (12). Concurrently, though, the water content increases more than threefold. The corresponding degree of chitin hydration, characterized by the water-to-chitin mass ratio, increases from about 2.2 in the rostrum to 3.3 in the untanned regions. Evidently, over this range, changes in the degree of hydration play a greater role than that of chitin content, leading to the observed stiffness reduction with increased distance from the beak tip.

Beak stiffness is undoubtedly influenced by both microarchitectural and molecular factors. With respect to the latter, stiffness is closely correlated to the incremental and complementary distributions of two biopolymers—chitin, a polysaccharide of β1,4-linked *N*-acetylglucosamine, and a family of His- and dopa-containing proteins— and the extent to which they are hydrated or cross-linked. Although the proteins have yet to be fully characterized, the dipeptide His-dopa and His-dopa cross-links (Fig. 3A and fig. S5) attest to the frequent proximity of the two amino acids in squid beaks. In this respect, squid beaks are reminiscent of sclerotized insect cuticle. Both contain catechols (peptidyl-dopa and N-acetyldopamine (NADA) (13), proteins with His-rich domains (14), catechol-His cross-links (15,16) and a reinforcing chitin network (17,18). However, there are also substantial differences. In contrast

to the low–molecular weight NADA used by insects, the catecholic functionality in beaks (dopa) is tethered to a protein backbone, imposing steric and diffusional constraints on compositional gradients. With three distinct ingredients (NADA, chitin, and protein), insects can and do vary NADA independently of the other two ingredients (19). This process is severely limited with protein-tethered dopa. In a protein with a fixed proportion of its tyrosyl residues modified to dopa, a gradient of dopa necessarily includes a gradient of protein and vice versa. There are cases in which the Tyr residues in a protein are differentially modified to dopa over the range of its expression in a tissue. For example, mussels have an increasing gradient of dopa/Tyr in *Mytilus* foot protein-1 along the proximal-to-distal axis of its distribution in each byssal thread (20). In squid beaks, the dopa/Tyr ratio in precursor proteins is not yet known; however, the pigment/protein ratio remains roughly constant.

The most notable discovery of this study is the importance of water content in defining the stiffness gradient. Complete desiccation by freeze-drying virtually eliminates the gradient, narrowing stiffness to between 5 and 10 GPa. On the basis of the inverse distributions of chitin and protein, the stepwise desolvation of chitin may be accomplished by a titrated admixture of dopa-containing beak proteins. Catechols are known to have a potent dehydrating effect on various biopolymers, attributable to their hydrophobicity (21,22) and/or hydrogen-bonding ability (23). However, the occurrence of cross-links, such as dopa-His cross-links, and the incremental deposition of cross-linked multimers that constitute the tanned pigment are also likely to reduce solvation (24,25). Absent from our squid beak analyses are cross-links between protein and chitin; whether this means that the two macromolecules are not covalently cross-linked or that the cross-links are not captured by phenylboronate remains to be determined. Although both noncovalent and covalent factors are important for controlling the hydration of squid beaks, it is not possible at this time to resolve the magnitude of their respective contributions.

The importance of water in the functional properties of biomolecular materials is widely recognized. Equally important, though subtler, is the suggestion from this study that hydration of wet chitin can be exquisitely tuned from 20 to 70 wt% by association with a cross-linked His-rich protein. Given the available 100-fold range in stiffness, the nature of this association merits further careful study and may offer a new dimension of versatility to bio-inspired dopa-containing polymers (26,27).
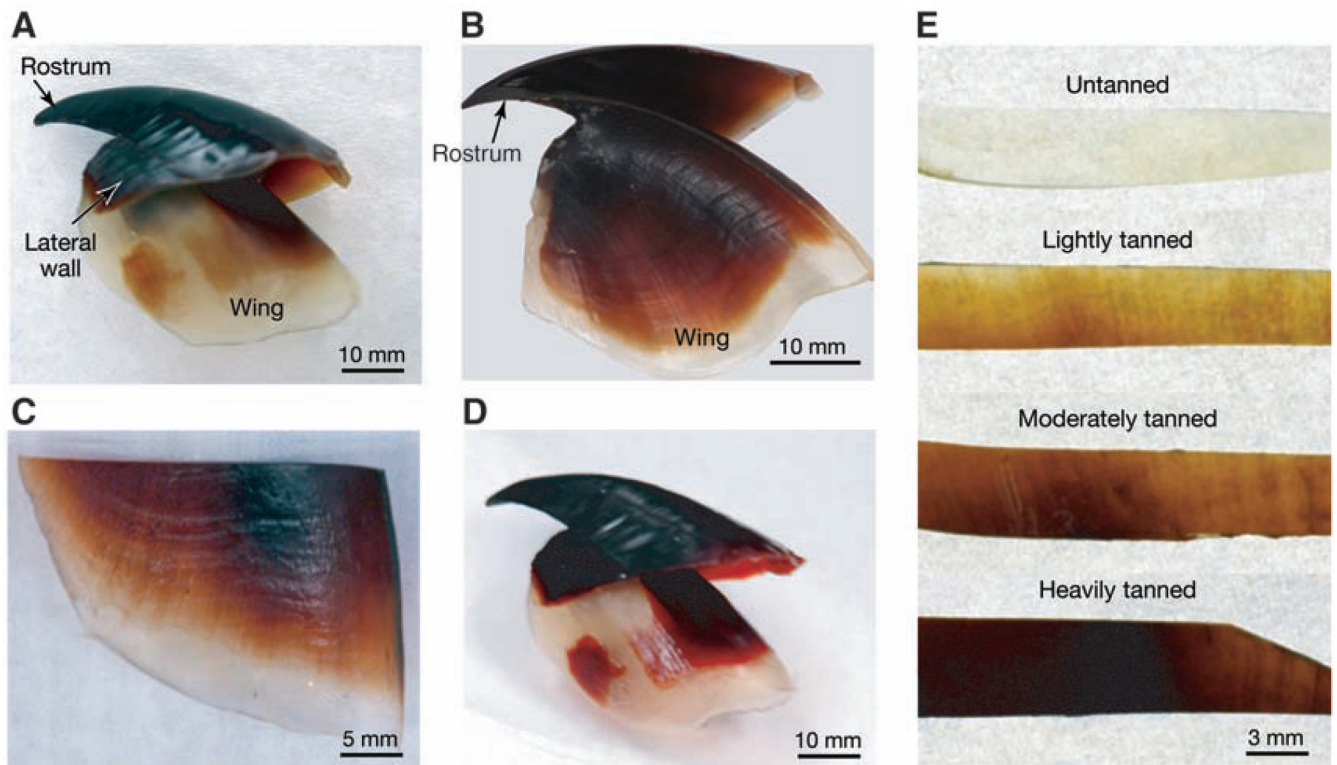
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Beak of the Humboldt squid *Dosidicus gigas*. (**A**) Side view of a full upper beak after removal
from the buccal mass showing natural pigmentation. (**B**) Split beak highlighting the relation
of the wing to the rostrum. (**C**) Dissected wing. (**D**) Beak in (A) after staining for dopa-
containing proteins with a catechol-specific reagent. (**E**) Series of sections representing
different shades of the pigmentation gradient.
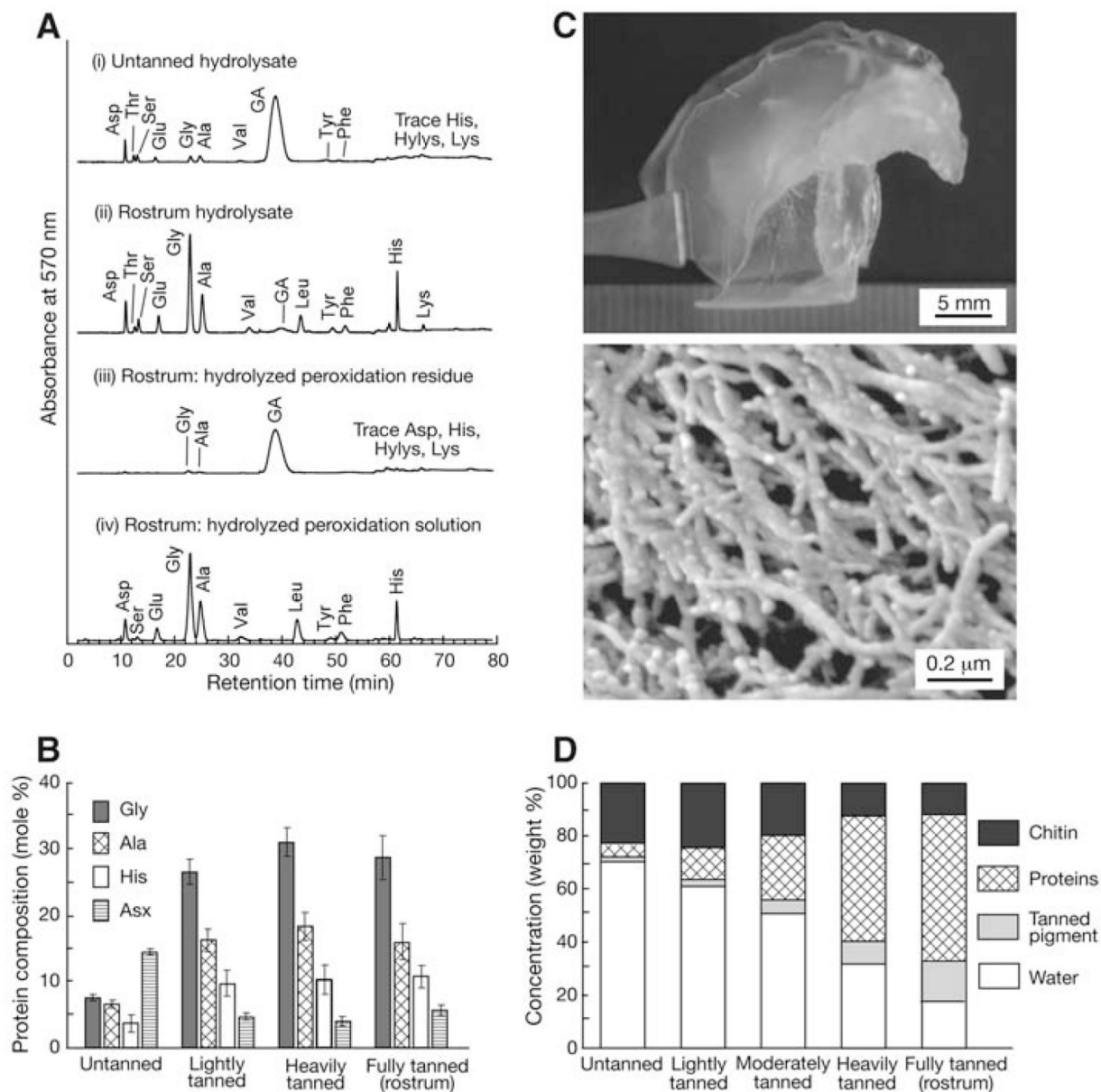
Figure 2.
Chemical and gravimetric analyses of squid beak cutouts. (**A**) Absorbance spectra of acid hydrolysates. GA, glucosamine; Hylys, hydroxylysine. (**B**) Compositions of the dominant amino acids in the hydrolysates. (**C**) Optical image of the whole beak after alkaline peroxidation (top), and high-magnification scanning electron image of the chitin fiber network in the rostrum (bottom). (**D**) Chitin, protein, and pigment contents ascertained from gravimetric measurements.
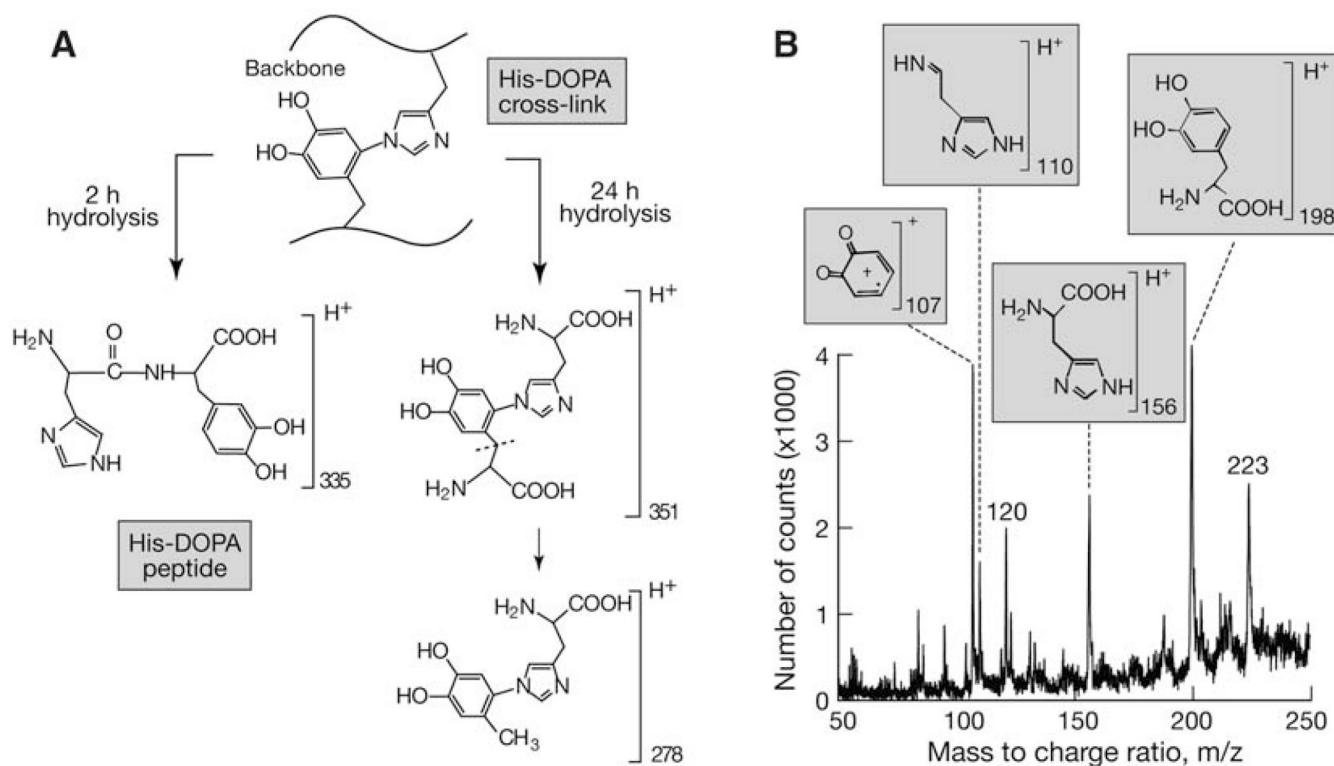
Figure 3.
Dopa and cross-link derivatives. (**A**) His-dopa peptide (*m/z* 335) and His-dopa cross-link (*m/z* 351) captured by phenylboronate chromatography of a hydrolyzed beak. (**B**) Prominent ion fragments in acidinsoluble black pigment, obtained by laser desorption/ionization–time-of-flight mass spectrometry.
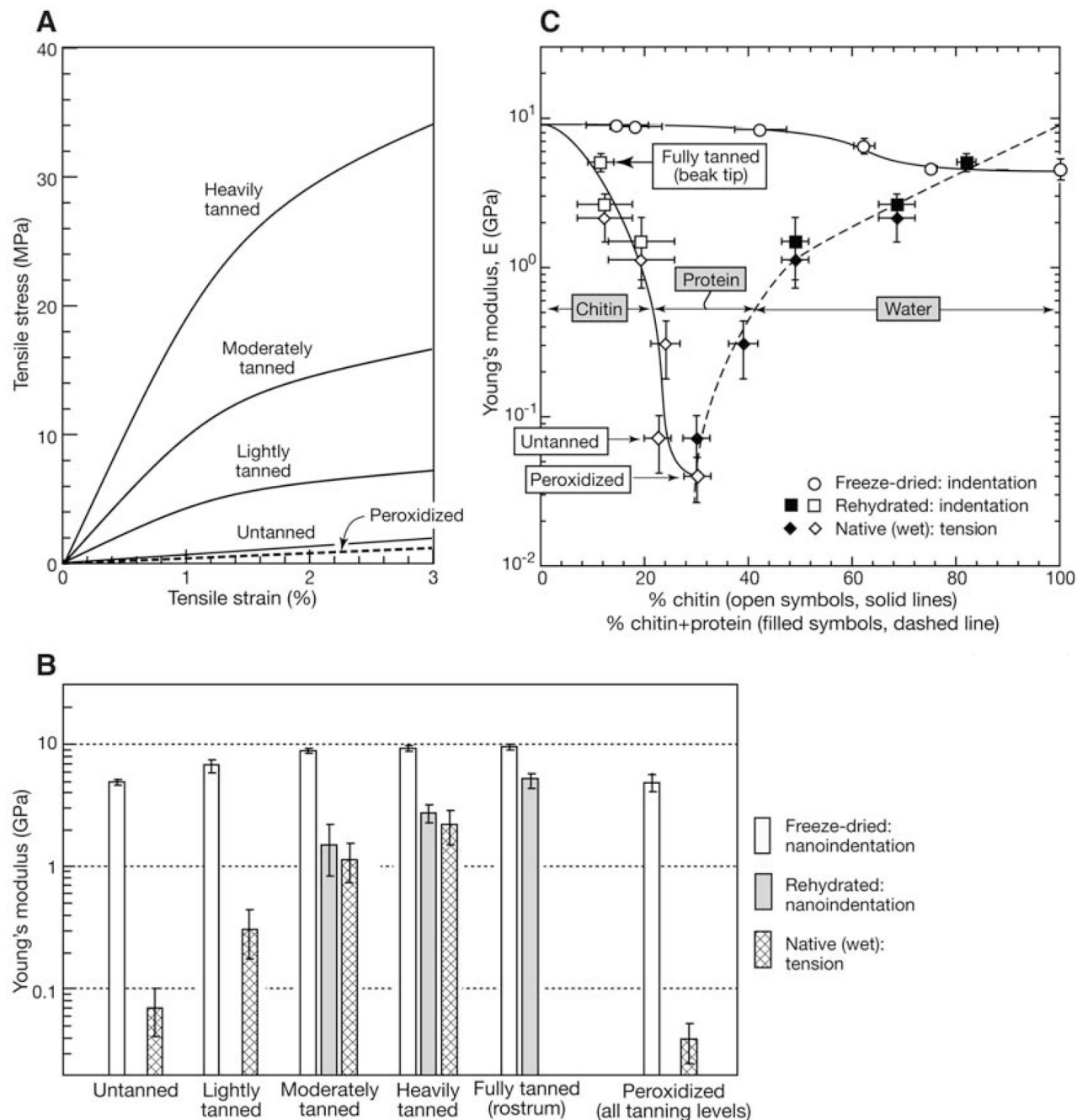
Figure 4.
Mechanical properties of squid beaks. (**A**) Typical tensile stress/strain curves for hydrated beak specimens. Tests could not be performed on the most heavily tanned material at the rostrum because of its size and shape. (**B**) Summary of Young's moduli obtained for dry and hydrated coupons by tensile testing and/or nanoindentation. Nanoindentation tests proved useful for measuring *E* for all but the two softest states; in untanned and lightly tanned rehydrated samples, the displacements necessary for reliable modulus measurement exceeded the accessible range of the instrument. Tests on peroxidized samples showed no effect of the initial pigmentation level, and thus all data have been combined (far right). Error bars indicate 1 SD.

(**C**) Young's modulus plotted against both chitin content and combined chitin/protein content in both dry and hydrated coupons. Error bars indicate 1 SD.