EXOTIC COLLAGEN GRADIENTS IN THE BYSSUS OF THE MUSSEL
MYTILUS EDULIS

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Summary

Byssal threads of the common mussel *Mytilus edulis* contain collagenous molecules from which two pepsin-resistant fragments have been isolated and characterized. These show a complementary distribution along the length of the thread, such that one predominates distally (Col-D) and the other proximally (Col-P). Both fragments contain three identical α-like chains with molecular masses of 50 kDa (Col-P) and 60 kDa (Col-D) and have typically collagenous amino acid compositions; for example, 35% glycine and almost 20% proline plus 4-trans-hydroxyproline. Hydroxylysine and 3-hydroxyproline were absent. Col-P sequences are also typical of collagen in consisting of tandem repeats of the triplet Gly-X-Y in which X and Y generally represent any amino acid. When proline occurs, it is hydroxylated to 4-trans-hydroxyproline only in the Y position. Seven instances where X is glycine have been detected in Col-P. Specific polyclonal anti-Col antibodies were used to isolate the precursors of Col-P and Col-D from the mussel foot. PreCol-P has a molecular mass of 95 kDa and contains 36% glycine but a lower imino acid content (13%). It has a complementary distribution with another precursor (preCol-D, 97 kDa) along the length of the foot. The two precursor compositions suggest resilin-like and silk-fibroin-like structures, respectively, in the noncollagenous domains of preCol-P and preCol-D. Immunogold labelling studies indicate that Col-P is associated with the coiled fibers of the inner core in the proximal portion of the thread, whereas Col-D is localized to the straight fiber bundles of the distal thread as well as to the outer core of the proximal thread.

Key words: byssus, collagen, gradient, *Mytilus edulis*, mussel, amino acid sequence.

Introduction

Collagens are highly diversified structural proteins derived chiefly from mesenchymal cells and employed in the scaffolding of connective and interstitial tissues. In some lesser known metazoans, collagens of ectodermal origin are used to construct materials outside the confines of the living organism (Adams, 1978; Gross, 1963), Gooseberry sawfly silk (Spiro et al. 1971), selachian egg capsules (Knight et al. 1993), worm cuticle collagens (Kramer et al. 1982; Mann et al. 1992), spongian (Garrone, 1978) and mussel byssus are but a few examples of such exoskeletal collagenous materials. With the possible exception of the worm cuticle collagens, none of these has been particularly well studied. Indeed, in most, the existence of collagen is based on little beyond amino acid analyses that have included the detection of hydroxyproline, staining of specimens for transmission electron microscopy and, occasionally, wide-angle fiber X-ray diffraction.

The byssus of the common mussel *Mytilus edulis* has intrigued biologists since the beginning of the century (Seydel, 1909). This can be at least in part attributed to the silken byssal threads (diameter 0.1 mm; length 2–4 cm) which a mussel can repeatedly form at a few minutes notice for attachment to a rock or other solid surface (Fig. 1). These threads were dubbed ‘collagenous’ on the basis of fiber X-ray diffraction (Rudall, 1955; Mercer, 1952), hydroxyproline content (Melnick, 1958), amino acid composition (Benedict and Waite, 1986; DeVore et al. 1984; Pujol et al. 1976) and shrinkage temperatures (Pikkarainen et al. 1968). Although the collective evidence presents a strong case, some of the extraordinary properties of byssus are hard to reconcile with ‘typical’ collagens. These include the high melting temperature, lack of banding patterns and resistance to denaturants, acids and proteases (Brown, 1950; Pikkarainen et al. 1968). Moreover, according to several lines of evidence, byssal threads are not uniformly collagenous throughout, i.e. the diffractive and mechanical properties typical of fibrous collagen are best reflected at the stiff distal end of the thread, whilst the more extensible or elastic proximal end seems to be disordered (Gathercole and Keller, 1975; Price, 1981; Smathers and Vincent, 1979; Vitellaro-

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This has led to the suggestion that protein gradients including a collagen gradient occur along the length of each byssal thread (Gathercole and Keller, 1975; Mascolo and Waite, 1986). Nothing is known at present about how such gradients might be contrived during thread formation.

Byssal threads are assembled by the mussel foot from granular precursors in a rapid process resembling polymer injection-molding (Waite, 1992). When a new thread needs to be made, fiber-containing granules are released from large holocrine glands and conducted through ciliated ducts into the ventral groove of the foot, which serves as the mold. There they coalesce into a thread that is shaped, and possibly oriented, by peristalsis in the groove. Before being disengaged from the groove, the thread is sized by a protective coating (Waite, 1992). Such processing of a diverse assortment of precursors into a natural polymeric material is an exquisite example of microengineering and, in our opinion, worthy of closer scrutiny.

Our aims in the present study were to establish the presence of collagen in the byssus, to isolate and characterize the collagen and its precursors, and to use immunochemical techniques to localize these in the byssal threads as well as in the foot of *Mytilus edulis*. Our findings suggest that two unique collagens exist in byssal threads. One of these, Col-D, prevails in the *distal* portion of each thread, while the other, Col-P, predominates in the *proximal* thread. Viewed over the entire thread, the distribution of the two collagens is roughly complementary.

**Materials and methods**

Mussels (*Mytilus edulis* L.) were collected locally from Roosevelt Inlet near the mouth of the Broadkill River, DE, USA, and maintained in an aquarium with running sea water at 15–20 °C. Fresh byssal threads were harvested every other day and stored in distilled water at −20 °C prior to use.

*Extraction of collagens from byssal threads*

Byssal threads were homogenized to a purée in 5 % acetic acid with 4 mol l−1 urea using frosted-glass tissue grinders (Kontes, Vineland, NJ, USA) and centrifuged at 31000 g (average force) for 30 min at 4 °C (Miller and Rhodes, 1982; Benedict and Waite, 1986). Following a brief rinse, the pellet was resuspended in 0.5 mol l−1 acetic acid, pH 2.6, containing between 1:10 and 1:30 pepsin:insoluble protein (Boehringer-Mannheim, Indianapolis, USA) and digested for 48 h at 4–7 °C, whereupon it was again centrifuged at 17000 g for 30 min. The supernatant was collected and dialyzed against 0.1 mol l−1 sodium borate, pH 8.0, to precipitate collagens preferentially whilst inactivating pepsin. Precipitates were harvested by centrifugation (31000 g for 60 min) and redissolved in 0.5 mol l−1 acetic acid. Residual insoluble substances were removed by centrifugation. Collagens were again precipitated, this time by adjusting pH to 7.0 with NaOH and centrifugation at 17000 g for 30 min. The pellet was collected and dialyzed against 0.1 mol l−1 sodium borate, pH 8.0, to precipitate collagens preferentially whilst inactivating pepsin. Precipitates were harvested by centrifugation (31000 g for 60 min) and redissolved in 0.5 mol l−1 acetic acid. Residual insoluble substances were removed by centrifugation. Collagens were again precipitated, this time by adjusting pH to 7.0 with NaOH and centrifugation at 17000 g for 30 min. The pellet was redissolved in 0.5 mol l−1 acetic acid with 4 mol l−1 urea and prepared for high-performance liquid chromatography (HPLC). Since the pellet is only partially soluble in acetic acid with urea, it typically required centrifugation on an Eppendorf microcentrifuge (10 min at 15 000 revs min−1) prior to application to a C-8 column for HPLC. The 250 mm×4.6 mm column consisted of RP-300 Aquapore (Applied Biosciences Inc.) and was eluted with a linear gradient (15 % to 28 %) of aqueous acetonitrile with 0.1 % trifluoroacetic acid (Waite et al. 1985). Eluent was monitored continuously at 230 nm, and 1 ml fractions were collected and assayed by amino acid analysis and electrophoresis following freeze-drying.

To determine the distribution of pepsin-resistant collagens in different parts of the byssal thread, about 300 threads measuring 2–4 cm were carefully collected from mussels maintained at 4–8 °C. The threads were then sectioned into five segments: the plaque and the cuff, the distal (rigid) and proximal portions of the thread and the transitional region between the distal and proximal regions. Similar segments were pooled, homogenized and treated with pepsin, as above, to release sufficient protein for visualization following polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (see below).
**Preparation and purification of peptides**

Trypsin (Boehringer-Mannheim, Indianapolis, USA) digestion of byssal collagen was performed using the following reaction conditions. About 1 mg of Col-P was dissolved in 1 ml of 50 mmol l⁻¹ Tris–HCl with 10 mmol l⁻¹ CaCl₂ buffered to pH 7.5 at a protease:protein mass ratio of about 1:10, with constant stirring at 22–24 °C for 18h. The progress of digestion in each case was monitored by removing 5 μl samples at 3 h intervals for inspection by sodium dodecylsulfate gel electrophoresis (see below). The digestion was terminated by the addition of 0.3 ml of glacial acetic acid, followed by freezing (−80 °C) and lyophilization. The freeze-dried residue was dissolved in 0.5 ml of 5% acetic acid. Cyanogen bromide peptides were generated from purified Col-P and types I and II calf skin collagen (Ethicon, Morristown, NJ, USA) by incubation (4h for types I and II collagen; 48h for Col-P) in 70% trifluoroacetic acid containing a 100-fold excess (relative to methionine) of CNBr at 30 °C. Cleavage was terminated by dilution with a 10-fold excess of cold distilled water, freezing at −80 °C and lyophilization to dryness (Bornstein and Piez, 1965). CNBr peptides were comparatively mapped by electrophoresis (see below). Resolution of the tryptic peptides was achieved by reversed-phase HPLC using a 250 mm×4.6 mm Phenomenex or Mircrosorb C-18 column (Rainin Instruments, Woburn, MA, USA). The eluting solvent was a linear gradient of aqueous acetonitrile (0% to 25%) with 0.1% trifluoroacetic acid.

**Amino acid analysis and sequencing of peptides**

Peptides and proteins were hydrolyzed in 6 mol l⁻¹ HCl with 10% phenol and 10% trifluoroacetic acid in vacuo at 150 °C for 20 and 40 min to correct for the losses of certain amino acids (Tsugita et al. 1987). Amino acid analysis was by ion-exchange HPLC and a ninhydrin-based detection system (Beckman System 6300 autoanalyzer) using a gradient program described previously (Waite, 1991). Hydroxylated derivatives of Pro and Lys were separately quantified by the same program. The amino acid sequence of protein and peptides was derived by automated Edman degradation using a Poron Instruments microsequencer (Porton, Tarzana, CA). phenylthiohydantoin (PTH) derivatives of amino acids (Tsugita et al. 1989). Specific polyclonal antisera against purified Col-P were raised under contract (Cambridge Research Biochemicals, Wilmington, DE, USA) in three female New Zealand rabbits according to standard immunization protocols. Antibody titer was tested by dot blots, and specificity was determined by immunoblotting following electrophoretic transfer and visualized by a secondary goat anti-rabbit Fc antiserum coupled to alkaline phosphatase (Blake et al. 1984). Another polyclonal antiserum ostensibly against byssal collagen was donated by Dr C. V. Benedict (Farmington, CT, USA). It was found to be preferentially reactive towards Col-D and was used at titers of 1:10⁵ in immunoblots.

**Identification of precursors in the foot tissue**

Mussel foot tissues were examined for the presence of collagen precursors and prefabricated collagen gradients. Freshly amputated mussel feet were frozen on glass plates at −80 °C. Pigmented epithelial and muscular layers were flayed from the frozen feet with a scalpel. Each foot was then serially sectioned into 1–2 mm slices (as in Fig. 7, for example). Each slice was individually homogenized in 20 volumes of 5% acetic acid, microfuged (5 min) to remove insoluble material, and digested with pepsin (1:20 enzyme to tissue wet mass) for 2h at 4 °C. Pepsin digestion was terminated by adjusting the pH to 7.0. Parallel samples were homogenized in 5% acetic acid with inhibitors (0.01% pepstatin, 0.01% leupeptin, 1 mmol l⁻¹ iodoacetate and 0.1 mmol l⁻¹ phenylmethyl-
sulfonylfluoride) at a 1:20 wet mass to volume ratio. The acid-soluble materials were clarified by microcentrifugation (5 min at 14 000 revs min^{-1}) and precipitated by the addition of cold perchloric acid (to 1%). Precipitated material was redissolved in cold 5% acetic acid. Protein precursors were separated by SDS polyacrylamide gel electrophoresis and identified by immunoblotting following electrophoretic transfer to Immobilon P. Parallel Immobilon panels were set aside for band excision and amino acid analysis, as described above.

**Immunogold electron microscopy**

Small pieces of the proximal and distal portions of byssal threads were dissected from mussels maintained at 4–6°C. These samples were fixed in 2.5% glutaraldehyde in 0.1 mol l^{-1} cacodylate/HCl, pH 7.3, followed by post-fixation in 1% osmium tetroxide in the same buffer. After dehydration, samples were embedded in Epon-812 resin and sectioned at 50 nm with a diamond knife (DuPont Biomedical Products, Wilmington, DE, USA) on a Reichert model E ultramicrotome. Ultrathin sections mounted on nickel grids were treated with 10% hydrogen peroxide, blocked with 2% bovine saline albumin and 1% cold fish gelatin in phosphate-buffered serum, and incubated with specific polyclonal antisera diluted 1:100 according to procedures recommended by Hyatt (1984). The sections were then washed and incubated with 15 nm immunogold particles (Electron Microscopy Sciences, Fort Washington, PA, USA). The gold particles bind to the F\text{c} domain of the primary antibody, thereby creating an electron-dense tracker for its presence. After extensive washing, the sections were counterstained with uranyl acetate and examined under a Zeiss model 902 transmission electron microscope.

**Results**

Soluble proteins are at best poorly extractable from mature byssal threads. To remedy this, we attempted an extended pepsin digestion of mechanically disrupted threads. Pepsin is typically indiscriminate in its digestion of proteins, with the notable exception of the triple helical domain of native collagen. Prolonged pepsin digestion releases at least two collagenous fragments, Col-P and Col-D, each with three identical \( \alpha \)-chains with molecular masses of 50 kDa and 60 kDa, respectively. It is noteworthy that the ratio of extractable Col-P:Col-D varies according to the temperature of the sea water to which the mussels were introduced; for example, at warm temperatures (18–20°C), the ratio was 1:0.5, whereas at 4–8°C it was 1:2. Our experience suggests that, at the lower temperatures, Col-D-containing byssal precursors are less effectively cross-linked and thus more easily extracted.

To improve the yield of pure Col-P, which is normally poorly cross-linked and thus more easily extracted, Col-P was purified to homogeneity using preferential extraction and reversed-phase HPLC (Figs 2, 3). Col-D was analyzed from protein electroblotted to Immobilon. The availability of an anti-Col-P antibody enabled us to improve the yield of pure Col-P, which is normally poorly cross-linked and thus more easily extracted.

Specific polyclonal antibodies were successfully raised against Col-P in rabbits. In dot blot tests, these reacted strongly (titer 1:10^3 dilution) with Col-P but not perceptibly with Col-D, bovine collagen types I and II or any of the standard proteins. The availability of an anti-Col-P antibody enabled us to improve the yield of pure Col-P, which is normally poorly cross-linked and thus more easily extracted.
Exotic collagen gradients in the mussel

Exotic collagen gradients in the mussel

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Fig. 3. HPLC profiles of pepsin-solubilized byssus on a C-8 reversed-phase column. (A) Extract of byssal threads formed at 18–20˚C. (B) Extract of threads formed at 4–8˚C. Peaks containing Col-P and Col-D were identified by SDS polyacrylamide gel electrophoresis of the fractions under the peak. Differences in the elution times probably reflect the degree of pepsinization into the collagenous core.

to address two questions concerning the distribution of Col-P within the byssal thread and the distribution of its precursor, preCol-P, within the foot. Byssal threads (secreted at 4–8˚C) 2–4 cm in length were cut into five segments, and segments from equivalent positions in about 300 threads were pooled, ground up and digested with pepsin. The pepsin-resistant proteins from each segment are shown in Fig. 6 after SDS–PAGE. The distribution of Col-D and Col-P resembles a pair of complementary gradients: the former decreasing as one moves longitudinally from the adhesive plaque to the cuff, whilst Col-P content increases over the same course (Fig. 6). Do these gradients pre-exist in the secretory foot tissues, or are they ‘manufactured’ during thread formation? To determine this, a frozen mussel foot approximately 2 cm in length was sectioned transversely with a single-edged razor into segments of equal length. Proteins extracted from each segment with or without pepsin treatment were analyzed following separation by electrophoresis and electroblotting. The results shown in Fig. 7 make three significant points. First, that the anti-Col-P antiserum recognizes a preCol-P weighing approximately

Table 1. Amino acid composition of byssal thread, Col-P, preCol-P, Col-D and preCol-D

<table>
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<th>Amino acid</th>
<th>Thread*</th>
<th>Col-P</th>
<th>PreCol-P</th>
<th>ΔP</th>
<th>Col-D</th>
<th>PreCol-D</th>
<th>ΔD</th>
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<td>37.5</td>
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</tbody>
</table>

ΔD and ΔP represent deduced differences between each precursor and its pepsin-resistant fragment.

Amino acids are listed in order of elution time, and all values are reported in residues per 1000 and have a standard error of about 5%.

*Benedict and Waite (1986).*
95 kDa; second, that Col-P can be generated from preCol-P by pepsin treatment, and third, that Col-P or preCol-P shows a gradient in the foot that mirrors its gradient in the threads. Also evident, but without the immunoblot illustration, is the complementary gradient of preCol-D and pepsin-derived Col-D. Anti-Col-D antiserum was of similarly high titer (approximately 10⁵), showing a strong preferential binding to Col-D over Col-P. PreCol-D has an apparent molecular mass of 97 kDa (Fig. 7). It appears that three of these chains are combined in the native precursor.

Amino acid analyses of preCol-D and preCol-P were performed on hydrolysates of these proteins following electrophoretic transfer to Immobilon. Their compositions resemble those of Col-P and Col-D in some respects, although the former has notably lower levels of glycine, alanine and serine than its precursor; Col-D, in contrast, has higher levels of glycine, hydroxyproline and glutamine/glutamate than its precursor. The differences in composition between the collagenous and non-collagenous domains of the preCols are best assessed by subtracting the composition of Col-P or Col-

Col-P N terminus

Partial pepsin cleavage:
A-A-R-A-N-A

Complete pepsin cleavage:
G-G-P-N-P-G-N-S-G-S-P-G-Q-P-G-P-Q-G-S-P-

Col-P tryptic peptide sequences

<table>
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<tr>
<th>Fraction</th>
<th>Sequence</th>
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<td>A'</td>
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<tr>
<td>B'</td>
<td>P-G-N-P-G-N-P-G-Q-P-G-N-P-G-T-P-G-K</td>
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<tr>
<td>A</td>
<td>G-Q-P-G-Q-P-G-Q-P-G-Q-P-G-G-P-A-G-G-Q-P-G-K......</td>
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<tr>
<td>B</td>
<td>G-A-P-G-T-E-G-E-P-G-P-G-C......</td>
</tr>
<tr>
<td>C</td>
<td>G-P-A-G-S-Q-G-P-S-G-C......</td>
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<tr>
<td>D</td>
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<td>E</td>
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<td>J</td>
<td>G-P-I-G-P-S-G-P-S-G-A-P-G-D-N-G-P-N-G......</td>
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Fig. 5. Amino acid sequence of Col-P N termini and tryptic peptides (see Fig. 4). P refers to 4-trans-hydroxyproline.
Exotic collagen gradients in the mussel

The results of this study indicate unequivocally that the byssus of Mytilus edulis contains collagenous proteins. This corroborates suggestions made earlier on the basis of wide-angle X-ray diffraction and detection of hydroxyproline-containing proteins in pepsinized byssus (Benedict and Waite, 1986; Rudall, 1955). Byssal collagen is, however, by no means identical with type I collagen, as DeVore et al. (1984) have concluded. Its pepsin-resistant domain is only half the size of pepsin-resistant fibrillar collagen types I–III and lacks 3-trans-hydroxyproline, hydroxylysine and, according to another study, aldmine and aldol-derived cross-links (van Ness et al. 1988). It is also unusual in containing several instances of tandem Gly–Gly sequences, although these have also been reported in invertebrate collagens from Nereis virens and Riftia pachyptila cuticles (Sharma and Tanzer, 1983; Gaill et al. 1991; Mann et al. 1992) and in sea urchins (Exposito et al. 1992). Apart from the obvious homology of having a glycine at every third residue and frequent imino acids at positions X and Y, byssal collagens have no match with any known collagen sequence.

In contrast to the secretion of interstitial procollagens by fibroblasts, byssal collagens are stored in and secreted from large (approximately 1–2 μm diameter) granules produced by the ‘collagen gland’, a large holocrine gland in the interior of the mussel foot (Pujol et al. 1972; Vitellaro-Zuccharello, 1980). Whether the 95 kDa preCol-P subunit isolated from foot tissue represents a comparable byssal procollagen is not known, but this seems unlikely in view of its composition. More probably, preCol-P is used without much proteolytic processing. The speed with which new threads must be made (2–5 min) may preclude the trimming necessary for the conversion of other procollagens to tropocollagen. Our results, however, do suggest that, whatever happens during its conversion from preCol-P to proximal byssal thread, there remains a tidy central Col-P domain that is unmodified by cross-links and excisable with pepsin from byssus (see Fig. 9). The same cannot be said of Col-D which, except in conjunction with cold shock, is extracted at very low yields. Only a little can be said about the domains flanking Col-P and Col-D. First, they must be cross-linked; second, the deduced amino acid composition of ΔP is 40% Gly, 13% Ala, 11% Ser and little to no Hyp. In the case of ΔD, Gly and Ala each contribute more than 30%, whereas Pro content is low and Hyp is absent (Table 1). It is noteworthy that ΔP and ΔD compositions are reminiscent of resilin (Seifert and Gallop, 1966) and silk fibroin (Rudall, 1962), which are elastic and stiff proteins, respectively (Table 2). Quite possibly then, the pepsin-labile flanking domains of preCol-D may be fibroin-like; by the same token, the flanking domains of preCol-P may resemble an elastic protein such as resilin. At this stage, such associations must be viewed as tentative at best, since composition does not reveal protein structure.

Discussion

Fig. 6. SDS polyacrylamide gel electrophoresis of the pepsin-resistant byssal collagens Col-P and Col-D showing their differential distribution over the length of a typical thread. Lanes A–E contain pepsinized protein derived from plaques (A), rigid portions (B), the transition from rigid to extensible portion (C), extensible portions (D) and cuffs (E). The lane marked ‘pure’ was loaded with a purified sample of Col-P. Both collagens were identified in parallel by western blotting with their specific respective antisera and amino acid analysis.

Ultrastructural and immunogold labelling studies were carried out on the proximal and distal portions of byssal threads. Proximally, the thread is elastic and flattened with a corrugated surface, while distally it is rigid and cylindrical with a smooth outer surface. Sagittal sections examined by transmission electron microscopy show a homogeneous electron-dense outer sheath surrounding an inner composite core consisting of fiber bundles anisotropically distributed in an amorphous matrix, with fibers greatly outnumbering matrix (Fig. 8E). In proximal thread, the core consists of distinct outer and inner morphologies. In the outer core, as in distal portions, discontinuous straight fibers are embedded in a matrix (Fig. 8A,C). In contrast, the inner core contains coiled fibers (Fig. 8B,D). Immunogold studies suggest that anti-Col-P antibodies are localized with a high density on the coiled fibers (Fig. 8B), whereas anti-Col-D is associated chiefly with the straight fibers of the outer core (Fig. 8C). In the distal portion of the thread, however, only anti-Col-D antibodies are observed bound to bundled fibers (Fig. 8F). Anti-Col-P is not bound in the distal thread. Neither of the antisera bound to the cuticular sheath.

D from that of its respective precursor. This calculation can only be made following the conversion of ‘residues per thousand’ to ‘residues per molecule’ or part of a molecule as the case may be (see Materials and methods). Table 1 shows the difference compositions for ΔD and ΔP. ΔD is predicted from this to consist of more than 60 mol% equimolar glycine and alanine. Serine, alanine and glycine (approximately 40%) account for more than 60 mol% of ΔP.

Apart from the obvious homology of having a glycine at every third residue and frequent imino acids at positions X and Y, byssal collagens have no match with any known collagen sequence.

Discussion

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Curiously, the existence of a major β-protein motif in distal byssus was predicted by Rudall (1955) using wide-angle fiber X-ray diffraction. In presenting his results, he mentions that they ‘suggest that there is another protein (or another phase of the collagen structure) in the byssus threads’. Future molecular studies of the complete preCol sequences should be able to elaborate on the validity of this hypothesis.

The possible inclusion of silk-like and resilin-like domains in the collagenous tensile element of byssus begs the following question. Why do mussels use collagen at all? We can think of one reason. Since true underwater silks are not known to exist in nature, a collagenous domain may be necessary to endow byssal precursors with the capacity for fibrillogenesis. The reason for amalgamating collagen with silk- and resilin-like domains is more subtle and is probably rooted in the concept of composite materials; for example, ‘it is a common principle that two or more components may be profitably combined to form a composite material so as to make best use of the more favorable properties of the components while simultaneously mitigating the effects of some of their less desirable

![Diagram of Col-P and Col-D distribution](image)

**Fig. 7.** Distribution of Col-P and Col-D and their precursors in transverse foot sections. (A) Col-P and Col-D from pepsinized foot sections followed by SDS polyacrylamide gel electrophoresis (upper panel, lanes a–e), followed by pure Col-P and Col-D (lane f). (B) Precursors of Col-P and Col-D identified immunochemically from extracts of nonpepsinized foot sections followed by SDS polyacrylamide gel electrophoresis (lower panel). Lanes a–e stained for protein with Serva Blue R-250; lanes b′–e′ contain parallel samples, transferred to nitrocellulose and western-blotted with anti-Col-P. The fast-moving species in e′ are degradation products of preCol-P. The last two lanes contain, respectively, purified Col-P stained with Serva Blue R-250 (left) and western-blotted Col-P (WB) using anti-Col-P antisera.
Each byssal thread functions under the somewhat paradoxical expectation that it be strong under tension and that it absorb shock. Collagen is as strong a material as silk, but silk is 30–40 times tougher, i.e. has a higher breaking strain energy density (Denny, 1988). No doubt, in distal thread the presence of silk would toughen the collagen. In proximal thread, extensions of more than 100% (Smeathers and Vincent, 1979) are carried by the elastic domains whereas, at much greater strains, the collagen and silk-like network would limit the extensibility of the composite. Indeed, the breaking energy of byssal threads is reported to be $12.50 \times 10^6 \text{J m}^{-2}$, which is intermediate...
between the breaking energies of tendon (\(2 \times 10^6\) to \(5 \times 10^6\) J m\(^{-3}\)) and silk (\(50 \times 10^6\) to \(180 \times 10^6\) J m\(^{-3}\)) (Denny, 1988).

The rough correlation of Col-P and Col-D gradients in byssal threads with the extensible and stiff portions, respectively, is intriguing. Fig. 9 presents a simplistic model of collagen distribution in byssus that reconciles many of the experimental observations. It is simple because it does not faithfully reflect the extent of overlap in the distribution of Col-P and Col-D that is apparent from Fig. 6. Although the distribution of the two is complementary to some extent, it does not appear to be symmetrically complementary, i.e. Col-D is detectable in all portions of the thread except the cuff, and Col-P does not appear to be present in rigid thread. If, for the sake of argument, the parent molecule of Col-P serves to provide the molecular extensibility of proximal thread, then why should Col-D continue to persist proximally? The results of immunogold labelling studies appear to shed some light on this. Col-D and Col-P are associated with straight and coiled fibers from the distal and proximal portions of the thread, respectively. However, some straight fibers, i.e. Col-D, are carried over into the proximal thread, where they are limited to the peripheral layer of the thread core just inside the cuticular varnish (Fig. 8C). One reason for this carry-over might be to provide shear stability between the rigid cuticular varnish and the extensible thread core. Without this, there is a risk of cuticular delamination during cyclic thread elongation/shrinkage, were it applied directly to the core. Perhaps preCol-

<table>
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<tr>
<th>Amino acid</th>
<th>Collagen type I (rat tail)</th>
<th>Silk fibroin (Braura truncata)</th>
<th>Resilin (Locust)</th>
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Values given in residues per 1000.

D has an elastic modulus intermediate between that of the varnish and the elastic thread components. This would then represent a second gradient perpendicular to the first, in which Col-D decreases as one moves from the cuticular varnish to the core of the proximal thread (Fig. 9). In contrast to the above, Bairati (1991) has proposed an alternative model of the proximal thread based on electron microscopical analysis that places the elastic elements just inside the varnish and the more rigid tensile fibers in the thread core.

The observation that matching complementary distributions of two byssal collagens were found along the long axis of byssal threads and the foot suggests that a compositional gradient has been predetermined and prefabricated in the collagen gland. Although it is premature to say how this has been achieved, there are two intriguing possibilities: (1) that collagen granules are titrated with different proportions of the two collagens along the length of the foot, and (2) that there are two different types of granules, i.e. one for preCol-D and the other for preCol-P, whose relative abundance varies according to their location in the foot. Ultrastructural studies of the collagen gland of M. galloprovincialis (Vitellaro-Zuccarello, 1980) support the presence of two major granule types; however, unpublished immunogold labelling studies of
collagen granules in our laboratory tend to support the former (X. Qin and J. H. Waite, unpublished observations). Until the granule content has been determined, this will remain speculative.

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