Biomechanical properties of fibers assembled from native sea cucumber (*Cucumaria frondosa*) collagen fibrils

Thomas J. Koob, Magdalena M. Koob-Emunds and Douglas Pringle
Skeletal Biology Section, Shriners Hospitals for Children, Tampa, Fl 33612

Echinoderms employ mutable collagenous tissues (MCTs) for a variety of functions including autotomy, locomotion, size and shape changes, energy-free postural control, defense, and feeding. These tissues have in common the ability to undergo rapid and reversible changes in their mechanical properties, alternating between stiff and compliant states over physiological time scales. Mutability derives from cell-mediated changes in interactions between parallel arrays of spindle shaped collagen fibrils: fibrils slide past one another in the compliant state, inter-fibril displacement is prevented in the stiff state. While the specific mechanisms underlying natural changes in inter-fibrillar interactions are not entirely understood, we have identified several proteins that are capable of acting on live dermis specimens[1] or isolated collagen fibrils[4,5]: these include cell-derived stiffening and plasticizing factors that act *in vivo* on the intact dermis MCT, stiparin, a glycoprotein that aggregates purified collagen fibrils, and stiparin-inhibitor, a sulfated polygalactose containing glycoprotein that prevents stiparin from aggregating purified fibrils. We have two objectives for the experiments reported here: the first is to develop a system to examine the endogenous MCT parameters that regulate inter-fibrillar interactions and thereby biomechanical properties in the sea cucumber dermis; the second is to explore the potential of utilizing fibers formed from native sea cucumber collagen fibrils for biomedical applications.

Intact, native collagen fibrils were isolated from the inner dermis from the two ventral interambulacra of *Cucumaria frondosa* as previously described[3] by washing 36 g of minced specimens three times for 30 min each in 250 ml deionized water, treating the washed specimens with 250 ml 4 mM ethylenediamine tretraacetic acid (EDTA) in 50 mM Tris-HCl, pH 8.0 for 24 hr, washing the chelated specimens three times for 30 min each in 250 ml deionized water, then slowly stirring the specimens in 500 ml deionized water for 24 hr; all steps were performed at 4°C. The fibril preparation was centrifuged at 160 x g for 30 min and the resulting suspension of fibrils was separated from the tissue by decanting the upper two thirds off the tissue pellet. Fibers were produced from the fibril preparation by dialyzing 10 ml of the suspension in 6.4 mm diameter SpectraPor 2 dialysis tubing against 0.5 M CH_3COOH for 24 hr at 4°C. The gel formed during dialysis was extruded into water, one end was gripped with a plastic hemostat, and the gel was slowly drawn out of the water and allowed to dry at 16°C for 16 hr. The mechanical properties of fibers were measured with uniaxial tensile tests to failure. Approximately 4 mm of the mid-portion of the fiber segment was hydrated in deionized water, the dry ends were secured to the materials testing system with compression clamps, and the segment was pulled until it ruptured. The force in Newtons (N) was recorded during the test and the maximum force achieved was taken as the tensile strength. For some experiments, the diameter of the hydrated portion of the fiber was measured microscopically before the test; the force at failure was normalized to the calculated cross-sectional area to compute the material strength in Pascals ($Pa = N/m^2$). The hydrated fibers were on average 0.38 mm in diameter. Analysis of proteins and proteoglycans associated with these fibers, as well as extracted fibers described below, was performed by extracting dried segments of the fibers in SDS/PAGE gel sample buffer (1 cm of fiber/100 µl buffer) and electrophoresing the extract directly on Novex 4-20% linear Tris-glycine PAGE pre-cast gels. Gels were stained with Coomassie brilliant blue for proteins and Alcian blue for proteoglycans and glycosaminoglycans.

The tensile strength of fibers produced directly from the fibril preparation by dialysis against acetic acid at pH 2.5 averaged 7.48 +/- 1.10 N (three separate experiments, n = 15). The material strength of these fibers was 101.6 +/- 14.7 MPa. The latter is similar to the tensile strength of tendon fibers at 100 MPa and that of artificial tendons produced from bovine molecular type I collagen polymerized with nordihydroguaiaretic acid at 90 MPa[2].

The basis for the cucumber fiber tensile strength was hypothesized to derive from the ionic interaction between positively charged collagen fibrils (pK of 3.5) and negatively charged chondroitin sulfate chains (pK of 1.0) covalently bound to the intact native fibrils. To examine this hypothesis, dry fibers formed in acetic acid were hydrated in solvents of varying composition for 16 hr at 16°C, they were then dried as described above and subjected to tensile tests after hydrating the mid-portion of the fiber in deionized water. The results are shown in Fig 1. Treating the fibers a second time in acetic acid had no effect on their properties. Incubating the fibers in deionized water lowered the tensile strength by 55%. Sterile filtered sea water lowered the tensile strength by 83%. The greatest reduction was caused by neutral phosphate buffer (0.1 M, pH 7.4) which lowered the tensile strength by over 95%. These data indicate that elimination of the positively ionized groups with sea water and phosphate buffer significantly lowered the tensile strength, thereby supporting the hypothesis that the basis for interfibrillar binding is the ionic interaction caused by the differential ionization of the collagen fibrils and constituent chondroitin sulfate chains.

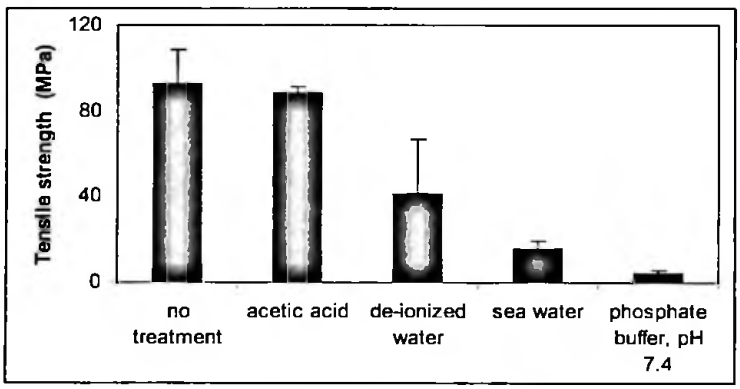

Figure 1. Tensile strength of fibers treated in the indicated reagents, dried and then tested with uniaxial tensile tests to failure. Values shown are means of 5 specimens +/- S.D.

Further support of this hypothesis was gained by adding purified chondroitin sulfate to the fibril preparation before fiber formation. Chondroitin 6-sulfate (Sigma Chemical Company, St. Louis, MO) was added to the fibril preparation at concentrations ranging from 0.05 to 3.0 mg/ml, the mixtures were allowed to equilibrate with gentle stirring for 1 hr, they were then loaded into dialysis bags and dialyzed against acetic acid to form fibers. Chondroitin sulfate caused a concentration dependent decrease in the tensile strength of the fibers (Fig. 2). Lowest tensile strength was achieved at a concentration of 0.5 mg/ml. The capacity of chondroitin sulfate to reduce the tensile strength of the fibers suggests that it competes with the endogenous, fibril-bound glycosaminoglycan for binding sites, and thereby lowers the number of effective cross-links that are responsible for the fiber's tensile strength. Further experiments using unsulfated chondroitin as the competitor will determine whether the sulfate group is responsible for cross-linking the fibrils. In addition, experiments examining the effects of related glycosaminoglycans such as dermatan sulfate, keratin sulfate and hyaluronic acid

may provide evidence for the role of the composition of the glycosaminoglycan disaccharide in mediating interfibrillar interactions.

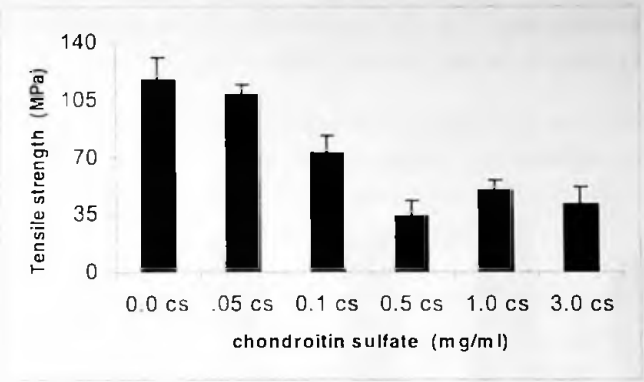

Figure 2. Tensile strength of fibers produced in the presence of varying concentrations of chondroitin 6-sulfate shown on the abscissa. Values are means of 5 specimens +/- S.D.

Production of mechanically testable fibers facilitated experiments to assess the potential role of matrix and cell derived protein factors on the interactions of collagen fibrils in the sea cucumber MCT. The initial approach was designed to examine the effects of removing non-fibrillar macromolecules from the assembled fibers. Fibers were incubated in solvents of varying composition, they were then dried and their tensile strengths were measured. Segments of the same fibers were extracted with SDS/PAGE gel sample buffer and the extract was electrophoresed on 4 – 20% Tris-glycine gels to determine the nature and extent of the proteins remaining in the fibers. Figure 3 shows the proteins remaining in the fibers after incubation in 0.5 M acetic acid, deionized water, sterile filtered sea water, 0.1 M phosphate buffer, pH 7.4, or Hanks balanced salt solution (HBSS) modified with addition of salts for sea water conditions. Acetic acid and deionized water did not appreciably affect the relative amount or composition of the proteins associated with the fibers. Sea water and phosphate buffer removed a substantial amount of these proteins. Hanks balanced salt solution treated fibers retained the least amount of fiber associated proteins.

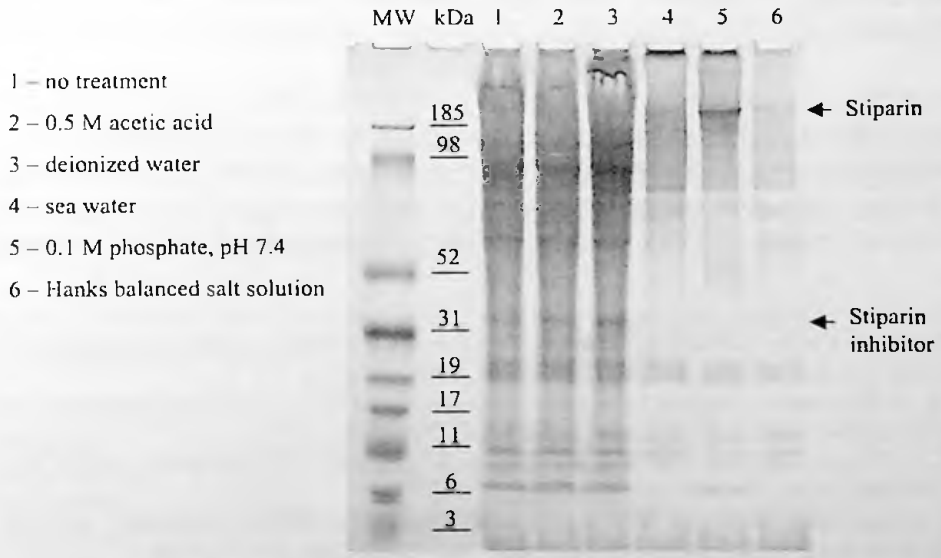

Figure 3. SDS/PAGE analysis of proteins remaining in the fibers after incubation in the indicated solutions.

The tensile properties of the fibers incubated in the solutions described above are shown in Fig. 4. The tensile strength was not affected by any of the incubations, despite the reduction in amount of associated proteins in the fibers as shown above. These results suggest that the basis for the intermolecular interactions responsible for the biomechanical properties of the fibers is likely the collagen fibrils and covalently bound chondroitin sulfate.

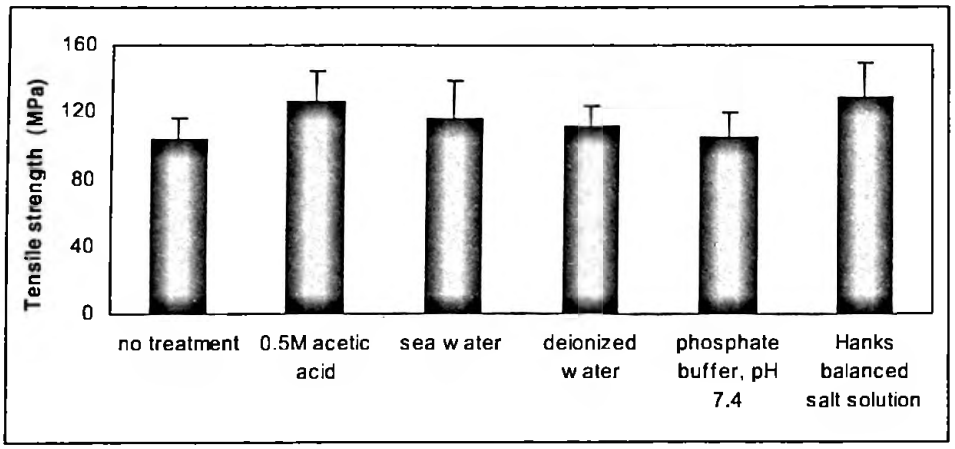

Figure 4. Tensile strength of fibers incubated in the solutions noted on the abscissa. Following incubation, the fibers were dried , re-equilibrated in acetic acid, then dried again. Values shown are means +/- S.D. for 5 specimens.

Taken together the experiments described here establish that a model system can be fabricated to examine the parameters that mediate specific interactions between native collagen fibrils that are involved in determining the tensile properties of echinoderm MCTs and artificial fibrous materials intended for biomedical applications. For MCTs, it will be important first to ascertain the ionic conditions that best replicate the in situ milieu, particularly with respect to divalent anions that might participate in interactions between chondroitin sulfate chains. Future experiments will investigate the effects of including purified extracellular macromolecules, such as stiparin and stiparin inhibitor, and cell derived stiffening and plasticizing factors on the biomechanical properties of the fibers.

The tensile strength of these fibers is particularly intriguing from a biomedical perspective since they are as strong as tendon and ligament fibers without the need for exogenous cross-linking agents. Mimicking the chemistry responsible for the fiber's tensile strength while at the same time formulating it to be stable at neutral pH could prove beneficial for designing biologically based artificial fibrous materials for a variety of applications. Funded by Shriners Hospitals for Children, award 8610.

1. **Koob, T.J., M.M. Koob-Emunds and J.A. Trotter.** Cell-derived stiffening and plasticizing factors in sea cucumber (*Cucumaria frondosa*) dermis. *J. Exp. Bio.* 202: 2291-2301, 1999.
2. **Koob, T.J. and D. J. Hernandez.** Material properties of polymerized NDGA-collagen composite fibers: development of biologically based tendon constructs. *Biomaterials* 23: 203-212, 2002.
3. **Trotter, J.A., F.A. Thurmond and T.J. Koob.** Molecular structure and functional morphology of echinoderm collagen fibrils. *Cell Tiss. Res.* 275: 451-458, 1994.
4. **Trotter, J.A., G. Lyons-Levy, D. Luna, T.J. Koob, D.R. Keene and M.A.L. Atkinson.** Stiparin: A glycoprotein from sea cucumber dermis that aggregates collagen fibrils. *Matrix Biol.* 15: 99-110, 1996.
5. **Trotter, J.A., G. Lyons-Levy, K. Chino, T.J. Koob, D.R. Keene and M.A.L. Atkinson.** Collagen fibril aggregation-inhibitor from sea cucumber dermis. *Matrix Biol.* 18: 569-578, 1999.