# FACTORS FROM SEA CUCUMBER (*CUCUMARIA FRONDOSA*) DERMIS AFFECTING COLLAGEN FIBRIL AGGREGATION

Magdalena M. Koob-Emunds[1], John A. Trotter[2] and Thomas J. Koob[1]

[1]Skeletal Biology, Shriners Hospital for Children, Tampa, FL 33612
[2]Department of Cell Biology and Physiology, University of New Mexico, Albuquerque, NM 87131

Echinoderm catch connective tissues are able to rapidly and reversibly change length and mechanical properties during normal movements and in response to a variety of environmental cues. The basis for this ability is that the extracellular matrix is composed of discontinuous collagen fibrils that can slide past one another in the compliant state but are prevented from doing so in the stiff state. While the mechanisms responsible for mediating the reversible interactions between collagen fibrils in situ, and thereby tissue compliance, have not been unequivocally identified, we have discovered in the dermis of the sea cucumber *Cucumaria frondosa* two cell derived regulatory proteins, one of which ('stiffener') causes live dermis specimens to become stiff, while the other ('plasticizer') causes these specimens to become compliant (Koob et al., *J. Exp. Biol.* 202; 2291-2301, 1999). In addition, two glycoproteins isolated from the dermis, stiparin and a stiparin inhibitor, have been shown to affect aggregation of intact native collagen fibrils in vitro (Trotter et al., *Matrix Biol.* 15, 99-110, 1996; Trotter et al., *Matrix Biol.*, in press, 1999). The present report describes the discovery of two additional factors from the dermis that mediate collagen fibril aggregation in vitro.

Sea cucumbers (*Cucumaria frondosa*) were obtained from the near shore waters of Frenchman Bay either by trawling or diving. Dermis from the two ventral interambulacra was prepared and extracted as previously described (Koob et al., op. cit.). Freeze-thaw extracts of the inner and outer dermis were fractionated over a HiTrapQ anion exchange column and Sephacryl S-100 HR size exclusion column. Fractions were tested for effects on both the compliance of live specimens in bending tests as previously described (Trotter and Koob, *J. Exp. Biol.* 198, 1951-1961, 1995) and for the capacity to influence aggregation of isolated native collagen fibrils. Two distinct preparations of collagen fibrils were used: standard native fibrils with associated matrix macromolecules, and stripped fibrils lacking non-covalently bound macromolecules, including stiparin, which were prepared by extracting tissue with 3 M guanidineHCl prior to fibril isolation. Isolated collagen fibrils remain in suspension in water, but aggregate in the presence of a variety of agents. Standard fibrils, which have stiparin bound to them, aggregate in 0.4M NaCl, 20 mM TrisHCl, pH 8.0. Stripped fibrils do not aggregate in the 0.4 M NaCl buffer. Stiparin causes their aggregation in the 0.4 M NaCl buffer. Stiparin inhibitor prevents this stiparin-induced aggregation. Divalent cations cause aggregation of both standard and stripped fibrils in 20 mM TrisHCl, pH 8.0. Aggregation assays were performed by diluting the fibrils in water to 60 μg collagen/ml with the appropriate buffer (standard fibrils – water; stripped fibrils – 0.4 M NaCl, 20 mM TrisHCl, pH 8.0). Aliquots of fractions from the chromatographic separations were added and the solution was brought to 0.4 M NaCl. The samples were gently mixed and fibril aggregation was assessed by visual inspection. Fibrils remain as a cloudy suspension in the absence of aggregating agents; fibrils form a condensed clot when aggregated.

Extracts of the inner dermis were chromatographed on a HiTrapQ anion exchange column and the fractions were tested for fibril aggregating activity as well as effects on tissue compliance in bending tests. Fraction 6 caused stripped fibrils to aggregate but had no effect on tissue compliance. The previously identified stiffener eluted in fractions 7 – 9 and also induced fibril aggregation. Fractions 10 – 16, which contain stiparin, aggregated stripped fibrils. Fractions 6 - 9 were further fractionated by size exclusion chromatography (Fig. 1). Fractions 8 and 9 induced stripped fibrils to aggregate. Fractions 13 and 14, in which the stiffener elutes, also caused stripped fibrils to aggregate.

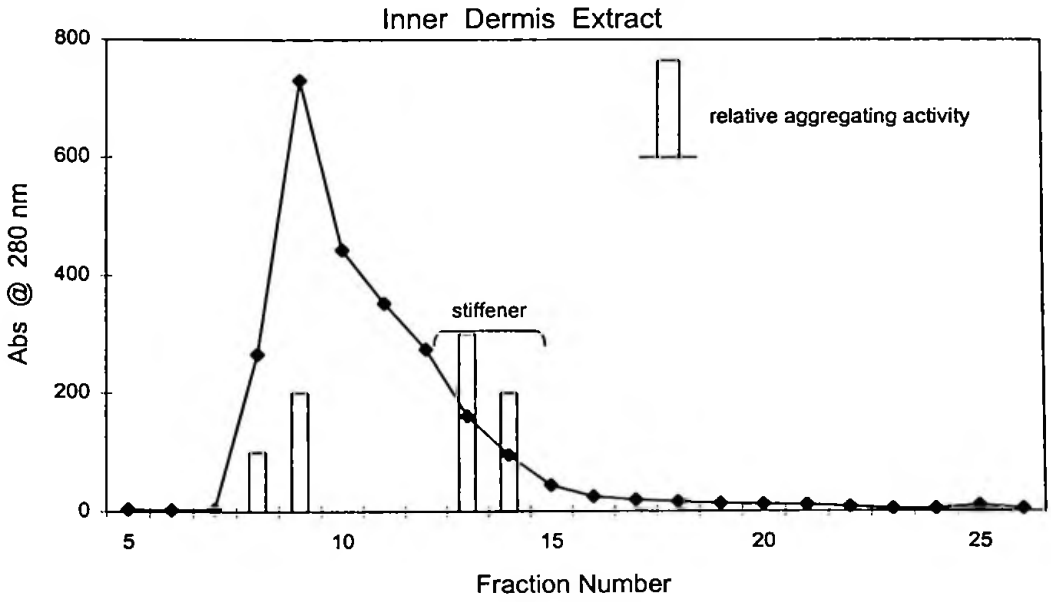


Figure 1. Collagen fibril aggregating activity in fractions from Sephacryl S-100 size exclusion chromatography of anion exchange fractionated inner dermis extracts. The freeze-thaw extract of the inner dermis was first chromatographed over Q Sepharose. Fractions containing fibril aggregating activity were collected and chromatographed on a Hi-Prep 16/60 Sephacryl S-100 HR column. Line shows absorbance at 280 nm. Bars show relative fibril aggregating activity. Aggregation assays were performed with stripped fibrils; 200 µl of fractions 5 through 26 were added to a 0.4 M NaCl, 20 mM TrisHCl, pH 8.0 solution containing 60 µg of stripped fibrils. Bending tests performed on these fractions showed the previously identified stiffener eluted in fractions 13 and 14 as indicated. Fractions 8 and 9, which contained the fibril aggregating factor, did not affect tissue compliance in bending tests; these fractions did not stiffen compliant specimens nor did they plasticize stiff specimens.

Extracts from the outer dermis were similarly fractionated by anion exchange (HiTrapQ) and size exclusion (Sephacryl S-100) chromatography. The plasticizer eluted in fractions 6 and 7; neither fraction caused stripped fibrils to aggregate. A fibril aggregating factor eluted from the HiTrapQ column in fraction 5. This fraction did not affect tissue compliance in bending tests.

Proteins in fraction 5 were further fractionated by size exclusion chromatography. The factor causing stripped fibrils to aggregate eluted from the Sephacryl column in fraction 9 (Fig. 2).

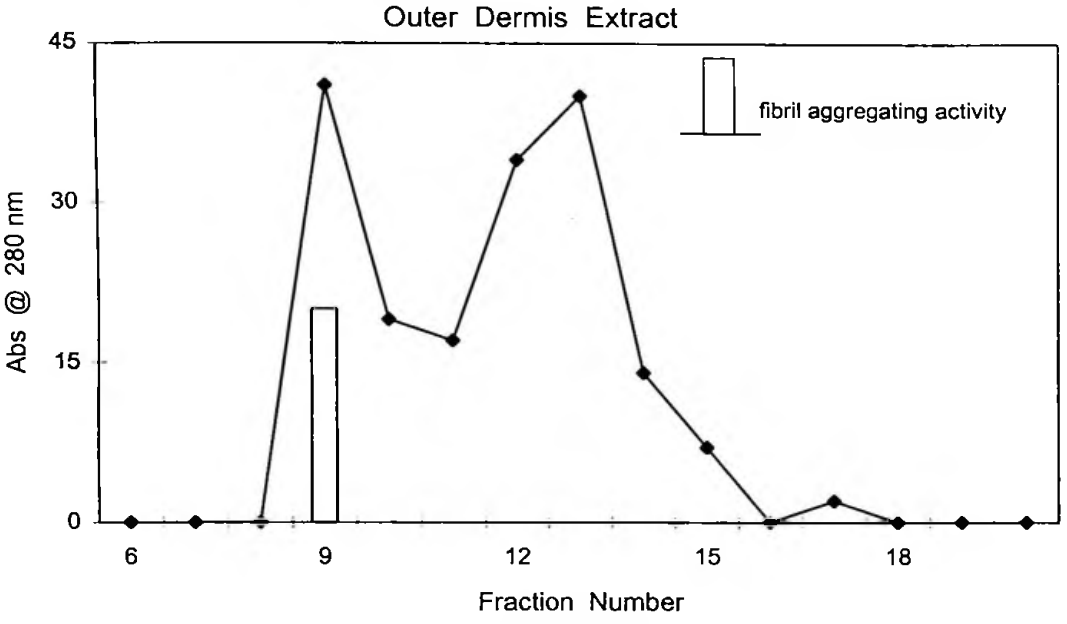


Figure 2. Collagen fibril aggregating activity in fractions from Sephacryl S-100 size exclusion chromatography of anion exchange fractionated outer dermis freeze-thaw extracts. Extraction, chromatography and aggregation assays were performed as described in the legend to Fig. 1. Fibril aggregating activity was assessed with stripped fibrils. Fractions 9, which contained the fibril aggregating factor, did not affect tissue compliance in bending tests; this fraction did not stiffen compliant specimens nor did it plasticize stiff specimens.

The plasticizing factor from the outer dermis renders live cucumber specimens compliant, allowing the collagen fibrils to slide past one another with minimal tensile loads (Koob et al., op. cit.). We hypothesized that this factor would directly inhibit interaction of isolated collagen fibrils in the in vitro aggregation assays. For these experiments, the effects of chromatographic fractions on stiparin induced fibril aggregation were assessed. Freeze-thaw extracts were prepared and fractionated according to established chromatographic methods for the purification of the plasticizer. Fractions 7 - 9 from the HiTrapQ column inhibited stiparin induced aggregation of stripped fibrils; bending tests determined that fractions 8 and 9 contained the plasticizer as previously described. Fractions 7 - 9 were combined and chromatographed on a Sephacryl S-100 gel filtration column. Fractions 12 – 14 contained the fibril aggregation inhibiting activity (Fig 3). These fractions did not affect the bending properties of live specimens. The plasticizer, which eluted in fractions 18 – 20, did not inhibit stiparin induced fibril aggregation.

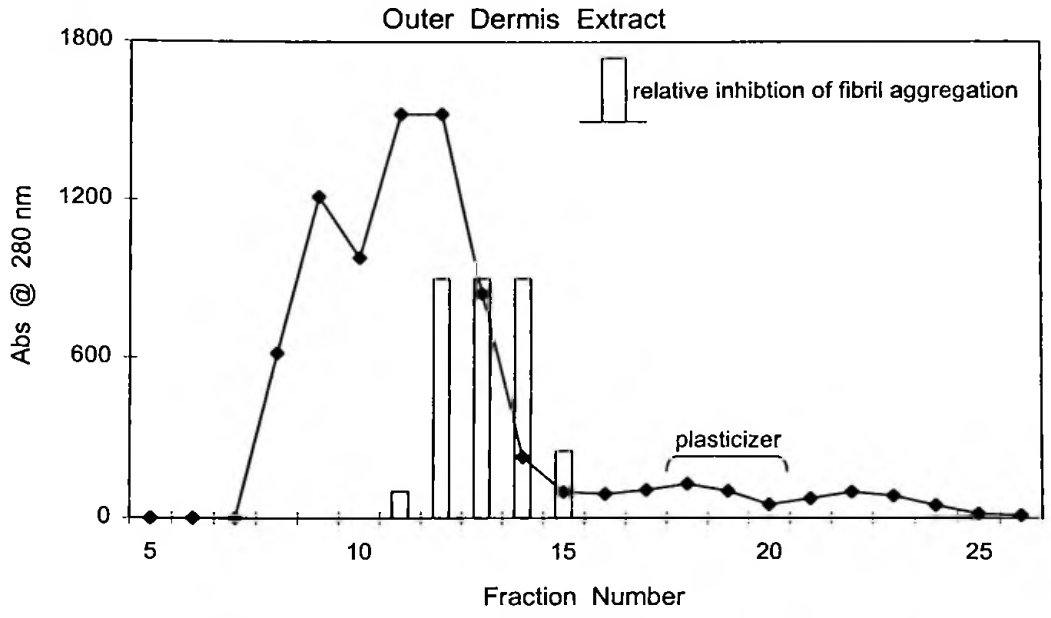


Figure 3. Inhibiting activity on stiparin induced collagen fibril aggregation in fractions from Sephacryl S-100 size exclusion chromatography of anion exchange fractionated freeze-thaw extracts of outer dermis. Extraction and chromatography were as described in the legend to Fig. 1. Aggregation assays were performed with purified stiparin and stripped fibrils: 200 µl of each fraction were added to 60 mg of stripped collagen fibrils in 0.4 M NaCl, 0.2 M TrisHCl, pH 8.0; 2 µg of purified stiparin was then added. Bending tests showed that fractions containing the aggregation inhibitor had no effect on tissue compliance. The plasticizer eluted in fractions 18 to 20 as indicated.

The effects of fractions 12 – 14 on the various means of aggregating collagen fibrils in vitro were examined. The following results were obtained: the factor inhibited stiparin induced aggregation of stripped fibrils but failed to prevent NaCl induced aggregation of standard fibrils; the factor inhibited calcium induced aggregation of stripped fibrils; the factor did not inhibit aggregation of stripped fibrils caused by purified stiffener; the factor bound to standard fibrils, but not to stripped fibrils.

The present observations bring the number of factors that are able to affect in vitro collagen fibril aggregation to six: stiparin, stiparin inhibitor, stiffener, and the three new factors described here. Stiparin, stiffener, and the two factors from inner and outer dermis cause stripped fibrils to aggregate. Stiparin inhibitor and one factor from the outer dermis inhibit stiparin induced fibril aggregation. Whether all of these factors are directly involved in the mutability of the cucumber dermis connective tissue is presently unknown. Only the stiffener is able to affect both tissue compliance in bending tests and aggregation of isolated collagen fibrils. Conversely, while the plasticizer is able to regulate tissue compliance in live specimens, it fails to inhibit fibril aggregation in vitro as shown here. These results implicate a complex array of intracellular and extracellular molecules in the generation and alteration of the biomechanical properties of the dermis. Supported by the National Science Foundation IBN-9723260 (JAT) and The Shriners of North America #8610 (TJK).