IDENTIFICATION OF STIFFENING AND PLASTICIZING FACTORS IN SEA CUCUMBER (CUCUMARIA FRONDOSA) DERMIS

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Certain echinoderm connective tissues can rapidly alternate between stiff and compliant states in response to a variety of environmental and mechanical cues (for recent review see Wilkie, in Echinoderm Studies 5, Ed. Jangoux & Lawrence, 1996). Regulation of tissue stiffness in situ is mediated by resident neurosecretory cells through an as yet undetermined mechanism affecting extracellular matrix mechanical properties. Like all echinoderm tissues so far investigated, the dermis of the sea cucumber, Cucumaria frondosa, responds to changes in extracellular calcium, becoming compliant in the absence of calcium and stiff when calcium levels are returned to normal. Previous studies have demonstrated that this experimental modulation of tissue stiffness, results from a positive effect of extracellular Ca^{2+} not on the extracellular matrix directly, but rather on calcium-channel mediated secretion of cytoplasmic granule-bound molecules (Trotter & Koob, J. Exp. Biol. 198, 1951-1961, 1995). In addition, we have presented preliminary evidence for an organic stiffening agent released by cells (op. cit.). The present report describes the identification of this stiffening agent and of an organic plasticizing molecule.

Cucumaria frondosa were obtained from September through November and maintained in the flow-through sea water tanks at MDIBL. All specimens were prepared from the dermis of the two ventral interambulacra that lack podia. The white inner dermis was used to prepare extracts containing stiffening activity, which was bioassayed as previously described (Trotter & Koob, Bull. MDIBL 34, 6-9, 1995). The black outer dermis from which the epidermis had been removed was used to prepare extracts containing plasticizing activity. The principal test solutions used in these studies were MOPS-buffered artificial sea water (ASW), which consisted of 0.5 M NaCl, 0.05 M $MgCl_2$, 0.01 M $CaCl_2$, 0.01 M KCl, and 0.01 M 3-(N-morpholino)propane sulfonic acid (MOPS), pH 8.0; and EGTA-ASW in which the $CaCl_2$ was replaced by 7.2 mM ethyleneglycol-bis-(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA). As previously established, ASW rendered freshly excised specimens of the inner dermis stiff, whereas these specimens incubated in EGTA-ASW were plastic.

For the preparation of freeze-thaw (FT) extracts, the dermis was cut into roughly 2 x 4 x 6 mm pieces and the dark pigmented outer dermis, including the epidermis, were separated from the white inner dermis with a razor blade. For isolation of stiffening agents, 20g of the inner dermis were finely minced into 1 mm^3 pieces and incubated at ambient sea water temperature in 5 volumes of EGTA-ASW for 5h with intermittent gentle agitation, after which the fluid was decanted and replaced by the same volume of fresh EGTA-ASW. The tissue was frozen in EGTA-ASW for at least 2h at -70°C, followed by incubation at sea water temperature until it was completely thawed. These steps were repeated for a total of five FT cycles. To prepare extracts of the outer dermis, the epidermis was scraped off with a razor blade and the remaining pigmented

part of the outer dermis was minced and extracted with ASW. All other steps were carried out as described above for the inner dermis, except that ASW was used instead of EGTA-ASW for the initial extract. The FT extracts were clarified by centrifugation at 27,000 x g for 30 min. These extracts were then fractionated by anion exchange chromatography, gel filtration chromatography, chromatofusing and hydrophobic interaction chromatography (described below). To assay the extracts and fractions for stiffening and plasticizing activity, four to five replicate specimens were tested in bending. All extracts and fractions to be tested were dialyzed into either EGTA-ASW (stiffener) or ASW (plasticizer) prior to the bending tests.

To prepare uniform test specimens for bending tests, the safety shields of single-edged razor blades were screwed together, either directly or with an intervening metal shim. This cutting apparatus was used to produce equivalent specimens from the white inner dermis that were approximately 3 cm long, 0.85 mm thick, and 1.7 mm wide. The long axis of each specimen was parallel to that of the animal. Gravity bending tests were performed as previously described (Trotter & Koob, Bull. MDIBL op. cit.). The time required for the specimen end to move a vertical distance of 4 mm was measured. All specimens tested were initially incubated for at least 90 min. in either ASW (for plasticizer) or in EGTA-ASW (for stiffener), before they were placed into test solutions. Another 90 min. incubation period in the test solution preceded the bending tests.

Stiffener: FT extracts of the inner dermis were initially applied to a 5 ml anion exchange HiTrap Q column (Pharmacia) in 0.8 M NaCl, 20 mM Tris-HCl, pH 8.0. Stiffening activity was found exclusively in the unbound fraction, whereas all the proteoglycans in the extract were bound to the column, but could be eluted in 3 M NaCl. Following dialysis to reduce NaCl to 0.05 M, the flow through was chromatographed over the same column using a linear gradient from 0.05 to 0.8 M NaCl in 20 mM Tris-HCl, pH 8.0. Bending tests showed that three 2ml fractions eluting between 0.2 and 0.3 M NaCl contained stiffening activity. These fractions were combined, dialysed against 0.8 M NaCl, 20 mM Tris-HCl, pH 8.0 and chromatographed over a 1.6 x 90 cm Sephacryl S-200 gel filtration column (Pharmacia). Bending tests showed that three 5 ml fractions eluting with K_{av} between 0.35 and 0.45 contained stiffening activity (Fig. 1A, fractions 19-21). SDS/PAGE analysis determined that only one protein with apparent molecular weight of approximately 38 kDa was common to these three fractions (Fig. 1B). Chromatofocusing and hydrophobic interaction chromatography confirmed that this protein contained the stiffening activity.

Plasticizer: Only the freeze-thaw extract of the outer dermis contained plasticizing activity; the initial ASW extract had no such activity. This FT extract was subsequently fractionated as described above for the stiffener. Plasticizing activity was found only in the 0.8 M NaCl flow through when first chromatographed on the HiTrap Q column. Only one 2 ml fraction at 0.26 M NaCl from the HiTrap Q displayed plasticizing activity in bending tests. This fraction was subsequently chromatographed on a 1.6 x 60 cm Sephacryl S-100 HR column in 0.8 M NaCl, 20 mM Tris-HCl, pH 8.0; 4 ml fractions were collected. Bending tests determined that one fraction eluting with a K_{av} 0.5 displayed plasticizing activity (Fig. 2, fraction 19). SDS/PAGE analysis of this fraction showed a single Coomassie staining band with an apparent molecular weight of approximately 10 kDa.

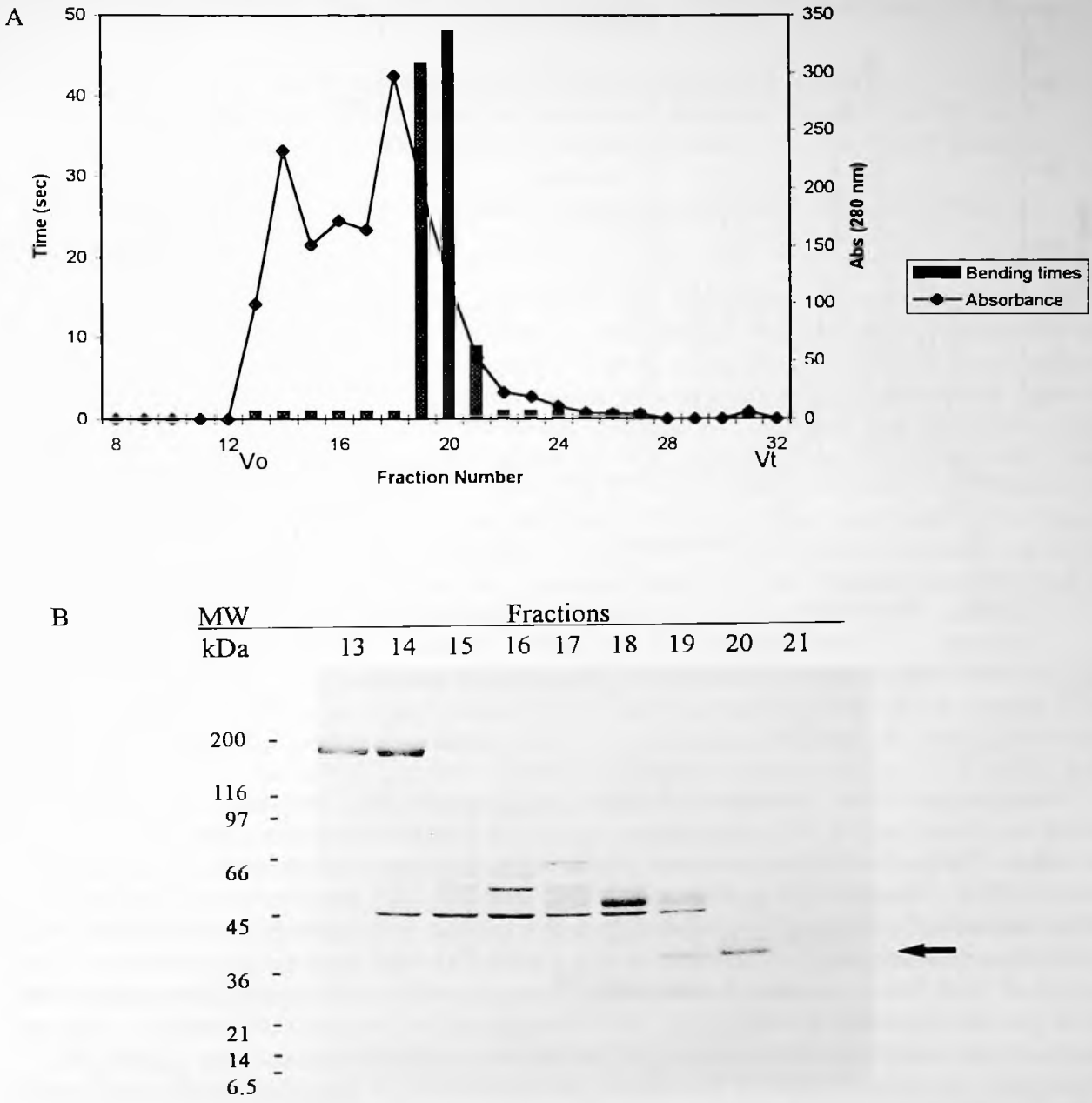

Figure 1. Purification of a stiffening factor from the inner dermis of Cucumaria frondosa. A) Chromatography of active fractions from anion exchange chromatography on Sephacryl S-200 HR. The bars show the results from the bending tests. n = 5 for each bar. B) 4-20% linear gradient SDS/PAGE of fractions from Sephacryl S-200 HR. Arrow indicates the protein with stiffening activity.

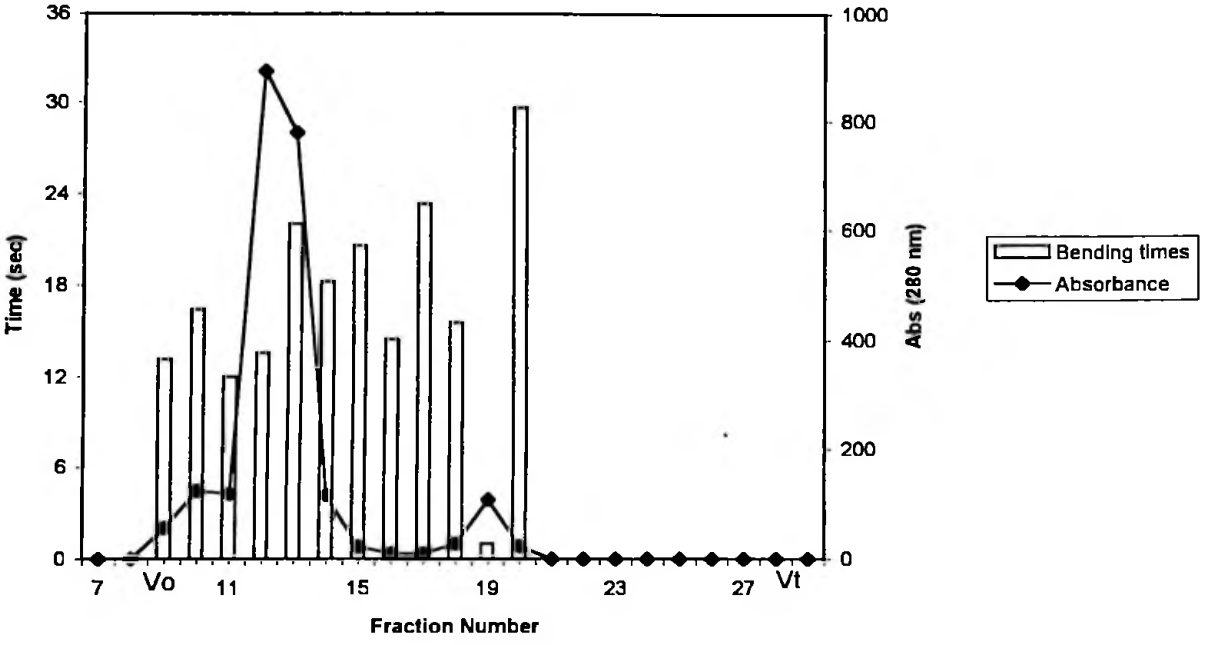

Figure 2. Purification of a plasticizing factor from the outer dermis of Cucumaria frondosa. Chromatography of the active fraction from anion exchange chromatography on Sephacryl S-100 HR. Bars show the results of the bending tests (n = 5 for each bar).

These results confirm and extend our previous observation that modulation of extracellular calcium is not required to alter the compliance of the sea cucumber dermis when live specimens are tested. The purified stiffener increased tissue viscosity in the presence of a calcium chelator (EGTA-ASW). Moreover, the purified plasticizer increased tissue compliance in the presence of normal amounts of calcium (ASW). Since both the stiffener and plasticizer were released only from tissues in which cells were lysed by five freeze-thaw cycles, it appears that significant amounts of these factors are stored within cells. Taken together, these observations suggest that cells in the sea cucumber dermis release either the stiffener or the plasticizer and that these act directly on the matrix macromolecules which mediate tissue mechanical properties.

The identification of these regulatory proteins and development of methods for isolating them in active form, as described here, will allow future experiments to be directed towards: 1) studying the mechanism by which they regulate viscosity of the extracellular matrix of the dermis both in the tissue and with isolated macromolecules; 2) characterizing their biochemical and biophysical properties that are functionally important; 3) delineating the physiological factors that control their secretion.

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