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New insights into the mutable collagenous tissue of *Paracentrotus lividus*: preliminary results*

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Abstract

The mechanically adaptable connective tissue of echinoderms (Mutable Collagenous Tissue—MCT), which can undergo drastic nervously-mediated changes in mechanical properties, represents a promising model for biomaterial design and biomedical applications. MCT could be a source of, or an inspiration for, new composite materials whose molecular interactions and structural conformation can be changed in response to external stimuli. MCT is composed mostly of collagen fibrils, comparable to those of mammals, plus a variety of other components, including other fibrillar structures, proteoglycans and glycoproteins. This contribution presents the preliminary results of a detailed analysis of MCT components in the sea-urchin *Paracentrotus lividus*, focusing on biochemical characterization of the fibrils and biomolecular analysis of the presumptive glycoproteins involved. The final aims will be to confirm the presence and the role of these glycoproteins in echinoids and to manipulate simpler components in order to produce a composite with mutable mechanical properties.

Key words: Mutable Collagenous Tissue, Echinodermata, Echinoidea, glycoproteins, mechanical properties

Introduction

Many echinoderm connective tissues undergo rapid and reversible changes in their mechanical properties (Motokawa 1984; Wilkie *et al.* 2004). The mutability of these tissues is facilitated by distinctive features of their collagen fibrils, which are much shorter than the length of the tissue in which they are found and lack permanent interfibrillar associations (Matsumura 1974; Trotter & Koob 1989; Trotter *et al.* 1996). The load-bearing potential of these tissues depends on the ability of adjacent fibrils to transfer stress via transiently established interactions. The transient nature of these associations accounts for the capacity of such tissues to become reversibly stiff or compliant (Wilkie *et al.* 1993).

MCT is composed mostly of collagen fibrils comparable to those of mammals, plus a variety of other components, including other fibrillar structures (fibrillin microfibrils), proteoglycans and glycoproteins. According to Trotter *et al.* (1996, 2003), the extracellular matrix of holothurians includes at least two important glycoproteins, *stiparin* and *tensilin*, that can modulate the aggregation of collagen

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fibrils and their capacity for reciprocal sliding and establishing interfibrillar links.

The aims of this paper are to present preliminary results concerning (1) the biochemical characterization (purification, extraction and quantification) of collagen and collagen fibrils from the peristomial membrane of the sea-urchin *Paracentrotus lividus*, and (2) the biomolecular characterization of tensilin from the peristomial membranes of *P. lividus* and *Strongylocentrotus purpuratus*.

Materials and Methods

Specimens of *P. lividus* were collected in the Ligurian Sea (Italy), and specimens of *S. purpuratus* were collected along the Connecticut coast (USA) and then delivered to Milan in RNA*later*® conservation solution. Peristomial membranes were dissected from the sea-urchin test, after carefully removing the external epidermis, and then were processed as follows.

Collagen fibril isolation. Collagen extraction was performed according to the protocol of Matsumura (1974). The disaggregation was carried out below 7°C. The tissue was suspended in a solution of 0.5 M NaCl, 0.05 M EDTA-Na, 0.1 M Tris-HCl buffer (pH 8.0) and 0.2 M b-mercaptoethanol. The suspension was stirred for 2 days and then filtered through a 60 mesh sheet of Nylon gauze. The filtered material contained fibrous components and was used for the preparation of collagen fibrils: it was dialyzed against 200 ml of 0.5 M EDTA-Na solution (pH 8.0) and successively against distilled water. The collagen solution was stored at -20°C on dry silica gel. Some samples were stirred continuously in 4 M guanidine chloride in 0.05 M sodium acetate (pH 5.8) or 0.1 N NaOH at 4°C for 24 h.

Collagen quantification. Collagen quantification was performed according to the protocol of Taşkiran *et al.* (1999). Sirius red F3BA, a strong anionic dye, stains collagen by reacting, via its sulphonic acid

TABLE 1. Primers used in amplification and sequencing of tensilin full-length from *S. purpuratus* and *P. lividus*. Sp_F and Sp_R, forward and reverse primers to amplify *S. purpuratus* full-length tensilin; S purp 5' AP and S purp 3' AP, forward and reverse adapter primers for the *in-vitro* translation; P liv ESON F1 and P liv ESON R1, first set of forward and reverse primers to amplify *P. lividus* full-length tensilin; P liv ESON F2, P liv ESON F3, P liv ESON R2 and P liv ESON R3, second set of forward and reverse primers to amplify *P. lividus* full-length tensilin.

Primer	Direction sequence
Sp_F	forward 5'—CACATC TACTCAGCACCATG
Sp_R	reverse 5'—CGTTGGTCGTGTGTGCTAAT
S purp 5'AP	forward 5'—ATATCTCGAGCGGCCGCTAGCTAATACGACTCACTATA-GGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTT-TAAGAAGGAGAGCCACC
S purp 3'AP	reverse 5'—AAATATTCAATAACAAAAAAATGTATCTTTACATT-TAGGTTTATTTAAATACCCGCACCAATTAGTGGTGATGGTGATGATG
P liv ESON F1	forward 5'—CAGTTATGAAGGTGAAAATCAC
P liv ESON F2	forward 5'—GGACGCTGACAACGAGGCA
P liv ESON F3	forward 5'—GTCCATGCAAAAAGCAGTTTTG
P liv ESON R1	reverse 5'—TTACCTCCTATCACATAAGTATC
P liv ESON R2	reverse 5'—GGTGTAGGGATGGAGCAGG
P liv ESON R3	reverse 5'—CTAGGTGAGTAGAAGAAGATGT

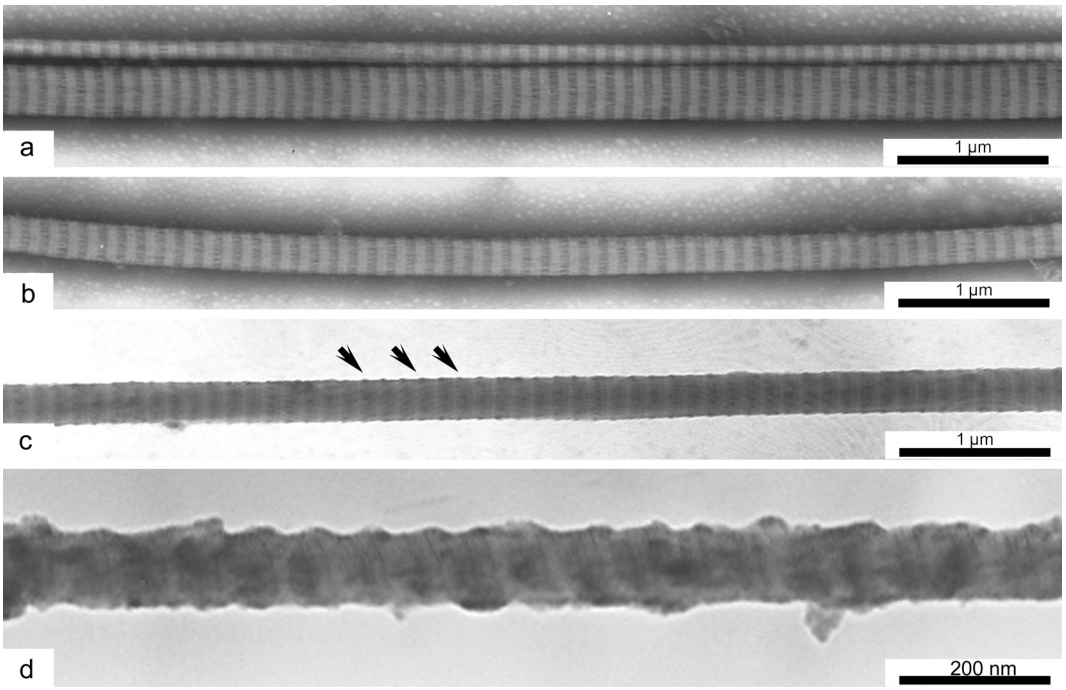

FIGURE 1. Transmission electron micrographs of extracted collagen fibrils from the peristomial membrane of the sea-urchin *P. lividus*. a, b, samples were negatively stained with PTA; c, extracted collagen treated with guanidine chloride; d, extracted collagen treated with NaOH solution. The collagen banding periodicity varied between 60 and 66 nm. Covalently bound PGs appear as black dots on collagen fibrils surface (arrow heads) in extracted collagen.

groups, with basic groups present in the collagen molecule. In the Sirius red assay the sample was lyophilized and added to a solution of 1mg/ml pepsin in 0.5 M acetic acid at 4°C for 3 days. Undigested material was removed by centrifugation. The supernatant was diluted with 1.8 ml of 0.5 mM Sirius red in 0.5 M acetic acid and incubated at room temperature for 20 minutes. The samples were centrifuged at 2500 g for 10 minutes and supernatant absorbance was read at 528 nm against 0.5 M acetic acid as blank. The assay was calibrated using Type I collagen purified from rat tail. The method sensitivity was found to be in the range of 5 to 40 mg/ml of collagen.

Histological techniques. Intact peristomial membranes were treated with 4 M guanidine chloride in 0.05 M sodium acetate (pH 5.8) or 0.1 N NaOH to remove proteoglycans and cells. Samples were continuously stirred in one of these two solutions at 4°C for 24 h, then embedded in paraffin wax. Sections 10 µm thick were stained with Milligan's trichrome or alcian blue at pH 2.5 (Sheehan & Hrapchak 1980) to determine if PGs and cells had been removed. Peristomial membrane in ASW was used as a control.

Biomolecular characterization of *Strongylocentrotus purpuratus* and *Paracentrotus lividus* tensilin. *S. purpuratus* full-length tensilin cDNA was isolated from the peristome tissue by means of RT-PCR using gene-specific primers designed on the sequence (acc. number: XM775549.2) available in the GenBank database (for primer sequences see Table 1). A second round of amplification was performed using an aliquot of the first reaction with two adapter primers (for primer sequences see Table 1) in order to include in the amplicon the 5' and 3' sequences necessary for the following in-vitro translation. The cDNA was gel-purified and in vitro translated using the EasyXpress Insect Kit II (QIAGEN) according to the manufacturer's specifications. In future work the protein will be tested for its collagen-aggregation capability.

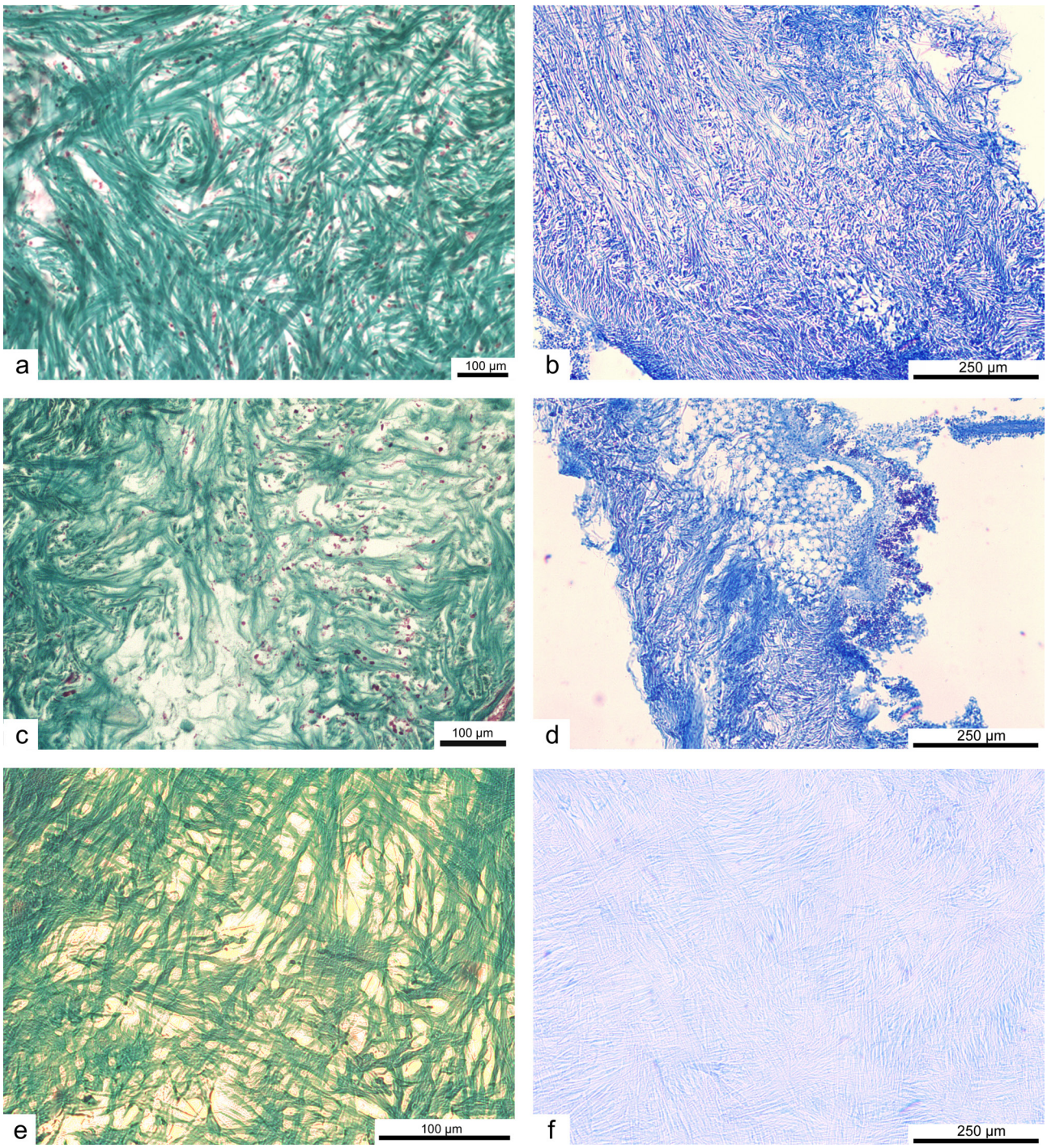

FIGURE 2. Histological sections of *P. lividus* peristomial membrane; a, c, e: stained with Milligan's trichrome stain (collagen fibres are stained blue and cellular elements are stained red), b, d, f: stained with alcian blue (collagen fibres are stained blue due to the presence of negatively charged PGs). a, b: ASW control; c, d: sample treated with guanidine chloride; e, f: sample treated with NaOH solution.

Partial *P. lividus* tensilin cDNA was amplified from the peristome tissue using primers (for primer sequences see Table 1) based on the EST clone available in the GenBank database (acc. number: AM565277.1).

Abbreviations. ASW, artificial sea water; EDTA, ethylenediamine-tetraacetic acid; EST, Expressed Sequence Tags; GAG, glycosaminoglycan; MCT, mutable collagenous tissue; ORF, open reading

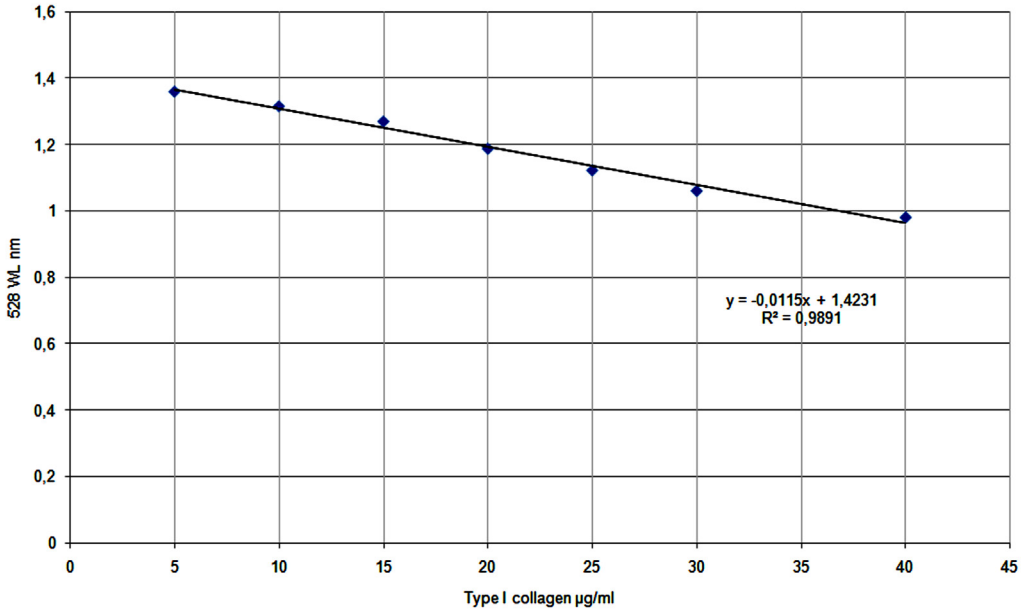

FIGURE 3. Standard curve showing the absorbance of Sirius red at 528 nm versus concentration of type I collagen. Each point is the mean of triplicate samples and the regression coefficient for the line is 0.989.

frame; PG, proteoglycan; PTA, phosphotungstic acid; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase-polymerase chain reaction; Tris, tris[hydroxymethyl]aminomethane; TIMP, tissue inibitors of metalloproteases; MMP, matrix metalloproteases.

Results and Discussion

Collagen extraction from the peristomial membrane of the sea-urchin *P. lividus* was performed according to the protocol of Matsumura (1974). TEM analysis of extracted collagen fibrils stained with PTA demonstrated the effectiveness of the collagen extraction protocol on the peristomial tissue of *P. lividus* (Fig. 1, a-b). The collagen banding pattern of negatively stained isolated fibrils from different individuals was measured. It varied between 60 and 66 nm (mean ± SD 64.5 ± 2.6; n = 35). These values are comparable to those found in the literature (Matsumura *et al.* 1973; Matsumura 1974; Trotter & Koob 1989) for isolated fibrils of sea-urchins and other echinoderms.

Matsumura *et al.* (1973, 1974) first showed that whole collagen fibrils can be isolated from sea cucumbers and starfish by exposure of tissues to a disaggregating solution containing 0.5 M NaCl, 0.2 M b-mercaptoethanol, 0.05 M EDTA, 0.1 M Tris HCl, pH 8.0. Matsumura's method was used subsequently to isolate fibrils from sea-urchin ligaments (Trotter & Koob 1989). We tested two different disaggregating solutions, which included 0.1 M or 0.2 M b-mercaptoethanol. We found that, although tissues began to disaggregate in 0.1 M b-mercaptoethanol, much more collagen could be extracted in 0.2 M b-mercaptoethanol.

The left column of Fig. 2 shows Milligan's trichrome-stained sections of peristomial membranes that were treated with guanidine chloride and NaOH solutions. Cellular material appears to have been unaffected by guanidine chloride but was removed entirely by NaOH.

The right column of Fig. 3 shows alcian blue-stained sections of peristomial membranes that were treated with guanidine chloride and NaOH solutions. Again, whilst guanidine chloride did not affect

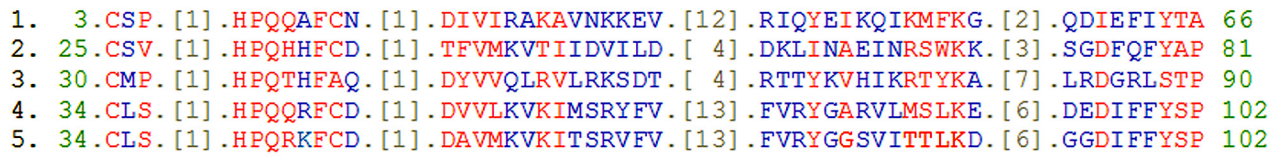

FIGURE 4. Alignment of a fragment of the two tensilin sequences cloned from *S. purpuratus* and *P. lividus* in comparison with known NTR domains of the TIMP-like subfamily in other species. 1. chain T, crystal structure of the Mt1–Mmp–Timp-2 Complex [*Bos taurus*]; 2. tensilin [*Cucumaria frondosa*]; 3. tissue inhibitor of metalloproteases, isoform B [*Drosophila melanogaster*]; 4. similar to tensilin [*Strongylocentrotus purpuratus*]; 5. EST tensilin-like [*Paracentrotus lividus*].

the intensity of staining of the collagen fibers, this was significantly reduced by NaOH. Alcian blue at pH 2.5 has a strong affinity for negatively charged GAGs and so can be considered an indirect measure of GAG and PG content. This is further confirmed by TEM analysis of extracted collagen fibrils treated with guanidine chloride and NaOH solutions and stained with cuprolinic blue (Fig. 1 c-d). Guanidine chloride removes non-covalently bound PGs from collagen fibrils, such as those of the echinoid spine ligament (Trotter & Koob 1989). Since it had no discernible effect on the peristomial membrane, it appears that covalently bound PGs dominate in this tissue.

Figure 3 shows the calibration curve of type I collagen, which indicates a straight-line relationship between absorbance and collagen concentration. The mean wet and dry weights of peristomial membranes of *P. lividus* were found to be 57.07±13.23 mg and 31.92±2.01 mg respectively. The mean total collagen extracted from a single peristomial membrane was found to be 615.04±254.72 mg/ml of solution (Table 2). This spectrophotometric quantification using the dye Sirius red confirmed the effectiveness of the extraction protocol. We found that from a single peristomial membrane of dry weight ca. 30 mg we could extract up to 1 mg of collagen, which is comparable with literature data concerning collagen quantification by different methods (*e.g.*, hydroxyproline test: Bergman & Loxley 1963). Sirius red is a reliable, easy and inexpensive method requiring low cost reagents and equipment and might be used for quantification of the total collagen content in tissues as an alternative to other more expensive methods.

Preliminary data were obtained on the biomolecular characterization of tensilin. We extracted the total mRNA from the peristomial membranes of *S. purpuratus* and *P. lividus* and successfully isolated the sequence encoding tensilin. *S. purpuratus* and *P. lividus* tensilin is characterized by the presence of a conserved domain of the TIMP-like subfamily (NTR domain, Fig. 4). Such domains are essential regulators of extracellular matrix turnover and remodeling. They form complexes with MMPs and

TABLE 2. Wet weight, dry weight, and collagen content of six peristomial membranes. Total collagen levels were expressed as mg/ml of collagen solution extracted from a single peristomial membrane.

Sample	Wet weight (mg)	Dry weight (mg)	Total collagen (mg/ml)
1	55.2	33.0	522.0
2	50.6	30.0	519.4
3	40.2	29.0	302.9
4	60.6	33.5	694.2
5	55.7	32.0	587.2
6	80.1	34.0	1064.6
Mean±SD	57.1±13.2	31.9±2.0	615.0±254.7

inactivate them irreversibly by non-covalently binding their active zinc-binding sites. This is consistent with Tipper and co-workers hypothesis that the tensilin of the holothurian *Cucumaria frondosa* works as a TIMP, inhibits the MMPs and causes the stiffening of the tissue (Tipper *et al.* 2003).

In the near future, we intend to investigate the collagen-aggregation ability of these proteins using techniques previously applied to other mutable collagenous structures (Trotter *et al.* 1996; Tamori *et al.* 2006).

This work is part of a wider project that is exploring the potential of MCT to inspire, and provide biomimetic materials for, economically relevant biotechnological and clinical applications that require the controlled and reversible plasticization and/or stiffening of connective tissues.

Acknowledgements

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