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Tensilin-like stiffening protein from *Holothuria leucospilota* does not induce the stiffest state of catch connective tissue

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Summary

The dermis of sea cucumbers is a catch connective tissue or mutable connective tissue that exhibits large changes in mechanical properties. A stiffening protein, tensilin, has been isolated from the sea cucumber *Cucumaria frondosa*. We purified a similar protein, *H*-tensilin, from *Holothuria leucospilota*, which belongs to a different family to *C. frondosa*. *H*-tensilin appeared as a single band with an apparent molecular mass of 34 kDa on SDS-PAGE. No sugar chain was detected. Tryptic fragments of the protein had homology to known tensilin. *H*-tensilin aggregated isolated collagen fibrils *in vitro* in a buffer containing 0.5 mol l^{-1} NaCl with or without 10 mmol l^{-1} Ca^{2+}. The activity of *H*-tensilin was quantitatively studied by dynamic mechanical tests on the isolated dermis. *H*-tensilin increased stiffness of the dermis in the soft state, induced by Ca^{2+}-free artificial seawater, to a level comparable to that of the standard state, which was the state found in the dermis rested in artificial seawater with normal ionic condition. *H*-tensilin decreased the energy dissipation ratio of the soft dermis to a level comparable to that of the standard state. When *H*-tensilin was applied on the dermis in the standard state, it did not alter stiffness nor dissipation ratio. The subsequent application of artificial seawater in which the potassium concentration was raised to 100 mmol l^{-1} increased stiffness by one order of magnitude. These findings suggest that *H*-tensilin is involved in the changes from the soft state to the standard state and that some stiffening factors other than tensilin are necessary for the changes from the standard to the stiff state.

Key words: catch connective tissue, mutable connective tissue, stiffness, echinoderm, sea cucumber, tensilin.

Introduction

Echinoderms show fast and large changes in the mechanical properties of their connective tissues (Wilkie, 2002; Wilkie et al., 2004). These unique tissues include catch connective tissue (mutable connective tissue) and contractile connective tissue. Catch connective tissue changes its passive mechanical properties but is not contractile in the physiological condition, unlike the contractile connective tissue of crinoids (Birenheide and Motokawa, 1996; Birenheide et al., 2000; Motokawa et al., 2004). Catch connective tissue was regarded as one of the main characteristics of members of the phylum Echinodermata and was believed to have played a key role in the success of this phylum (Motokawa, 1988). This tissue is mostly composed of extracellular materials such as collagen fibres and proteoglycans. Although the changes in the mechanical properties have been attributed to the changes in extracellular materials, the molecular mechanism of the changes is still poorly understood. The changes in mechanical properties are controlled through nerves. Neuropeptides that stiffen or soften the holothurian catch connective tissue have been found in sea cucumbers (Birenheid et al., 1998; Inoue et al., 1999). The scenario so far proposed is that nerves control the secretory activities of some cells whose secretion(s) causes changes in the number of cross-links between extracellular macromolecules and thus stiffens or softens catch connective tissues (Wilkie et al., 2004).

The holothurian dermis is a typical catch connective tissue that changes its mechanical properties in response to various stimuli. These include mechanical, photic, electrical and chemical stimulation (Motokawa, 1981; Motokawa, 1984b). Chemical stimulation by artificial seawater (ASW) with altered ionic composition was often used to induce stiffness changes because of its sustained effect and availability. ASW with an elevated concentration of K^+ (KASW) induces stiffening (Motokawa, 1984c; Trotter and Chino, 1997; Motokawa and Tsuchi, 2003), whereas ASW from which Ca^{2+} is removed

(Ca^{2+}-free ASW) induces softening (Motokawa and Hayashi, 1987; Trotter and Koob, 1995; Trotter and Chino, 1997; Koob et al., 1999; Szulgit and Shadwick, 2000; Motokawa and Tsuchi, 2003). These media induce rapid stiffening or softening that reaches the maximum or minimum stiffness value within 10 min and that value is maintained for not less than 1 h. The changes in the ionic environment are thought to have effects on cellular elements in the dermis rather than directly on its extracellular matrix. KASW is likely to stiffen the dermis through a membrane depolarization because the Triton-extracted dermis does not stiffen in response to KASW (Motokawa, 1994). The removal of Ca^{2+} is believed to soften the dermis by inhibiting the secretion of some stiffening factor(s) from the juxtaligamental cells in the dermis (Wilkie et al., 2004).

The viscoelastic nature of the dermis has been studied by carrying out various mechanical tests, and several mechanical models have been proposed (Motokawa, 1984b; Szulgit and Shadwick, 2000; Motokawa and Tsuchi, 2003). Among these studies, the one by Motokawa and Tsuchi (Motokawa and Tsuchi, 2003) is the most detailed. They carried out dynamic mechanical tests with a wide range of strain and strain rate on the isolated holothurian dermis. They concluded that the dermis assumed three different mechanical states that were distinguished not only by elastic properties such as elastic modulus but also by viscous properties and by strain-dependent behaviours. They referred to the dermis rested in ASW as in the standard state, that in KASW as in the stiff state, and that in Ca^{2+}-free ASW as in the soft state. The mechanical parameters of the standard state did not show the simple intermediate value between those of the soft state and the stiff state, which led the authors to suggest that the stiffening mechanism altering the soft state to the standard state may be different from that altering the standard state to the stiff state. They also showed that the extent of the difference in the elastic modulus was maximal when measured under the dynamic strain imposed at the frequency of 0.3 Hz.

To understand the molecular mechanism of the stiffness changes in catch connective tissues, it is necessary to identify the cell-derived stiffening or softening factors that directly act on the extracellular matrix. Recently, a protein, tensilin, a candidate for the cell-derived stiffening factor, was isolated from the dermis of the dentrochirotid sea cucumber *Cucumaria frondosa* (Koob et al., 1999; Tipper et al., 2003). Tensilin retarded the speed of sagging of the dermis held horizontally at one end to allow downward bending by gravity in Ca^{2+}-free ASW. This tissue-bending test gives no insight into standard mechanical parameters such as absolute stiffness and energy dissipation and thus we have no data to assess how much tensilin contributes to the extremely large changes in the mechanical properties of the dermis. A more quantitative mechanical test is necessary to understand the change in mechanical properties induced by tensilin. Moreover, we do not even know if tensilin has a role in the stiffness changes *in vivo* because tensilin has never been tested on dermis containing Ca^{2+}. As the dermis, whose mineral content is in equilibrium with seawater (Koizumi, 1935; Trotter et al., 1997), contains ~10 mmol l^{-1} Ca^{2+}, we might better reserve nominating tensilin as a candidate for stiffness-controlling proteins *in vivo* until it is proven to work under a natural ionic condition. We also do not know whether tensilin is a general stiffening factor within the class Holothuroidea because no attempts have been made to test it on the dermis of different holothurian species or to isolate similar proteins from other species.

In the present paper, we isolated a homologue of tensilin from the dermis of the sea cucumber *Holothuria leucospilota*, which belongs to the Aspidochirotida, a different family to that of the sea cucumber from which the known tensilin was obtained. Dynamic mechanical tests were employed to quantitatively describe the extent of stiffness and energy dissipation changes. The effect of the homologue of tensilin (*H*-tensilin) was examined under normal ionic conditions with and without Ca^{2+}. The present study showed that *H*-tensilin increased the stiffness in the soft dermis up to the stiffness level of the non-stimulated resting state (standard state), but it did not cause any further increase to reach the high level comparable to the stiffness found in the dermis in the stiff state.

Materials and methods

Animals and tissues

Specimens of the aspidochirotid sea cucumber *Holothuria leucospilota* Brandt were collected near Sesoko Marine Science Center, the University of the Ryukyus, Okinawa, Japan. They were shipped to Tokyo Institute of Technology and kept in an aquarium with closed circulating seawater at 20–23°C. The body wall is made of (starting from the outside) the pigmented epidermis, the thick dermis and the muscle layers, whose coelomic surface is covered by the coelomic epithelium. Unlike dendrochirotid sea cucumbers, the dermis has no apparent separation into outer and inner layers. We used the middle region in thickness of the dermis for both isolation of a stiffening factor and for mechanical tests. For mechanical testing, columnar test pieces were dissected out from the dorsal interambulacral region with a razor blade. Their long axis, along which tensile strain was applied, corresponded to the long axis of the animals. The size of the test pieces was 2×2×7 mm.

Dynamic mechanical test

Artificial seawater with normal composition (nASW) had the following composition: 0.50 mol l^{-1} NaCl, 50 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} KCl, 10 mmol l^{-1} $CaCl_2$ and 10 mmol l^{-1} 3-(*N*-morpholino)-2-hydroxypropanesulphonic acid (MOPS). In Ca^{2+}-free ASW, the $CaCl_2$ was replaced with 7.2 mmol l^{-1} ethyleneglycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). When the effect of elevated concentration of K^+ was investigated, a high K^+ stock solution (0.51 mol l^{-1} KCl, 50 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} $CaCl_2$ and 10 mmol l^{-1} MOPS) was added to the test solution to raise the KCl

concentration to 100 mmol l^{-1}. The pH of solutions used in mechanical tests was adjusted to pH 8.0.

Dynamic tests at the frequency of 0.3 Hz were performed on the isolated dermal pieces. As mentioned in the Introduction, the detailed mechanical dynamic tests covering a wide range of frequencies showed that the frequency of 0.3 Hz reveals the greatest differences between the stiffness value in the stiff state and that in the standard state (Motokawa and Tsuchi, 2003). The dermal pieces were subjected to sinusoidal tensile strain that ranged from 0 to 30% in a cycle. The stress at 0 strain was 0 at the beginning of the mechanical test. The details of the testing device have been described elsewhere (Motokawa and Tsuchi, 2003). As the maximal tensile stress occurred at the maximal strain in a cycle, the maximal stress divided by the maximal strain was defined as stiffness. The stiffness 500 s after the application of chemicals was compared with the value just before the application because the stiffness had reached a plateau or was close to a plateau 500 s after the application. The relative stiffness was calculated by dividing the value 500 s after the application by the value before the application. The mean values of stiffness were given in geometric means. The statistical differences between means of stiffness were tested by *t*-test on the log-transformed data. One cycle of deformation generated a hysteresis loop that was composed of a loading curve and an unloading curve. The energy dissipated corresponded to the area enclosed by the loop. It was expressed as a percentage of the deformation energy, which was the area under the loading curve (Motokawa and Tsuchi, 2003).

The dermis samples tested in Ca^{2+}-free ASW were incubated in the same solution at 4°C for 1 h and then at room temperature for another hour before use. The dermis tested in nASW was rested for 20 min in a trough filled with the same solution.

The experimental trough contained 0.9 ml of the bathing solution, either nASW or Ca^{2+}-free ASW, to which 0.1 ml of a test fraction was added. The test fraction was added after the stiffness value became steady (see Results). When the ionic composition of the fractions was different from that of the bathing solution, the fractions were dialyzed against the bathing solution before mechanical tests. The concentration of the purified stiffening factor used in mechanical tests was 1–3 μg ml^{-1}. The stiffness values given in Results were for a concentration of 3 μg ml^{-1}. The mechanical tests were performed at room temperature (20–27°C), which did not change more than 1°C during a test.

Purification of stiffening factor

The dermis was minced in two volumes of 2 mol l^{-1} NaCl, 10 mmol l^{-1} EGTA, 20 mmol l^{-1} Tris-HCl, pH 8.0. It was frozen at –80°C for at least 3 h and then thawed on ice. It was homogenized, frozen again and re-thawed, and was centrifuged at 27 000 ***g*** for 30 min at 4°C. The supernatant was used to purify the stiffening factor and the precipitate was used to isolate collagen fibrils (see below). The supernatant was precipitated with 60% saturated $(NH_4)_2SO_4$ and the precipitate was then dissolved in 0.5 mol l^{-1} NaCl, 2 mmol l^{-1} EGTA, 20 mmol l^{-1} Tris-HCl, pH 8.0 (Tris-HCl buffer) and dialyzed extensively against the same solution. After centrifugation at 300 000 ***g*** for 30 min, the supernatant was applied to an anion exchange chromatography column (Mono-Q; Pharmacia Biotech, Piscataway, NJ, USA) that had been pre-equilibrated with the same buffer. The column was washed with the same buffer, and stepwise NaCl elutions were successively applied (0.65, 1.0 and 2.0 mol l^{-1}) at a flow of 0.1 ml min^{-1}. The fraction with strongest stiffening activity (0.65–1.0 mol l^{-1} NaCl fraction; see Results) was then loaded onto a gel filtration column (Superose 6 HR 10/30; Pharmacia Biotech) equilibrated with Tris-HCl buffer. Stiffening activities of fractions obtained in all the purification processes were assayed by dynamic mechanical tests on the dermis in Ca^{2+}-free ASW. Protein concentrations were measured with a BCA protein assay reagent kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (Laemmli, 1970). The gels were stained with Coomassie Brilliant Blue R-250 to visualize proteins. To detect sugar chains, proteins in the gel were transferred (100 mA for 1.5 h) to polyvinylidine disulfide (PVDF) membrane, which was stained with a G. P. Sensor stain kit (Honen Corporation, Tokyo, Japan).

Partial amino acid sequences of stiffening factor

Purified stiffening factor was digested with trypsin (from bovine pancreas, TPCK-treated; Sigma, St Louis, MO, USA) and resulting fragments were applied to a reverse-phase high-performance liquid chromatography (HPLC) column (Inertsil 300C8; GL Science Inc., Tokyo, Japan) separated by application of a linear gradient of 10–60% acetonitrile in 0.1% trifluoroacetic acid. N-terminal amino acid sequences of undigested stiffening factor and a tryptic fragment were determined with a protein sequencer (PPSQ-10; Shimadzu, Kyoto, Japan). Obtained sequences were compared with a known sequence of tensilin from *Cucumaria frondosa* using the National Institute of Genetics website (www.nig.ac.jp).

Isolation of collagen fibrils and aggregation assay

The precipitate obtained from 10 g wet mass of dermis (see above) was suspended in 20 ml of 3 mol l^{-1} NaCl. The suspension was centrifuged at 27 000 ***g*** for 10 min at 4°C, and the precipitate was re-suspended in 3 mol l^{-1} NaCl. The cycle of precipitation and re-suspension was repeated four more times, and the precipitate was suspended in 20 ml of distilled water. The centrifugation and re-suspension in distilled water was repeated four more times and the precipitate was suspended in 40 ml of 3 mol l^{-1} guanidine-HCl. After the same centrifugation, the precipitate was suspended in Tris-HCl buffer. The centrifugation and re-suspension in the buffer was repeated three times to remove guanidine-HCl. Trypsin (1 μg ml^{-1}) was added to the suspension, and the suspension was incubated for 12 h at 25°C. Centrifugation and re-suspension in Tris-HCl buffer without trypsin was repeated three more times. We observed long fibres similar to reported

collagen fibrils (Trotter et al., 1995) under a light microscope. The fibrils were re-suspended in the test solution used in the following aggregation assay.

Suspension (20 μl) that contained collagen fibrils from 1.4 mg dermis was mixed with the same volume of test solution either in the presence of calcium (10 mmol l^{-1} $CaCl_2$, 0.5 mol l^{-1} NaCl, 20 mmol l^{-1}, Tris-HCl, pH 8.0) or in its absence (2 mmol l^{-1} EGTA, 0.5 mol l^{-1} NaCl, 20 mmol l^{-1} Tris-HCl, pH 8.0). Whether or not the collagen fibrils were aggregated was observed by eye (see Trotter et al., 1996).

Results

Purification and characterization of stiffening protein

The freeze–thaw extract showed slight stiffening activity. Separation on an anion exchange column yielded stiffening fractions eluted from the column with 1.0 mol l^{-1} NaCl (Fig. 1). They were further separated on a gel filtration column (Fig. 2), and a fraction was obtained with stiffening activity, which contained only a single major protein in SDS-PAGE. The apparent molecular mass from SDS-PAGE was 34 kDa, which is similar to the value of tensilin from *Cucumaria frondosa*. The apparent molecular mass of tensilin, a protein of 260 residues in the predicted mature form, is 33 kDa (Tipper et al., 2003). The yield of the purified stiffening protein from 1 g of dermis (wet mass) was 2.5 μg. The 34-kDa protein migrated at the same speed in SDS-PAGE regardless of the presence or absence of a reducing agent (Fig. 3A), suggesting that the protein is monomeric and contains little, if any, intrachain disulfide bonds. No sugar chain was detected by G. P. Sensor (Fig. 3B).

Partial amino acid sequences

The amino acid sequence of the N-terminus of the stiffening protein was Trp-Gly-Gln-X-Ser-X-Asn (X represents an unidentified amino acid). Among the tryptic fragments, we found one with the sequence Trp-Gly-Gln-His-Ser-Thr-Asn-His-Pro-Gln, which matched the N-terminus. Five of 10 amino acid residues of this sequence were identical to those of the N-terminal region of mature tensilin from *Cucumaria frondosa* (Tipper et al., 2003) (Fig. 4). Thus, we regarded the fragment as the N-terminus. Another tryptic fragment with 15 amino acid residues was sequenced; it had 33% homology to a known sequence of tensilin (Fig. 4). As discussed later, our data including the stiffening activity, molecular mass and amino acid sequences strongly suggested that the present protein from *Holothuria leucospilota* is a homologue of tensilin from *Cucumaria frondosa*. Thus, we called the stiffening protein from *Holothuria leucospilota* *H*-tensilin; the tensilin from *Cucumaria frondosa* is hereafter called *C*-tensilin.

Effect of H-tensilin on stiffness and dissipation ratio in Ca^{2+}-free dermis

In all the dermis pieces in Ca^{2+}-free ASW, stiffness decreased to about one third soon after the start of mechanical testing. Because the strain we used exceeded the maximum strain for induction of stress softening, this softening very likely corresponded to the stress softening characteristic of the dermis in the soft state (Motokawa and Tsuchi, 2003). The stiffness became constant or the rate of decrease became small in a few minutes. The stiffness 5 min after the onset of mechanical testing was 1.3 kPa (Table 1). The application of *H*-tensilin increased the stiffness whereas the addition of the same amount of Ca^{2+}-free ASW without *H*-tensilin did not change its stiffness (Fig. 5). The relative stiffness increased by 3–15-fold. The effect was apparent in a few minutes and the

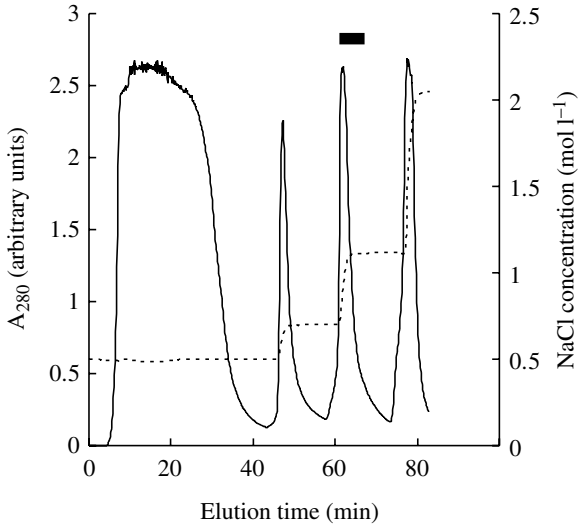

Fig. 1. Anion-exchange chromatography on a Mono-Q column of a freeze–thaw extract of sea cucumber dermis. The solid line shows absorbance at 280 nm; the dotted line shows NaCl concentration. The horizontal bar indicates fractions with strong stiffening activity.

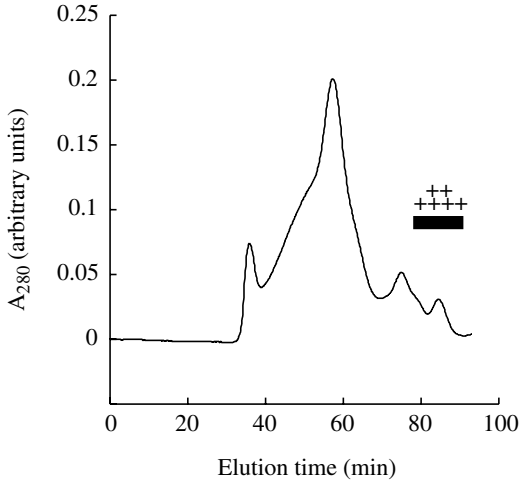

Fig. 2. Gel-filtration chromatography of the active fractions from the Mono-Q column marked by the horizontal bar in Fig. 1. Stiffening protein (*H*-tensilin) was eluted as the fourth small peak. The horizontal bar shows fractions with stiffening activity. Those with weak activity and with strong activity are indicated above the bar by '+' and '++', respectively.

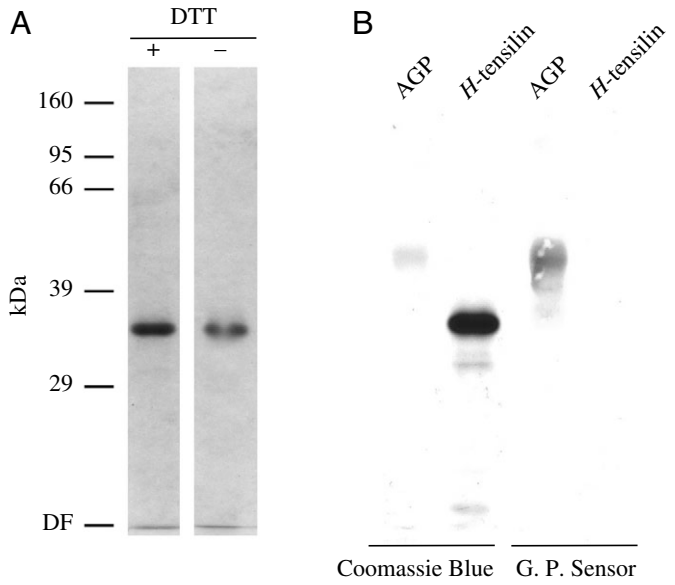

Fig. 3. SDS-PAGE (12.5%) of the purified stiffening protein (*H*-tensilin). (A) *H*-tensilin was run in the presence (left lane) and in the absence (right lane) of 0.1 mol l^{-1} dithiothreitol (DTT). Total protein of 0.5 μg was loaded in each lane. (B) Sugar chain detection. Alpha 1 acid glycoprotein (AGP; positive control; first and third lane) and the purified *H*-tensilin (second and fourth lane) were run on a gel under reducing condition, and the proteins were electroblotted on a PVDF membrane and stained with Coomassie Blue (first and second lane) or with G. P. Sensor stain (third and fourth lane). The same amount of protein was loaded in the first and third lane and in the second and fourth lane. Positions of molecular mass markers and dye front (DF) apply to both A and B.

Probable N-terminus	W G Q H S T N H P Q
C-tensilin (21–31)	Q C A G C S V K H P Q
Tryptic fragment	V E I L Q S E L D S T H Q M K
C-tensilin (43–58)	V T I I D V I L D R Q G G D K

Fig. 4. Peptide sequences of the probable N-terminus (first row) and a tryptic fragment (third row) of stiffening protein (*H*-tensilin). These sequences have homology to part of the sequence of *C*-tensilin (second and fourth row) obtained from *Cucumaria frondosa*. Identical amino acid residues are boxed.

stiffness reached a plateau in 4–10 min in most cases. After reaching a plateau, the stiffness changed little for up to 30 min. When *H*-tensilin was removed by washing with Ca^{2+}-free ASW, stiffness decreased to the level before the application of *H*-tensilin (Fig. 5B). The mean stiffness in *H*-tensilin was 8.9 kPa, which was statistically different from the value before application of *H*-tensilin (P<0.001, N=6) by *t*-test.

A single deformation cycle generated a hysteresis loop with a loading curve and an unloading curve. In Ca^{2+}-free ASW before the application of *H*-tensilin, the unloading curve did not follow the preceding loading curve; the stress in the unloading curve was much lower than that of the loading curve (Fig. 6A). Therefore, the dissipation ratio was as high as 52.3%

Table 1. *Stiffness (kPa) before and after application of H-tensilin and in artificial sea water (ASW) with elevated K^+*

	H-tensilin		
	–	+	K^+
Ca^{2+}-free ASW	1.3±0.15 (6)	8.9±0.14* (6)	–
nASW	16.6±0.55† (9)	16.6±0.54† (9)	123.0±0.38‡ (4)

Values are geometric means ± s.d. of log-transformed value (number of experiments). The mean values of + *H*-tensilin and K^+ are those 500 s after application of 3 μg ml^{-1} *H*-tensilin and 100 mmol l^{-1} K^+ media, respectively.

*Statistically different from the mean value before application of *H*-tensilin (P<0.001).

†Statistically different from the mean value in Ca^{2+}-free ASW without *H*-tensilin (P<0.001).

‡Statistically different from the mean values in normal artificial seawater (nASW) with and without *H*-tensilin (P<0.05) and from that in Ca^{2+}-free ASW with *H*-tensilin (P<0.01).

Table 2. *Dissipation ratio (%) before and after application of H-tensilin and in artificial sea water (ASW) with elevated K^+*

	H-tensilin		
	–	+	K^+
Ca^{2+}-free ASW	52.3±12.9 (6)	33.3±13.0* (6)	–
nASW	38.6±14.7 (9)	37.6±16.5 (9)	19.3±7.9† (4)

Values are arithmetic means ± s.d. (number of experiments). The mean values of + *H*-tensilin and 100 mmol l^{-1} K^+ are those 500 s after application of 3 μg ml^{-1} *H*-tensilin and 100 mmol l^{-1} K^+ media, respectively.

*Statistically different from the mean value before application of *H*-tensilin (P<0.05).

†Statistically different from the mean values in normal artificial seawater (nASW) with and without *H*-tensilin and from that in Ca^{2+}-free ASW with *H*-tensilin (P<0.05).

(Table 2). The addition of *H*-tensilin made the difference between the two curves smaller, and thus the dissipation ratio became smaller (Fig. 6B). The mean dissipation ratio was 33.3%, which was statistically different from that before application of *H*-tensilin by paired *t*-test (P<0.05, N=6) (Table 2).

Effect of H-tensilin on dermis in nASW

The dermis in ASW did not show stress softening at the onset of mechanical testing except in one sample. Other samples showed a slight decrease in stiffness or a transient increase in stiffness by ~2-fold for the first 10–15 min and then the stiffness became rather constant (Fig. 7). The mean stiffness in nASW was 16.6 kPa, which was statistically different from that in Ca^{2+}-free ASW without *H*-tensilin (P<0.001) by *t*-test (Table 1) but was not different from that in Ca^{2+}-free ASW with *H*-tensilin. The mean stiffness was greater than the highest stiffness value (13.5 kPa) of the *H*-tensilin-

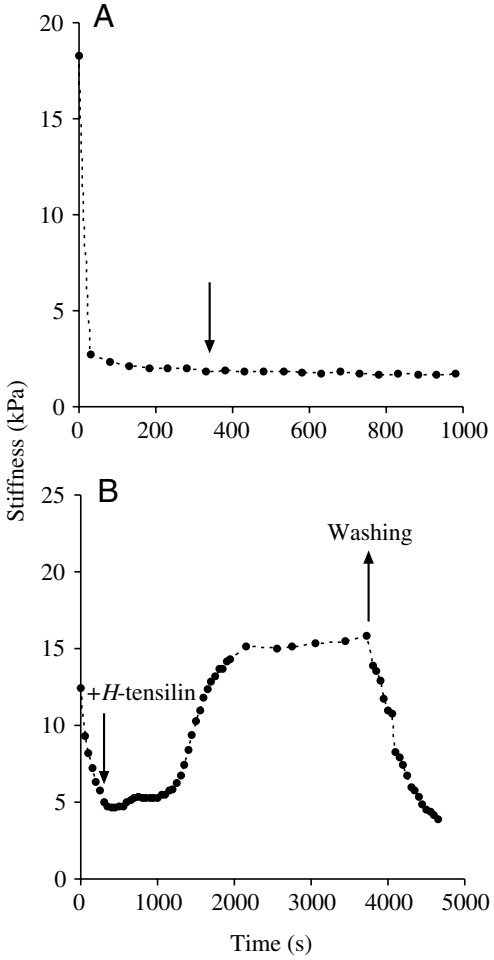

Fig. 5. Typical results of the mechanical test on the dermis in Ca^{2+}-free artificial seawater (Ca^{2+}-free ASW). (A) Mechanical test on a control dermis. The stiffness rapidly decreased at the very start of the dynamic test. Addition of 100 μl Ca^{2+}-free ASW (downward arrow) did not cause a change in stiffness. (B) Effect of 100 μl Ca^{2+}-free ASW containing *H*-tensilin (final concentration; 1 μg ml^{-1}) on the stiffness of the dermis. A rapid decrease in stiffness was observed at the start of the mechanical test, as in A. Application of *H*-tensilin (downward arrow) caused an increase in stiffness, and washing with Ca^{2+}-free ASW (upward arrow) caused a decrease in stiffening.

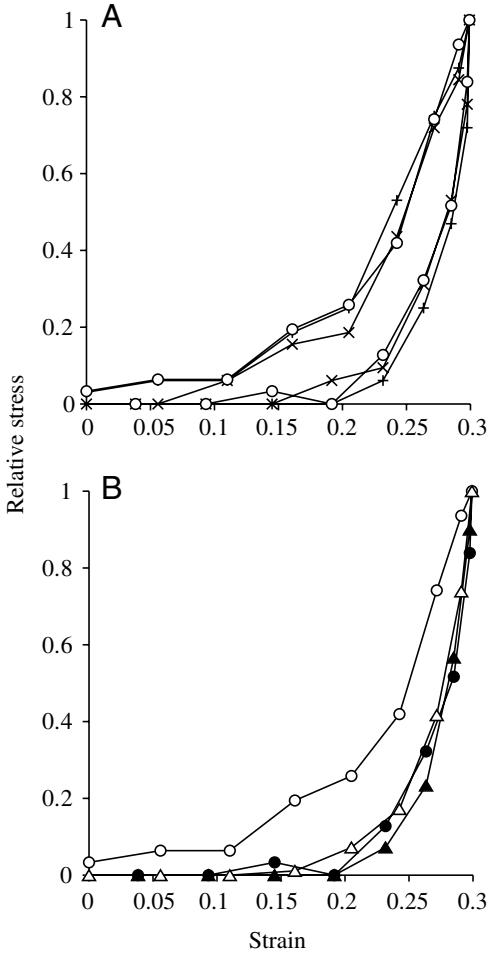

Fig. 6. (A) Stress–strain hysteresis loops obtained from three successive deformation cycles before application of *H*-tensilin in Ca^{2+}-free artificial seawater (Ca^{2+}-free ASW). The shape of the loops did not vary much from cycle to cycle. One of the loops (circles) is also shown, as a control, in B. The stress was normalized by the maximum stress in each hysteresis curve. (B) Typical hysteresis loops before (circles) and after (triangles) application of *H*-tensilin (3 μg ml^{-1}) in Ca^{2+}-free ASW. Empty symbols represent loading curves; filled symbols represent unloading curves. *H*-tensilin reduced the difference between loading and unloading curves.

treated dermis in Ca^{2+}-free ASW. Quite a large variation, ranging from 4.5 kPa to 98.7 kPa, was observed in nASW, as was reported previously (Hayashi and Motokawa, 1986; Motokawa, 1984c). The application of *H*-tensilin had no effect in eight out of nine samples tested (Fig. 7B). The mean stiffness after the application of *H*-tensilin did not differ from 1 before the application (Table 1). In one sample, *H*-tensilin increased the relative stiffness by 1.39-fold. This sample was exceptional both in responding to *H*-tensilin and in showing stress softening at the start of mechanical testing. The stiffness of this sample before the application of *H*-tensilin was 4.57 kPa, which was closest to the lowest value of 4.52 kPa in nASW.

The dissipation ratio of the dermis in nASW before application of *H*-tensilin was 38.6% (Table 2). The application of *H*-tensilin did not change the shape of the hysteresis curve and thus did not change the dissipation ratio: the mean ratio was 37.6%, which was not statistically different from the value before application of *H*-tensilin (Table 2). The dissipation ratios in nASW, with or without *H*-tensilin, were not statistically different from the ratio in Ca^{2+}-free ASW with *H*-tensilin.

Artificial seawater with the potassium concentration raised to 100 mmol l^{-1} was applied. This treatment caused a marked stiffness increase in the dermis that did not respond to *H*-tensilin (Fig. 7B). The stiffness reached a plateau about 500 s after the elevation of the concentration of K^+. A similar increase in stiffness in response to 100 mmol l^{-1} K^+ was also

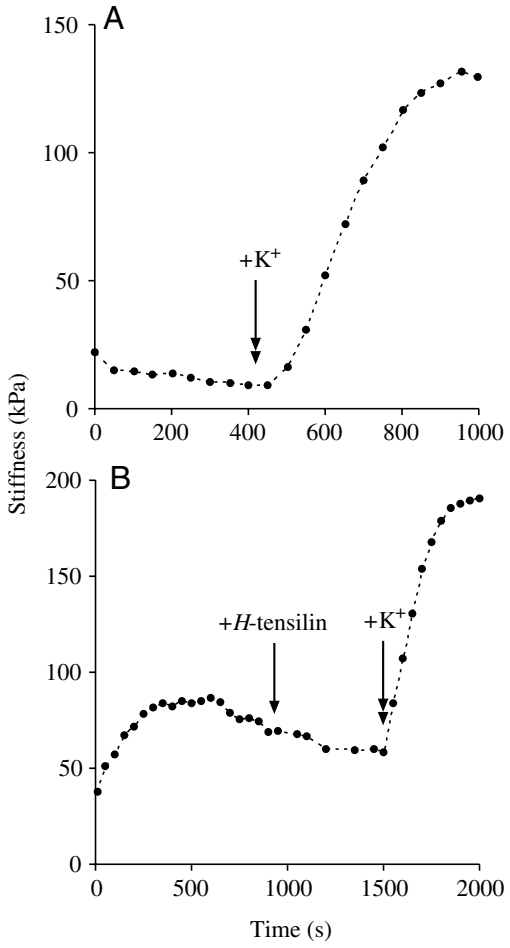

Fig. 7. Typical results of the mechanical test on the dermis in normal artificial seawater (nASW). (A) Effect of elevated K^+ (100 mmol l^{-1}) on the stiffness of the dermis. This sample showed a slight decrease in stiffness after the start of the mechanical test. Elevated K^+ concentration (double-headed arrow) caused an increase in stiffness. (B) Effect of *H*-tensilin (3 μg ml^{-1}) on the stiffness of the dermis. A transient increase in stiffness was observed in the first 10 min after the onset of the mechanical test. The application of *H*-tensilin (single arrow) did not induce stiffening, whereas the subsequent application of ASW with an elevated K^+ concentration (double-headed arrow) caused marked stiffening.

observed in the control dermis (Fig. 7A). The mean stiffness in the high K^+ solution was 123.0 kPa, which was statistically different from those in nASW with or without *H*-tensilin ($P<0.05$) by *t*-test and from that in Ca^{2+}-free ASW with *H*-tensilin ($P<0.01$) (Table 1). The mean dissipation ratio in the high K^+ solution decreased to 19.3%, which was different from means in nASW with *H*-tensilin and without *H*-tensilin by *t*-test ($P<0.05$) (Table 2).

Collagen fibril aggregation by H-tensilin

A clot of fibrils was formed in the collagen-suspension solution when it was mixed with an equal amount of buffer solution containing *H*-tensilin, whose concentration was 20 μg ml^{-1} after mixing. The fibril formation was observed both in media with Ca^{2+} and without Ca^{2+} (Fig. 8).

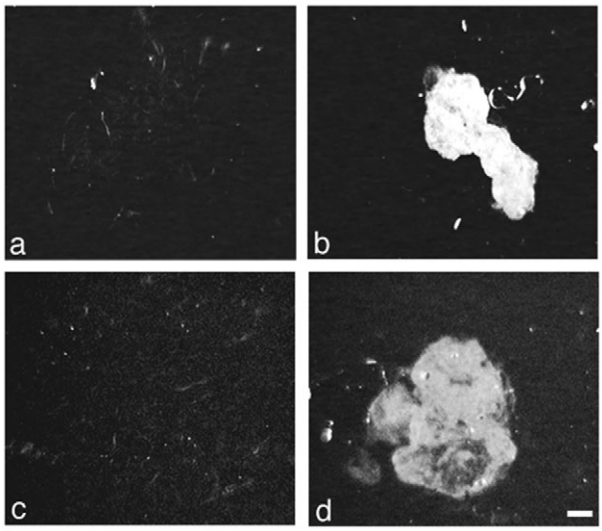

Fig. 8. Collagen fibril aggregation assay in (a,b) Ca^{2+}-containing medium and (c,d) Ca^{2+}-free medium. Addition of the medium containing *H*-tensilin to the suspension caused the aggregation of collagen fibrils into a clot (b,d) whereas addition of the medium alone did not cause the aggregation (a,c). These dark-field light micrographs were taken at low magnification. Concentrations of collagen fibrils were the same from a to d. Scale bar, 500 μm.

Discussion

H-tensilin is a homologue of C-tensilin

We have isolated from the sea cucumber *Holothuria leucospilota* a protein that stiffened the dermis in Ca^{2+}-free ASW. The protein induced aggregation of collagen fibrils *in vitro*. The protein seems to be a single chain because it behaved as a single band in SDS-PAGE with a reducing agent. It contained no detectable sugar chains. These characters, together with the apparent molecular mass and amino acid sequences, strongly suggested that the protein was a homologue of tensilin (*C*-tensilin) isolated from the sea cucumber *Cucumaria frondosa* (Koob et al., 1999; Tipper et al., 2003) and thus we called the present protein *H*-tensilin. Our finding that tensilin exists in two different holothurian families strongly suggests the wide distribution of tensilin in sea cucumbers. Although *H*-tensilin was similar in many respects to *C*-tensilin, they have a few different characters including the amino acid sequence. *H*-tensilin eluted from an anion exchanger at NaCl concentrations between 0.65 and 1.0 mol l^{-1} at pH 8.0 whereas *C*-tensilin eluted from anion exchange chromatography between 0.2 and 0.3 mol l^{-1} NaCl (Koob et al., 1999; Tipper et al., 2003). This difference suggests that *H*-tensilin is more negatively charged than *C*-tensilin at pH 8.0. Another difference was observed: *H*-tensilin had only a small number of intrachain disulfide bonds, if any, whereas *C*-tensilin had them in significant numbers judging from the migration speed in SDS-PAGE with or without a reducing agent (Tipper et al., 2003).

H-tensilin stiffened the dermis in the soft state

Motokawa and Tsuchi, based on their detailed mechanical tests in the holothurian dermis (Motokawa and Tsuchi, 2003),

distinguished three different mechanical states: the soft state, the standard state (intermediate state) and the stiff state. The soft state could be induced in isolated dermis by immersing it in Ca^{2+}-free ASW (Motokawa and Hayashi, 1987); most of the isolated dermal pieces in nASW were in the standard state; the stiff state could be induced in isolated dermis by immersion in ASW with elevated K^+ concentration. The soft state was distinguished from the standard state by low stiffness, a high dissipation ratio and stress softening. The present study confirmed these features. In Ca^{2+}-free ASW, every dermis showed stress softening at the start of mechanical testing and the stiffness was one-seventh of that in nASW. *H*-tensilin had a stiffening effect on the dermis in Ca^{2+}-free ASW, an effect that was already known for *C*-tensilin. The quantitative mechanical tests revealed that *H*-tensilin increased the dermal stiffness to the same level as that of the dermis in nASW and that *H*-tensilin decreased the dissipation ratio to the same level as that of the dermis in nASW. Therefore, *H*-tensilin converted the dermis in the soft state to the standard state.

The dermis is known to have a similar ionic environment to nASW (Trotter et al., 1997). The result that *H*-tensilin had no effect in nASW might infer that tensilin was not responsible for the stiffness changes *in vivo*. It should be noted, however, that Ca^{2+} depletion was not the essential condition for producing the soft state. The isolated dermis in nASW softened in response to various stimuli such as acetylcholine, holokinin, an endogenous peptide of sea cucumbers, and very strong mechanical stimulation (Motokawa, 1987; Motokawa, 1988; Birenheide et al., 1998), and thus the dermis could take the soft state with the presence of normal Ca^{2+} concentration in bathing media. The exceptional case in nASW of the present study did show that tensilin could exert effects, although not strong, under the normal ionic condition with Ca^{2+}. This exceptional sample in nASW was not only exceptional in responding to *H*-tensilin: it was the only sample that showed stress softening and was among the two dermis samples with exceptionally low stiffness in nASW. The stress softening and the low stiffness strongly suggested that it was in the soft state. The possibility has been discussed that, among the isolated dermis in nASW, some were in the soft state and some were in the stiff state, judging from the extremely wide range in the parameters of mechanical properties of the isolated dermis in nASW (Motokawa, 1984c). The wide range in stiffness was also observed in the present study. Thus, we concluded that tensilin had a stiffening effect on the dermis in the soft state irrespective of the presence or absence of Ca^{2+}.

H-tensilin did not produce the stiffest dermis

Based on the levels of stiffness and dissipation ratio and the absence of stress softening, we regarded the dermis in nASW as being in the standard state, with the exception of the one unusual sample discussed above. Instead of stress softening, transient stiffening was observed in nASW several minutes after the onset of dynamic tests in half of the samples. Similar stiffening was reported in other sea cucumbers in nASW (Shibayama et al., 1994). The dermis very likely took the cyclic stretching as mechanical stimulation and responded by stiffening (Motokawa, 1984a). *H*-tensilin had no effects on the dermis in the standard state; subsequent application of ASW with elevated K^+ concentration increased the stiffness by one order of magnitude. Therefore, *H*-tensilin could not account for the stiffness changes from the standard state to the stiff state. The result that *H*-tensilin did not change the dermis in the standard state to the stiff state strongly suggested that some factor(s) other than tensilin was needed to produce the stiffest dermis. The ASW with elevated K^+ was known to induce the stiff state through stimulating nervous or other cellular elements (Motokawa, 1994). The search for a cell-derived factor(s) other than tensilin looks promising; indeed, we have already isolated, from the present sea cucumber species, the fraction that had stiffening effects on the dermis in nASW (A.Y., M.T., K.O. and T.M., unpublished).

The present study showed that *H*-tensilin converted the dermis in the soft state to the standard state but it did not convert the dermis in the standard state to the stiff state. This finding, in turn, could be taken as the chemical evidence for the presence of the three different states that have been inferred through the mechanical studies (Motokawa and Tsuchi, 2003).

Molecular mechanism of stiffening by tensilin

The mechanical behaviour of the dermis in the soft state suggested the presence of two kinds of bonds between force-bearing molecules. The stress softening suggested the presence of bonds irreversibly broken by a large strain. After the stress-softening has completed, the stress–strain curves remained constant: in each cycle, the loading curve always showed higher stiffness than the unloading curve at the same strain. This result suggested the presence of bonds that were temporarily broken at stretching and were recovered when the tensile strain was removed (Motokawa and Tsuchi, 2003). The dermis in the standard state neither showed the stress softening nor the difference in the loading and unloading curves. This suggested that the bonds not broken by strain were dominant in the standard state. Either new bonds were introduced or the labile bonds were converted into nonlabile ones at the transition from the soft state to the standard state. We still do not know how tensilin exerted effects on these changes. Ca^{2+}-free ASW is believed to decrease stiffness by inhibiting the secretion of tensilin from juxtaligamental cells and thus decreasing the extracellular concentration of tensilin in the dermis (Wilkie et al., 2004). The ability to induce collagen-fibril aggregation *in vitro*, which was shown for *H*-tensilin (present study) and for *C*-tensilin (Tipper et al., 2003), might suggest that the aggregation and disaggregation of fibrils directly corresponded to changes in the bonds between force-bearing molecules. We should be cautious, however, as there is a report of a dermal protein, stiparin, with no stiffening effects on the holothurian dermis causing collagen-fibril aggregation (Koob et al., 1999).

Tipper et al. reported that the peptide sequence of *C*-tensilin had 30% identity to tissue inhibitor of metalloproteinases (TIMP) (Tipper et al., 2003). A sequence (His-Pro-Gln)

common to TIMP (Montagnani et al., 2001) was present in *C*-tensilin. *H*-tensilin also had this sequence in the probable N-terminal region. The stiffening activity of tensilin might be through the TIMP activity, but this has not been demonstrated yet. The present study, however, did not support this possibility. *H*-tensilin lacked two cysteine residues near its N-terminus, which were present both in TIMP and in *C*-tensilin (Montagnani et al., 2001; Tipper et al., 2003). The lack in *H*-tensilin was consistent with the result of SDS-PAGE showing little presence of disulfide bonds. In human TIMP-1, these cysteine residues near the N-terminus form intrachain disulfide bonds (Cys1–Cys70 and Cys3–Cys99) (Williamson et al., 1990) that were suggested to be important for TIMP activity (Gomis-Rüth et al., 1997). The location of cysteine residues in other TIMPs is conserved, and thus the disulfide bonds are probably conserved to play important roles in TIMP activities (Montagnani et al., 2001). The lack of such bonds in *H*-tensilin does not favour the idea that TIMP activities are involved in the rapid changes in dermal mechanical properties.

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