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A novel stiffening factor inducing the stiffest state of holothurian catch connective tissue

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SUMMARY

The dermis of sea cucumbers is a catch connective tissue or mutable collagenous tissue that shows large changes in stiffness. Extensive studies on the dermis revealed that it can adopt three different states having different mechanical properties that can be reversibly converted. These are the stiff, standard and soft states. The standard state is readily produced when a dermal piece is immersed in the sea water containing Ca^{2+}, whereas the soft state can be produced by removal of Ca^{2+}. A stiffening protein, tensilin, has been isolated from some sea cucumbers (*Cucumaria frondosa* and *Holothuria leucospilota*). Although tensilin converts the state of the dermis from soft to standard, it cannot convert from standard to stiff. In this study, we isolated and partially purified a novel stiffening factor from the dermis of *Holothuria leucospilota*. The factor stiffened the dermis in normal artificial sea water (ASW) but did not stiffen the soft dermis in Ca^{2+}-free ASW. It also stiffened the dermis that had been converted to the standard state in Ca^{2+}-free ASW by the action of tensilin. These results suggest that the factor produces the stiff dermis from the standard state but cannot work as a stiffener on the soft dermis. Its addition to longitudinal muscles of the sea cucumber produced no effects, suggesting that its effect is specific to the catch connective tissue. Its stiffening activity was susceptible to trypsin, meaning that it is a polypeptide, and its molecular mass estimated from gel filtration chromatography was 2.4 kDa.

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

INTRODUCTION

Catch connective tissues or mutable collagenous tissues of echinoderms can extensively change their mechanical properties such as elasticity and viscosity within a few minutes under the regulation of their nervous system (Motokawa, 1984; Motokawa, 1988; Wilkie, 2002). The tissues contain a large amount of the extracellular matrix, mainly consisting of collagen fibrils, proteoglycans and microfibrils (Trotter and Koob, 1989; Trotter et al., 1994; Thurmond and Trotter, 1996; Thurmond et al., 1997; Szulgit, 2007). The unique properties of these collagenous tissues might be due to lack of permanent associations between the collagen fibrils and the surrounding extracellular matrix because it is easy to isolate collagen fibrils from catch connective tissues – unlike collagenous tissues of adult vertebrates (Matsumura, 1974; Trotter and Koob, 1989; Trotter et al., 1994; Tamori et al., 2006; Szulgit, 2007). It seems that crosslinking of the collagen fibrils with adjacent ones and other components of the extracellular matrix is formed or broken during changes in the mechanical properties of catch connective tissues. The molecular mechanisms underlying the change are, however, not yet fully understood. The holothurian body wall dermis is a typical catch connective tissue that shows rapid and reversible changes in its mechanical properties in response to various stimuli. Extensive studies on the dynamic mechanical properties of the dermis of the sea cucumber *Actinopyga mauritiana* revealed that the tissue can adopt at least three different states (Motokawa and Tsuchi, 2003). These are stiff, standard and soft states, which can be distinguished by elastic and viscous properties and by strain-dependent behaviors. Dermis kept in artificial sea water (ASW) with normal ionic composition including Ca^{2+} (nASW) is in the standard state, that stimulated with acetylcholine or a high concentration of K^+ is in the stiff state, and that in the Ca^{2+}-free ASW (CFASW) is in the soft state. The mechanical parameters of the standard state are not simply the intermediate values between the stiff and the soft states, suggesting that the molecular mechanism converting the soft to the standard state is different from that converting the standard to the stiff state.

Protein factors that alter the dermal stiffness have been found in the body wall dermis of the dendrochirotid sea cucumber *Cucumaria frondosa* (Koob et al., 1999). One of them is tensilin, a 33-kDa protein that increases the stiffness of isolated dermis bathed in CFASW containing EGTA (Tipper et al., 2003). Thus tensilin works as a stiffener on the soft dermis. Tensilin binds to make crossbridges between collagen fibrils *in vitro*. The presence of a plasticizing protein has been also suggested (Koob et al., 1999). In these studies, the stiffness was measured by simple bending tests, which indicated that the factors make the tissue relatively stiffer or relatively softer. The dermis in CFASW is in a soft state; tensilin stiffens such dermis, but whether it stiffens to the standard state or to the stiff state is unknown. Based on these studies, Wilkie (Wilkie, 2005) presented a model of the molecular mechanism of the connective tissue catch. In his model, tensilin stiffens the tissue by forming crosslinks among collagen fibers, and a tensilin-specific protease induces destiffening.

However, we revealed that the mechanism suggested above is at best only part of the whole story (Tamori et al., 2006). We isolated a protein that is similar to tensilin of *Cucumaria frondosa* from the dermis of the aspidochirotid sea cucumber *Holothuria leucospilota*. Its molecular mass was estimated to be 34 kDa. It stiffened the dermis in the absence of Ca^{2+} and induced aggregation of isolated collagen

fibrils irrespective of the presence of Ca^{2+}. These properties resemble those of the tensilin isolated from *Cucumaria frondosa* (*C*-tensilin). Therefore, we regarded it as a counterpart of tensilin in this species and named it *H*-tensilin. Although *H*-tensilin increased the stiffness of the dermis in CFASW to a level comparable to that in the standard state in our dynamic mechanical test, it did not stiffen the dermis in nASW. Thus, *H*-tensilin increased the stiffness in the soft state to the standard state but did not cause any further increase to the stiff state. This result further supports the idea that the molecular mechanism for the stiffening from the soft state to the standard state is different from that from the standard state to the stiff state. Our recent work is also in agreement with this view. It showed that water exudes when the dermis of sea cucumbers stiffens from the standard state to the stiff state but not when the soft dermis in CFASW stiffens through the action of *H*-tensilin (Tamori et al., 2010). Thus, some unknown factor other than tensilin seems to be needed to produce the stiff state.

During the purification process of *H*-tensilin, we found a fraction that increased the stiffness of the dermis in the presence of Ca^{2+} but did not do this in its absence. This factor was hypothesized to be a factor involved in the change of the state from standard to stiff. In the present work, we partially purified this novel stiffening factor. Our data showed that it was a small polypeptide having a molecular mass of *ca*. 2.4 kDa.

MATERIALS AND METHODS

Animals, tissues and dynamic mechanical test

Specimens of the sea cucumber *Holothuria leucospilota* Brandt were collected and kept in an aquarium, as described previously (Tamori et al., 2006). The body wall dermis was dissected and used for isolation of a stiffening factor and collagen fibrils, purification of *H*-tensilin and dynamic mechanical tests. For the mechanical test, the dermal pieces from the dorsal interambulacral region were used. The size of the pieces was 2 mm×2 mm×7 mm or 0.5 mm×1 mm×7 mm. The larger and the smaller ones were used in experimental troughs, whose capacities were 1 ml and 0.2 ml, respectively. Their long axis, along which tensile strain was applied, corresponded to the long axis of the sea cucumbers. The size of the dermal piece did not affect the results obtained. Longitudinal muscles of the body wall were also dissected, cut into strips of ~15 mm×3 mm×3 mm and used to see the effects of the isolated factor on them.

Dynamic mechanical tests of the dermal pieces in nASW and CFASW were performed as described previously (Tamori et al., 2006). In brief, sinusoidal perturbation at the frequency of 0.3 Hz was imposed on the isolated dermal pieces, and the tensile force was monitored. The maximal tensile stress in one stretch–compression cycle occurred at the maximal strain. The maximal stiffness of the sample in one cycle was proportional to the maximal tensile stress, and thus the maximal tensile stress was used as the measure of stiffness. The relative stiffness was the stiffness 500 s after the application of a test fraction that was normalized to the stiffness just before the application of a fraction. A test fraction was added to the trough that contained either 0.9 ml or 0.18 ml of the bathing solution (nASW or CFASW). The added fraction was 0.1 or 0.02 ml in volume, which was one-tenth the capacity of the trough. The nASW contained 10 mmol l^{-1} $CaCl_2$. In CFASW, $CaCl_2$ was replaced by 7.2 mmol l^{-1} EGTA. The concentration of *H*-tensilin used in the mechanical tests was 3 μg ml^{-1}. The mechanical tests were performed at room temperature (20–27°C), which did not change by more than 1°C during any one mechanical test. The data analyzed with statistical tests follow a normal distribution because the test of goodness of fit accepts the null hypothesis except the relative stiffness in CFASW with *H*-tensilin purified by a Superose 12 column. Therefore, statistical differences between means were tested using *t*-tests, except the statistical difference of the relative stiffness before and after the addition of *H*-tensilin, which was tested by sign tests.

Contraction of muscle

Isometric force generated by the longitudinal muscle was measured with a force transducer (LVS-20GA, Kyowa, Japan). One end of the sample was fixed to a holder in an experimental trough filled with 0.9 ml of nASW. The other end was connected to the force transducer by means of a silver chain. When the effects of the isolated factor were tested, 0.1 ml of the fraction was added to the trough. Acetylcholine chloride (Nacalai Tesque, Japan) was added to the trough to a final concentration of 10^{-5} mol l^{-1}.

Purification of *H*-tensilin and isolation of a novel stiffening factor

H-tensilin was purified from the dermis of *Holothuria leucospilota* as described previously (Tamori et al., 2006). In brief, the dermis was first homogenized in 2 mol l^{-1} NaCl, 10 mmol l^{-1} EGTA, 20 mmol l^{-1} Tris-HCl, pH 8.0. Its supernatant after centrifugation was precipitated with 60% saturated $(NH_4)_2SO_4$. The precipitate was dissolved in 0.5 mol l^{-1} NaCl, 2 mmol l^{-1} EGTA, 20 mmol l^{-1} Tris-HCl, pH 8.0 and dialyzed against the same solution. In some experiments, $(NH_4)_2SO_4$ precipitation was eliminated. The crude extract was then applied to a Mono-Q chromatography column (Pharmacia Biotech, USA) equilibrated with the same solution. The flow-through fraction showed stiffening activity of the dermis immersed in nASW (see Results), and therefore it was used for further isolation of the novel stiffening factor (see below). By contrast, stepwise elution fractionation between 0.65 mol l^{-1} and 1.0 mol l^{-1} NaCl was used for purification of *H*-tensilin. This fraction was further applied on a Superose 6 HR 10/30 gel-filtration column (Pharmacia Biotech), and the *H*-tensilin fraction was collected.

The flow-through fraction of the Mono-Q chromatography was applied on a Superose 12 10/300 GL gel-filtration chromatography column (Pharmacia Biotech), and stiffening activities of the obtained fractions were tested in nASW to find the fractions containing the novel stiffening factor.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed using 12.5% gels under reducing conditions, and the gels were stained with Coomassie brilliant blue R-250, as described previously (Tamori et al., 2006). Protein concentration was determined as described previously (Tamori et al., 2006) using bovine serum albumin as a standard.

Collagen aggregation assay

Collagen fibrils were isolated from the dermis, and the aggregation assay was performed in the buffer containing 0.5 mol l^{-1} NaCl with or without 10 mmol l^{-1} Ca^{2+}, as described previously (Tamori et al., 2006).

Treatment with trypsin

To see whether the novel stiffening factor was susceptible to trypsin, the active fraction after the Superose 12 chromatography was treated with trypsin (Sigma, T-1426). Trypsin at the concentration of 0.5–5 μg ml^{-1} was added to the fraction and kept at room temperature for 10 min. The digestion reaction was terminated by the addition of soybean trypsin inhibitor (Sigma, T-9003). Soybean trypsin inhibitor at the concentration of 0.5, 2 and 5 μg ml^{-1} was used for the fraction treated with 0.5, 2 and 5 μg ml^{-1} trypsin, respectively.

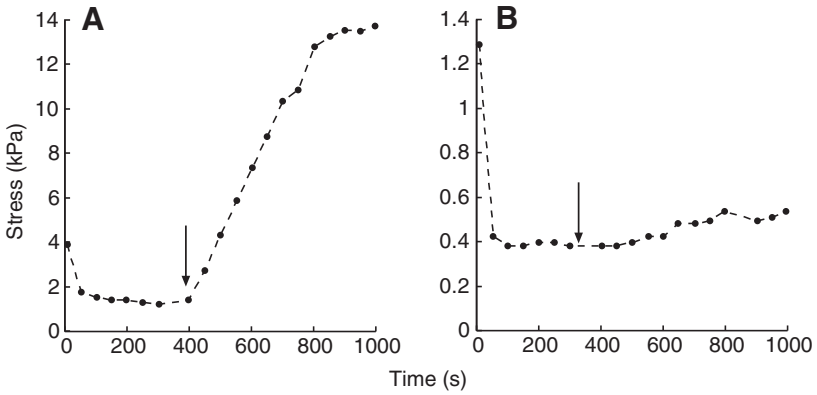

Fig. 1. Typical changes in the mechanical properties of dermal pieces in response to the addition of the flow-through fraction of the Mono-Q chromatography (indicated by the arrows). (A) In nASW containing Ca^{2+}, where the dermis was in the standard state, its stiffness markedly increased in response to the addition of the fraction. (B) In Ca^{2+}-free ASW, where the dermis was in the soft state, little increase in stiffness occurred in response to the addition of the fraction.

As a control experiment, a mixture of trypsin and soybean trypsin inhibitor was added to the fraction and kept at room temperature for 10 min. The trypsin concentration effective in suppressing the activity of the active fraction varied in different batches of the fraction. In the control experiment, the same trypsin concentration as that which suppressed the fraction of the same batch was used.

RESULTS

Isolation of the stiffening factor

During the purification of *H*-tensilin, we kept other fractions in Mono-Q and Superose 6 chromatography, and tested all of them to determine whether they had any effects on the dermis soaked in nASW (in the presence of Ca^{2+}). We found that the flow-through fraction of the Mono-Q chromatography dramatically stiffened the dermis (Fig. 1A). The addition of the fraction increased its stiffness by 3- to 10-fold. The mean relative stiffness in nASW containing the fraction was significantly greater than the value before its addition (Table 1; $P<0.05$). The fraction, however, had little stiffening activity on the dermis immersed in CFASW (Fig. 1B). The stiffening factor in this fraction thus differs from tensilin, which has strong stiffening activity in CFASW but not in nASW.

The active fraction was then loaded on a Superose 12 gel-filtration column (Fig. 2A, continuous line). The stiffening activity appeared in fractions of relatively low molecular mass (horizontal bar in Fig. 2A). The estimated molecular mass was *ca*. 2.4 kDa (Fig. 2B), which was much smaller than that of *H*-tensilin (34 kDa). A typical change in the mechanical properties of the dermis in response to the addition of the active fraction in nASW is shown in Fig. 3A. Shortly after the addition, the stiffness of the dermis began to increase. The stiffness reached a plateau within 10 min in most cases. The active fraction increased the stiffness of the dermis in nASW by 1.6- to 4.3-fold in 500 s. The mean relative stiffness 500 s after its addition was 3.35 (s.d.=1.21; N=5). This value was significantly greater than the one before the addition (Table 1, $P<0.05$). The fraction had no stiffening activity on the dermis in CFASW (see Fig. 6B). The mean relative stiffness 500 s after application of the fraction in CFASW was 0.70 (s.d.=0.16; N=5), which was significantly different from the corresponding value in nASW (Table 1). When *H*-tensilin purified as described in the previous report (Tamori et al., 2006) was applied to the same Superose 12 column, the stiffening activity in the Ca^{2+}-free condition appeared earlier (Fig. 2A, dotted line, arrow 'a'). This peak fraction stiffened all the five samples tested (Table 1). A sign test showed that the increase is statistically significant. Another absorbance peak appeared as indicated by arrow 'b' in Fig. 2A, but this peak fraction did not show any stiffening activity in CFASW (Table 1). Gel electrophoresis in the presence of SDS (Fig. 2A, inset) showed a band of *ca*. 34 kDa in the fraction of arrow 'a' but did not show any bands in the fraction of arrow 'b'. We concluded that the previously reported *H*-tensilin preparation contained some other components and that the peak indicated by the arrow 'a' in Fig. 2A was the real *H*-tensilin component.

We tried to concentrate the stiffening factor by the use of a commercially available ultra-filtration device, Vivaspin 500 (molecular weight cut off=3000; Sartorius AG, Germany), after the Superose 12 chromatography. When the concentrate was appropriately diluted and applied onto the dermal piece in nASW, its stiffness increased dramatically (Fig. 3B). The increase in stiffness was statistically significant (Table 1; $P<0.05$). By contrast, addition of the filtrate did not show any effects (Fig. 3C). The mean relative stiffness of the dermal piece applied with the concentrate was significantly greater than the value applied with the filtrate (Table 1; $P<0.05$). Thus, the factor can be concentrated with this device, suggesting that it is not a small bioactive molecule such as acetylcholine. Hereafter, in this paper, we call this factor the 'novel stiffening factor' (NSF).

Table 1. Relative stiffness of dermal samples after application of fractions

	In CFASW	In nASW
Flow-through[1]	1.29 and 0.65 (2)	5.52±3.15* (5)
NSF[2]	0.70±0.16 (5)	3.35±1.21*,[a] (5)
H-tensilin[3]	7.86±8.75* (5)	–
Peak 'b' fraction[4]	0.87±0.09 (3)	–
Concentrate[5]	–	3.55±2.30*,[b] (7)
Filtrate[5]	–	0.60±0.21 (3)
NSF + trypsin[6]	–	0.72±0.09[c] (4)
NSF + inactivated trypsin	–	1.65±0.50 (4)

Values are 'means ± s.d. (number of experiments)' except 'flow-through in CFASW.' The relative stiffness is the stiffness 500 s after the application of the fraction divided by the value just before its application.

[1]Flow-through fraction from Mono-Q chromatography.

[2]Novel stiffening factor (active fraction from Superose 12 chromatography).

[3]Fraction shown by arrow 'a' in Fig. 2A.

[4]Fraction shown by arrow 'b' in Fig. 2A.

[5]Concentrate or filtrate of the NSF-containing fraction by the use of Vivaspin 500.

[6]Trypsin concentration was 0.5–5 μg ml^{-1}.

*Statistically different from the value before application of the fraction ($P<0.05$).

[a]Statistically different from the value in CFASW ($P<0.01$).

[b]Statistically different from 'filtrate' ($P<0.05$).

[c]Statistically different from the control value given in the line below ($P<0.05$).

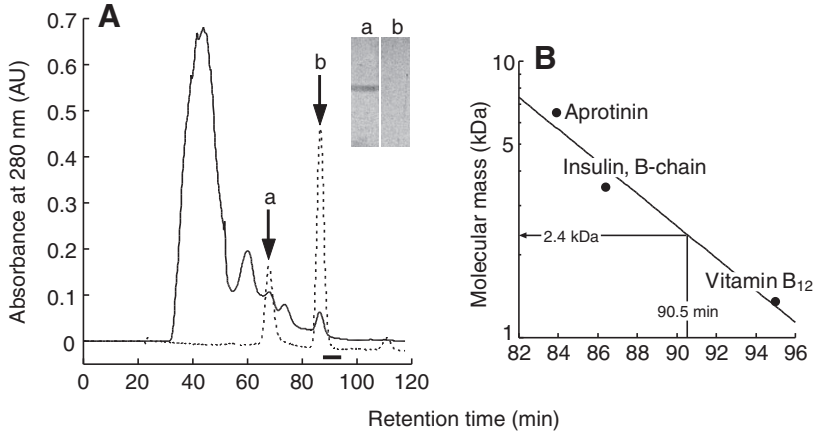

Fig. 2. Superose 12 gel-filtration chromatography. (A) Changes in the absorbance at 280 nm when the flow-through fraction of the Mono-Q chromatography (continuous line) or *H*-tensilin purified as previously reported (dotted line) was loaded. In the former case, the stiffening activity in nASW was detected in the fraction indicated by the black bar (88–93 min). In the latter case, two main peaks were detected ('a' and 'b'). The stiffening activity in Ca^{2+}-free ASW was shown in the peak 'a'. The peak 'b' fraction had no stiffening activity in Ca^{2+}-free ASW. The inset shows the result of SDS-PAGE of the two peak fractions. The 34-kDa protein band was detected in the peak 'a' fraction. (B) Estimation of the molecular mass of the unknown stiffening factor. Aprotinin (Sigma, A-1153), oxidized insulin B-chain (Sigma, I-6383) and vitamin B_{12} (Sigma, V-2876) were used as markers. The estimated molecular mass was 2.4 kDa.

Characterization of the stiffening factor

By using the active fraction of Superose 12 chromatography, we tried to define some characteristics of NSF. Even after a treatment at 80°C for 3 min, NSF retained the stiffening activity of the dermis (data not shown). However, when NSF was treated with trypsin, it lost the stiffening activity (Fig. 4A). As a control experiment, we tested trypsin inactivated by soybean trypsin inhibitor. NSF retained the activity through this treatment (Fig. 4B). The effective concentration of trypsin to the different batch of active fraction was variable, but the fraction mixed with inactivated trypsin always retained stiffening activity in the control experiment irrespective of the concentration of trypsin (N=4). The mean relative stiffness of the dermal piece applied with trypsin-treated NSF was significantly less than that of the corresponding control (Table 1; P<0.05). These results, including the estimation of the molecular mass from the retention time in gel-filtration chromatography, suggested that NSF was a small heat-stable peptide consisting of *ca*. 20 amino acid residues.

We investigated the effects of NSF on the longitudinal body-wall muscle (Fig. 5). The NSF in an amount sufficient to increase dramatically the stiffness of the dermis did not have any effects on the muscle (N=3). But acetylcholine, which also increases stiffness of the dermis (Motokawa, 1987; Motokawa and Tsuchi, 2003), caused contraction of the muscle at a concentration of 10^{-5} mol l^{-1}. The active tension generated by acetylcholine was 11.7±6.8 mN (mean ± s.d.; N=3). The results indicate that NSF is not a neurotransmitter that causes muscle contraction, such as acetylcholine.

In view of Motokawa and Tsuchi's conclusion (Motokawa and Tsuchi, 2003) that holothurian catch connective tissue can adopt three different states and the assumption that tensilin is involved in the conversion of the state from soft to standard, dermis in the standard state can be produced by the addition of tensilin in the Ca^{2+}-free condition. We investigated whether NSF could convert the dermis from the standard to the stiff state. The result is shown in Fig. 6A. At the beginning of the mechanical test, the dermis was immersed in nASW. When the bathing medium was changed to CFASW, the dermis softened. Addition of *H*-tensilin to the soft dermis in CFASW (double-headed arrow in Fig. 6A) increased the stiffness by 4- to 8-fold. The stiffness became almost constant *ca*. 10 min after the addition of *H*-tensilin. After the stiffness reached a plateau, NSF was applied (single-headed arrow in Fig. 6A), and it caused a further increase in stiffness. The addition of *H*-tensilin and the following addition of NSF invariably caused a two-step increase in stiffness in all the dermis tested (Table 2, Experiment A). By contrast, when NSF was first added to the soft dermis in CFASW (Fig. 6B, single-headed arrow), no increase in stiffness was observed. However, the following addition of *H*-tensilin (double-headed arrow in Fig. 6B) dramatically increased stiffness in all three samples tested (Table 2, Experiment B). Thus, NSF seems to increase stiffness of the dermis in the standard state only; it does not have any effects on the stiffness of the dermis in the soft state.

When *H*-tensilin was added to a suspension of collagen fibrils isolated from the dermis, aggregates of the fibrils formed both in

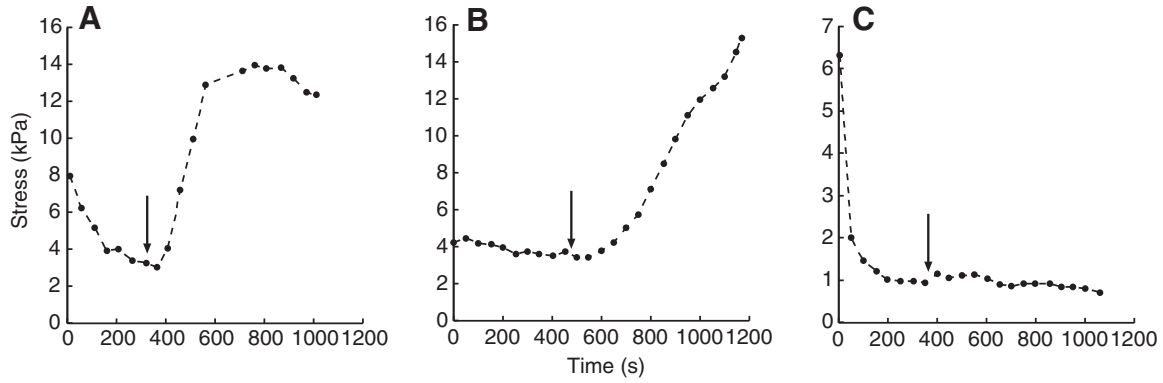

Fig. 3. Typical changes in the mechanical properties of dermal pieces immersed in nASW. (A) When the fraction of the Superose 12 chromatography indicated by the black bar in Fig. 2A was added to the dermis (arrow), the stiffness increased. (B) The active fraction of the Superose 12 chromatography was concentrated with a Vivaspin 500 ultra-filtration device. When the concentrate was added to the dermis (arrow), the stiffness increased. (C) However, when the filtrate was added to the dermis (arrow), the stiffness did not increase.

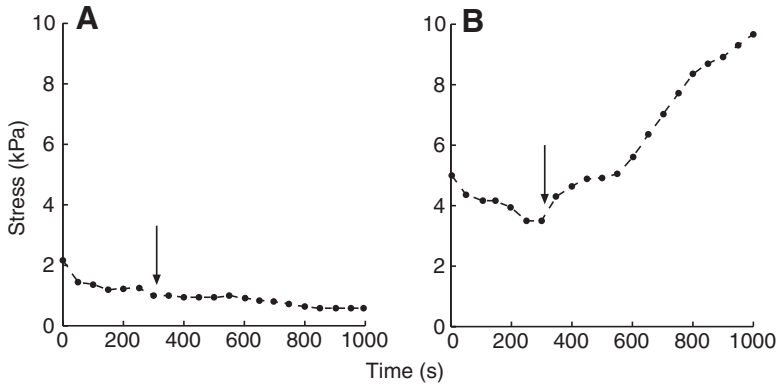

Fig. 4. The effect of trypsin (0.5 μg ml^{-1}) treatment on the activity of the stiffening factor. (A) When a trypsin-treated fraction of the stiffening factor was added to the dermis immersed in nASW (indicated by arrow), the stiffness did not increase. (B) When the fraction of the stiffening factor was first treated with trypsin inactivated with soybean trypsin inhibitor and then added to the dermis (arrow), the stiffness increased – as in the case when the fraction was not treated with trypsin.

the buffer with Ca^{2+} and that without Ca^{2+} (Tamori et al., 2006). By contrast, addition of NSF did not induce such aggregation either in the buffer with Ca^{2+} or that without Ca^{2+} (data not shown). The results suggest that NSF stiffens the dermis by a molecular mechanism different from that of tensilin.

DISCUSSION

Tensilin and the novel stiffening factor

In our previous work, we found that *H*-tensilin increased the stiffness of the dermis in the absence of Ca^{2+} but did not do this in the presence of Ca^{2+} (Tamori et al., 2006). In the present study, we found a factor that increased the stiffness in the presence of Ca^{2+} (Fig. 1). Although the effects appear similar, the conditions under which they are active are very different, suggesting that they are caused by different substances. Indeed, the factors showed different characteristics in column chromatography experiments. Under the standard condition of our purification procedure (0.5 mol l^{-1} NaCl, 2 mmol l^{-1} EGTA, 20 mmol l^{-1} Tris-HCl, pH 8.0), *H*-tensilin was absorbed to the Mono-Q anion exchanger, whereas the currently identified stiffener was not. In the Superose 12 gel filtration chromatography, the retention time of the stiffener was much longer than that of *H*-tensilin (Fig. 2). Its estimated molecular mass was *ca*. 2.4 kDa, which was less than one-tenth that of *H*-tensilin (34 kDa) (Tamori et al., 2006) and *C*-tensilin (33 kDa) (Tipper et al., 2003). In addition, it was heat-stable, unlike *C*-tensilin (Trotter and Koob, 1995). Despite these differences, it seemed to be a polypeptide, as is tensilin, as it was susceptible to trypsin (Fig. 4).

A few substances other than tensilin are known to increase the stiffness of the holothurian dermis. They are acetylcholine and NGIWYamide, which is a neuropeptide isolated from a sea cucumber (Birenheide et al., 1998). These substances stiffen the dermis in nASW (Motokawa, 1987; Birenheide et al., 1998), but the present study excluded the possibility that the present stiffening factor is either of them. Like the present factor, acetylcholine and NGIWYamide should be heat-stable. However, other characters distinguish the present factor from these substances. The estimated molecular mass of the present stiffening factor is larger than that of these substances. Its susceptibility to trypsin digestion also showed that it is neither acetylcholine nor NGIWYamide. Moreover, the present factor did not induce contraction of the longitudinal body wall muscle, unlike acetylcholine (Welsh, 1966) and NGIWYamide (Inoue et al., 1999). Thus, the identified factor is a 'novel stiffening factor' (NSF) that, to our knowledge, has never been reported before. To get more information about this factor, we are trying to get a larger and purer sample.

Role of the factors in the three-state model

Based on detailed mechanical tests in the holothurian dermis, Motokawa and Tsuchi (Motokawa and Tsuchi, 2003) proposed that holothurian catch connective tissue can assume three different states, distinguished by their mechanical properties such as elasticity and viscosity. The soft state could be obtained by putting a dermal sample in a Ca^{2+}-free environment (Motokawa and Hayashi, 1987). The standard state could normally be obtained when the sample was immersed in normal sea water. The stiff state occurred when it was put in artificial sea water with an elevated K^+ concentration. Our previous work revealed that *H*-tensilin did not increase the stiffness of the dermal piece in the standard state in the presence of Ca^{2+}, whereas it did in the soft state to a level comparable to that of the standard state in the absence of Ca^{2+} (Tamori et al., 2006). Thus, *H*-tensilin does not account for the stiffness change from the standard state to the stiff state, suggesting that factors other than tensilin are needed to produce the stiff state. In the present work, we found NSF increased the stiffness in the presence of Ca^{2+}. Although we have not yet fully clarified its identity, it could be the candidate for the factor involved in the stiffness change from standard to stiff. Indeed, even in the absence of Ca^{2+}, it increased the stiffness of the dermis when the standard state had been produced in advance by the addition of *H*-tensilin (Fig. 6). We are currently attempting to identify it.

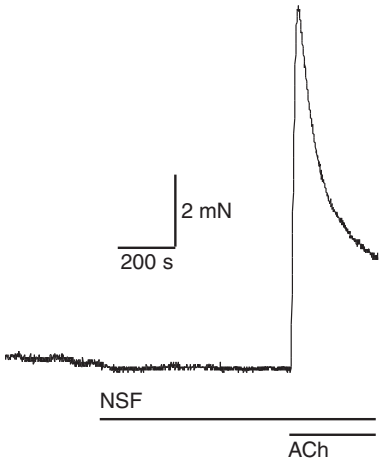

Fig. 5. Change in tension of a strip of longitudinal muscle immersed in nASW. The horizontal bars indicate the presence of chemicals. When NSF that can dramatically increase the stiffness of the dermis was added, tension development did not occur at all. Following the addition of acetylcholine (ACh; 10^{-5} mol l^{-1}), tension then developed.

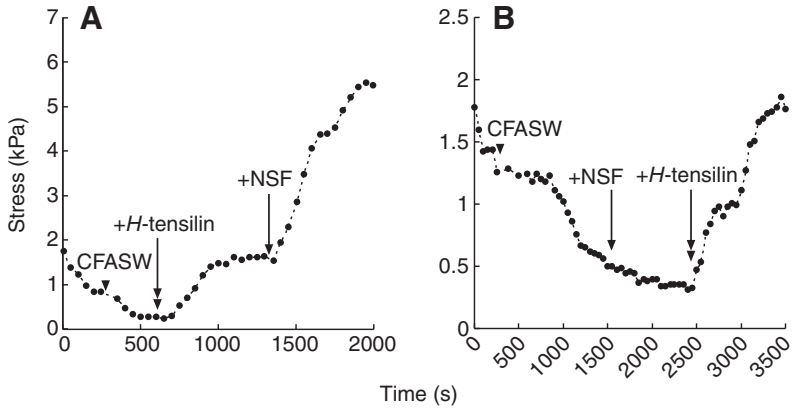

Fig. 6. Changes in the mechanical properties of dermal pieces in response to NSF and *H*-tensilin. (A) The dermal piece was immersed initially in nASW. The surrounding solution was then changed to CFASW, and the dermis became softer. When *H*-tensilin (3 μg ml^{-1}) was added (double-headed arrow), the stiffness increased to some extent. The subsequent addition of NSF (single-headed arrow) dramatically increased the stiffness. (B) After the surrounding solution was changed to CFASW, NSF was added (arrow). The stiffness did not increase in this case. The subsequent addition of *H*-tensilin (double-headed arrow; final concentration, 3 μg ml^{-1}) caused a dramatic increase in the stiffness.

The present work, together with our other recent work, strongly suggests that the molecular mechanisms for stiffening from the soft state to the standard state and from the standard state to the stiff state are different. Our recent work showed that the latter is associated with water exudation, whereas the former is not (Tamori et al., 2010). These results supported Motokawa and Tsuchi's three-state model (Motokawa and Tsuchi, 2003) rather than Wilkie's two-state model (Wilkie, 2005) in which tensilin is regarded as the only stiffener.

In addition to stiffening factors such as tensilin and NSF, factors involved in the reverse changes (plasticizing factors) should exist in catch connective tissues. A plasticizing protein has been partially purified from the outer dermis of the sea cucumber *Cucumaria frondosa* (Koob et al., 1999). As it decreased the stiffness of the dermis in ASW containing Ca^{2+}, it should be effective for the dermis in the standard state. In other words, it should be the factor that turns the state from standard to soft. Other factors that turn the state from stiff to standard might also exist.

Mechanism of the stiffness change by NSF and other factors

The details of the molecular mechanisms underlying the stiffness change of echinoderm catch connective tissue are not yet understood. The dermis of the sea cucumber is composed mainly of extracellular materials such as collagen fibrils and microfibrils. The stiffening activity of NSF is either due to direct action on the extracellular materials or due to action on some cells controlling the dermal stiffness. NSF did not correspond to the known neurotransmitters that stiffen the dermis. Nevertheless, we cannot rule out the possibility that NSF acts on cells. Although this kind of problem could be avoided by the use of dermal samples whose cellular activities have been destroyed by detergent treatment or freeze–thaw procedures, we did not employ them because such procedures might also affect extracellular mechanisms. Especially in the present case, investigation of NSF required dermis to be in the standard state induced by tensilin, and the effect of NSF could be observed only in preparations in which both the tensilin-dependent process and the NSF-dependent process were intact. Because the applicability of results obtained by the cell-disrupted dermis would be uncertain, we used only dermis containing living cells and regarded the determination of the site of action of NSF as being beyond the scope of the present study. The present results suggest, however, that the stiffening action of NSF is directly on the extracellular matrix because, in the presence of active tensilin and EGTA, NSF increases dermal stiffness, whereas the known cellular processes involved in secretions that stiffen the dermis are Ca^{2+} dependent (Trotter and Koob, 1995). Therefore, we confine the following discussion to the possible extracellular effects of NSF and tensilin during the increase in stiffness.

Tensilin was found to stiffen the holothurian dermis in the absence of Ca^{2+}. When it was added to dispersed collagen fibrils isolated from the dermis, the fibrils aggregated (Tipper et al., 2003; Tamori et al., 2006). This suggests that tensilin stiffens the dermis by forming crosslinks between collagen fibrils in the dermis. By contrast, NSF did not form such aggregates either in the presence or the absence of Ca^{2+}. This indicates that its stiffening mechanism is different from that of tensilin. However, we should not rule out possibilities that NSF crosslinks collagen fibrils directly or indirectly by means of, for example, microfibrils. In our procedure for isolating collagen fibrils, the dermis homogenate was treated with trypsin (Tamori et al., 2006). As microfibrils are susceptible to trypsin digestion (Thurmond et al., 1997), they were eliminated from our collagen fibril preparations. Furthermore, trypsin might have partially digested collagen fibrils of the holothurian dermis. If the factor binds to microfibrils or a part of collagen fibrils that is removed by trypsin, no change could be observed in the collagen aggregation assay experiments. Thus, further study, including further purification of NSF, is necessary to understand its stiffening mechanism.

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Table 2. Effects of the successive application of NSF and *H*-tensilin on the relative stiffness of dermal pieces in CFASW

	Experiment A		Experiment B	
	Agent	Relative stiffness	Agent	Relative stiffness
First application	*H*-tensilin	3.70±1.75	NSF	0.65±0.18
Second application	NSF	1.42±0.97	*H*-tensilin	4.38±1.47

In experiment A, *H*-tensilin was first applied and then NSF was applied, as shown in Fig. 6A, whereas, in experiment B, the order of their application was reversed, as shown in Fig. 6B. The relative stiffness is the stiffness 500 s after the application of an agent divided by the value just before its application, and values are means ± s.d. (*N*=3 for both Experiment A and Experiment B).

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