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Physicochemical and Biochemical Characterization of Collagen from *Stichopus* cf. *horrens* Tissues for Use as Stimuli-Responsive Thin Films

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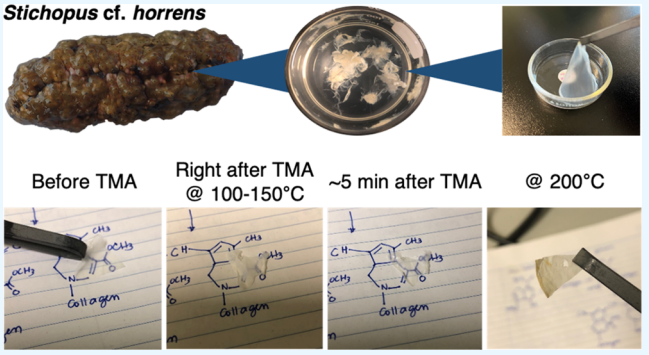
ABSTRACT: The mutable collagenous tissue (MCT) of sea cucumber, with its ability to rapidly change its stiffness and extensibility in response to different environmental stress conditions, serves as inspiration for the design of new smart functional biomaterials. Collagen, extracted from the body wall of *Stichopus* cf. *horrens*, a species commonly found in the Philippines, was characterized for its suitability as stimuli-responsive films. Protein BLAST search showed the presence of sequences commonly found in type VII and IX collagen, suggesting that *Stichopus horrens* collagen is heterotypic. The maximum transition temperature recorded was 56.0 ± 2 °C, which is higher than those of other known sources of marine collagen. This suggests that *S. horrens* collagen has better thermal stability and durability. Collagen-based thin films were then prepared, and atomic force microscopy (AFM) imaging showed the visible collagen network comprising the films. The thin films were subjected to thermomechanical analysis with degradation starting at >175 °C. At 100−150 °C, the collagen-based films apparently lose their translucency due to the removal of moisture. Upon exposure to ambient temperature, instead of degrading, the films were able to revert to the original state due to the readsorption of moisture. This study is a demonstration of a smart biomaterial developed from *S.* cf. *horrens* collagen with potential applications in food, pharmaceutical, biomedical, and other collagen-based research.



INTRODUCTION

Collagen is the most abundant protein component of the extracellular matrices (ECMs) of most organisms.[1,2] Its biocompatibility, low immunogenicity, and high biodegradability have led to its widespread use in food, cosmeceutical, pharmaceutical, biomedical, and other industries.[3] The increase in industrial collagen warrants the need to find alternatives to the commonly used mammalian sources.[4,5] Many studies have already shown that marine invertebrates (e.g., sponge, jellyfish, mussels, and sea cucumbers) can be abundant sources of high-quality collagen. Marine-derived collagen also offers advantages, such as availability, easier processing and extraction techniques, lower inflammatory response, and good metabolic compatibility.[6] Sea cucumbers have been one of the most studied potential sources of marine-derived collagen because their entire body walls are made up of structural components comprising collagen, proteoglycans, and glycoprotein.[1,7]

Another unique characteristic of sea cucumbers is their mutable collagenous tissues (MCTs), which allow the organism to instantaneously change the stiffness and extensibility of their body walls.[8,9] These changes can be triggered by environmental stresses or external stimuli, such as an increase in temperature, pH, ionic composition, or in the presence of predators.[10,11] Marine collagen, with its unique MCT properties, was also reported as a sustainable and green source of native fibrillar collagen in the production of thin membranes for regenerative biomedical applications.[6] Generally, the MCTs of echinoderms are known to produce fibrillar collagen membranes that have ultrastructural and mechanical properties comparable to physiological connective tissues.[12] Thus, many studies focused on the underlying mechanisms behind the mutability of collagenous tissue to help in the design of novel bioinspired materials.[13] Collagen-based membranes have porous and three-dimensional (3D) structures that are easily modifiable depending on the targeted use and application.[12]

Stichopus cf. *horrens* is a sea cucumber species commonly found in the Philippines and other western and central Pacific

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countries.[14] The characterization of collagen from other sea cucumber species has already been conducted, but there are only limited reports on the specific properties of the collagen from *Stichopus horrens*. Furthermore, *S. horrens* was observed to exhibit a swifter response to external stimuli, where its body wall "melts" into a viscous mass upon exposure to stress, which also required further investigation.

In this study, collagen was extracted from adult *S.* cf. *horrens*, and its biochemical and physicochemical properties were characterized and compared to commercial rat tail collagen. To demonstrate the feasibility of utilizing *S. horrens* collagen in the preparation of biomaterials, thin films were developed using the solvent casting method. The collagen-based thin film was tested for its potential stimuli-responsive properties using thermomechanical analysis (TMA). This study shows that *S.* cf. *horrens* is a good alternative source of high-quality collagen that has properties comparable to those of mammalian-derived collagen. Furthermore, *S. horrens* collagen also has distinct properties that have not yet been observed in other sea cucumber species, which can be useful in the development of new bioinspired smart materials.

RESULTS AND DISCUSSION

Protein Identification and Sequence Analysis. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profile of pepsin-solubilized collagen (PSC) is shown in Figure 1. Polypeptide strands α1 (100 kDa), α2 (150 kDa), and β (250 kDa) were assigned based on rat tail collagen profiles and apparent molecular weight. Low-molecular-weight peptides below 30 kDa were observed but were not excised for trypsin digestion.

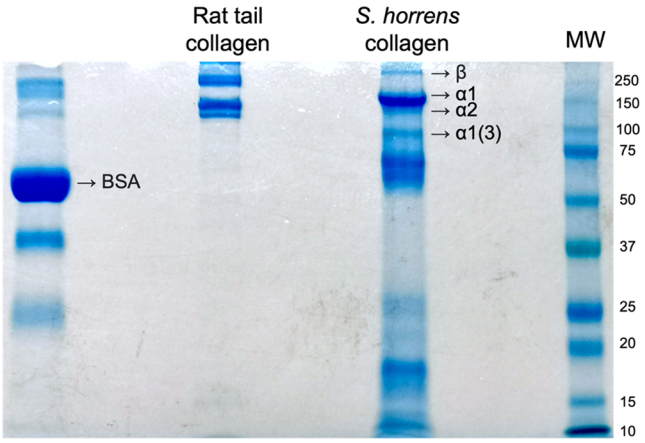

Figure 1. SDS-PAGE of rat tail collagen and *S. horrens* collagen. The labeled bands for the *S. horrens* samples were excised for in-gel digestion and subsequent liquid chromatography-mass spectrometry (LC-MS) analysis.

To confirm and identify *S. horrens* collagen types, mass spectrometry (MS)-based protein identification was performed. Briefly, proteins in PSC extracts were subjected to in-solution and in-gel tryptic digestion, and the resulting peptides were analyzed by tandem mass spectrometry. MS^2 data were searched against the genome of *Apostichopus japonicus* as *S.* cf. *horrens* genome data are currently unavailable. Protein matches and peptide sequences obtained from fragmentation data are summarized in Table 1. Collagen types were determined by running the obtained sequences through protein BLAST. The proteins identified through in-solution digestion were compared in parallel to those identified from the in-gel digestion. Matches obtained from the search resulted only in collagen or collagen-related proteins with repeating proline units, such as TNL-like C1q domain motif-containing proteins. As such, low-molecular-weight bands were determined to be nonspecific cleavages of the proteins due to overdigestion. The total peptide count per collagen type is presented in Table S1.

Table 1. Total Peptides and Proteins Verified from MS^2 Sequencing Done through Mascot

Sequence	Protein match	Type via BLAST	Species database
GPPGEDGDEGP GPTGAPAGPR	collagen	VII (anchoring)	*A. japonicus*
GPAGPTGPTGVP GPAGAPG	TNL-like C1q domain motif cntg protein	C1q/type I	*Pandoravirus inopinatum*
GPAGPAGQTGP AGPKGDR	collagen triple helix repeat protein		*Oryzihumus leptocrescens*
GSTGTQKGDNI IGPPGPPGDI	Pro-α1 type V/XI collagen	V/XI (fibrillar)	*Pagrus major*

Majority of the collagen peptides identified in this study are from fibrillar collagens, α1(I) and α1(III), which give an overall structure to the dermis of the animal.[15,16] Early studies of collagen were mostly based on SDS-PAGE band patterns, which leads to the conclusion that only type I collagen is present in sea cucumbers.[17,18] However, recent proteomic studies found the heterotypicity of collagen molecules in *A. japonicus*, indicating the presence of not only fibrillar collagen types but also Fibril Associated with Collagen Interrupted Triple Helices (FACITS).[19] Similar patterns were observed in *S. horrens* collagen extracts as the α1(IX) unit and type VII peptides were identified. These nonfibrillar constituents may contribute to the fluidity of *S. horrens* dermis.[20]

Different types of collagens are also known to be distinct in structure, physical properties, tissue distribution, and functions.[21,22] Type IX collagen FACITS are commonly found in surfaces of cartilage collagen fibrils,[19,23] which could also interact with fibrillar collagens, linking collagen fibers with other ECM components.[24] Meanwhile, type VII collagen, the major component of anchoring fibrils, plays an essential role in the stabilization and strengthening of connective tissues.[25] The maintenance of the structural properties of the sea cucumber body wall may be related to the diversity of its collagen composition;[19,26] thus, further proteomic studies are warranted.

Physicochemical Characterization. The average fiber diameter for the extracted collagen was estimated by measuring 100 individual fiber diameters using ImageJ software, (Figure 2). The average diameter of the *S. horrens* collagen fibers was approximately 145 ± 31 nm and ranged from 70 to 270 nm (Figure 2c). This is larger compared to the reported values for type I collagen fibrils from bovine skin, which ranges from 60 to 100 nm.[27] The maximum absorbance of collagen extracted from various sea cucumber species is generally reported to be around 220 nm.[18] The measured maximum absorbance for *S. horrens* collagen is 212 nm (Figure S1). This characteristic absorbance is attributed to the presence of multiple peptide chains of collagen.[28,29] A lesser intense peak at 280 nm signifies the presence of smaller amounts of aromatic functional groups, such as phenylalanine and tyrosine.[18]

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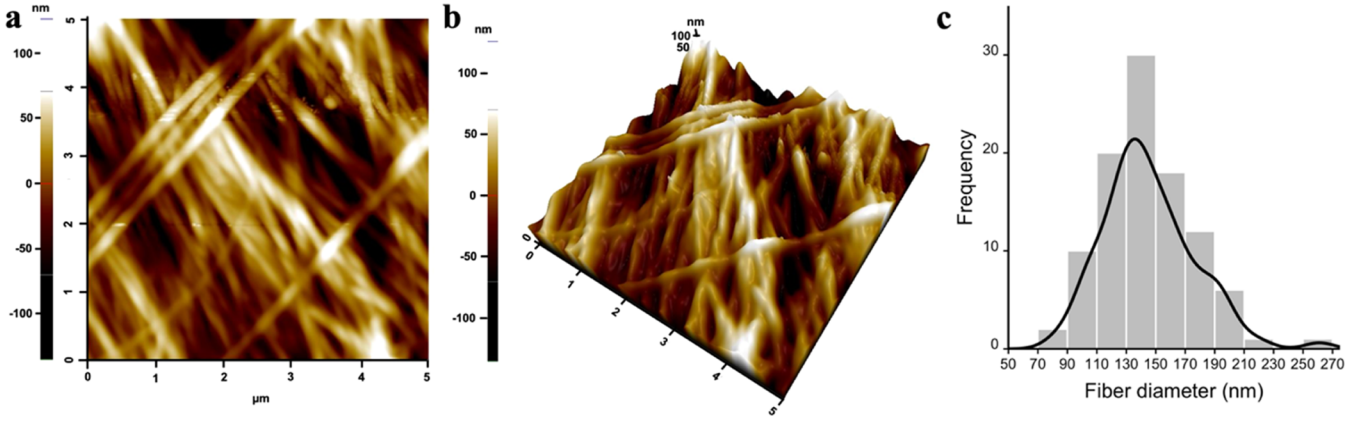

Figure 2. Atomic force microscopy (AFM) images of collagen from *S. horrens* were deposited on mica discs. The (a) two-dimensional (2D) and (b) 3D view of the images at a 5 μm scale with the (c) histogram data of the average fibril diameter analysis.

The susceptibility of collagen to heat is another factor to consider when determining its potential application. The maximum transition temperature (T_m) of the *S. horrens* collagen and the commercial rat tail tendon collagen were measured by differential scanning calorimetry (DSC). The T_m of 62.5 ± 2 °C for the rat tail collagen was consistent with other collagen from mammalian sources. In contrast, *S. horrens* collagen exhibited a T_m of 56.0 ± 2 °C (Figure 3). Lower thermal stability of marine-derived collagen is commonly reported in related studies, especially those obtained from sea cucumbers, such as *Stichopus japonicus* with a T_m of 35.0 °C[30] and *Stichopus monotuberculatus* with a T_m of 30.0 °C.[18] The T_m of collagen correlates to its stability and durability since this is the temperature when half of the triple helix structure denatures into random coils.[18] But these experiments were conducted using microcalorimetric and/or DSC methods on dissolved collagen.[18,30] There were studies that also utilized dried extracted collagen, which reported T_m values of 57.0 °C for *S. japonicus*,[7,15] close to the obtained T_m for *S. horrens*. Other marine collagen sources, such as the Asian sea bass and Nile tilapia skin, have thermal transitions completed at 29.0[31] and 50.6 °C,[32] respectively. Despite that, the thermal stability for *S. horrens* collagen was close to that of the rat tail tendon collagen, which is notably uncharacteristic of the common marine-sourced collagen.[6] The high thermal stability of *S. horrens* collagen can be attributed to several factors. Besides the amino acid composition, specifically, the hydroxyproline and proline contents, environmental conditions, and body temperature are also considered determinant factors for the thermal sensitivity of collagen fibrils from marine sources.[6,18]

Another feature of collagen is the presence of characteristic quarter repeats called D-bands, which result from the interactions between the collagen molecules.[15] The D-banded fibrils were observed in the scanning electron microscopy (SEM) images of the collagen samples at 50 000× magnification with an average distance between the fibrils of 47.9 ± 0.005 nm (Figure 4). This value is lower than the reported distance in rat tail tendon and bovine skin fibrils of 64 and 71 nm, respectively.[27] Other studies have reported that fibrils that were prepared for electron microscopy have lower and more varied D-values due to dehydration during sample preparation and are usually in the range of 55−65 nm.[33]

Stimuli-Responsive Properties of the Collagen-Based Thin Films. Initial observations showed that the lower concentrations (0.5 and 1.0 mM) did not form strong enough films for further analysis. Therefore, only 1.5 mM thin films were further characterized. The Fourier transform infrared (FT-IR) spectra of the collagen and collagen-based thin films are presented in Figure S2. The characteristic peaks of collagen, 3341 cm^{-1} (amide A), 1657 cm^{-1} (amide I), 1553 cm^{-1} (amide II), and 1241 cm^{-1} (amide III), are present in both samples. The AFM images of the thin films showed a dense network of collagen fibrils embedded within the solvent matrix with an average diameter of 260 ± 40 nm, taken from 100 individual fibrils (Figure 5).

A 1 × 1 $in.^2$ portion of the thin films was subjected to increasing temperatures with a constant applied force of 0.1 N (Figures 6a and S3a). Prior to TMA, the thickness of the films was measured to be 0.0451 ± 0.0005 mm. At 100 °C, the film turned opaque in comparison to its original translucent appearance (Figure 6b). The films also became brittle, losing their initial soft film-like property (Figure S3b). Upon exposure to ambient temperature (25 °C), the thin films gradually reverted to their original form (translucent and soft) after at least 5 min (Figure S3c). The dimension change of the membrane did not drastically decrease as the temperature approached 100 °C, suggesting that the degradation stage is not yet achieved. The same reversible response was also observed when the thin films were subjected to 150 °C, with their dimensions maintained, implying the stability of the materials at a higher temperature.

After the analysis at 200 °C, the thin films showed initial signs of degradation with slight discoloration and apparent dimension change starting at 175 °C (Figures S3d and S4a). A further increase in the temperature already induced degradation of the material. The DSC profile of the films supports the stimuli-responsive transition at around 100−150 °C and the degradation above 180 °C (Figure S4b). Subsequent AFM images also showed that the collagen fibrils are still present and intact after TMA (Figure 7), suggesting that this collagenous network is flexible enough to accommodate the removal and readsorption of water. This implies the structural integrity of the collagen-based films, even though there were observable physical changes after being subjected to heat.

Marine organisms as an alternative source of collagen have been explored because of their availability, easy extraction process, minor quality control regulations, minimal inflammatory reactions, and metabolic compatibility.[34] Thus, characterization studies of collagen from various well-known sea cucumber species, such as *S. japonicus*,[15,35] *Cucumaria*

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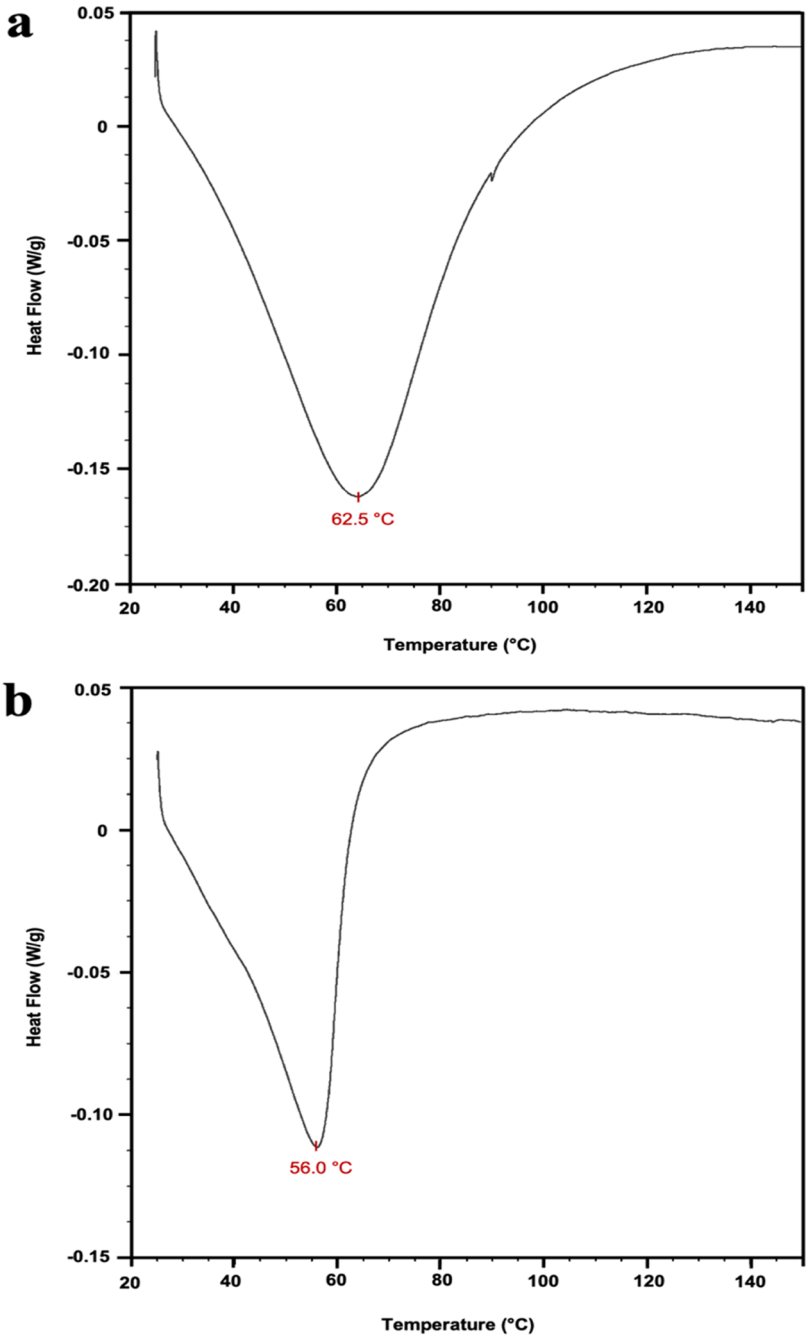

Figure 3. DSC thermograms of the (a) commercial rat tail tendon and (b) collagen from *S. horrens*.

frondosa,[36,37] *S. monotuberculatus*,[18,38] and *A. japonicus*,[26,39] were made available. Distinct characteristics were identified among sea cucumber collagen, wherein the thermal stability and denaturation temperature of marine collagen were found to be generally lower compared to mammalian-derived collagen.[6] Particularly, its triple helical structure makes it susceptible to heat, which is a significant factor in the determination of its potential application.[40,41]

Both the high thermal stability and heterotypicity of *S. horrens* collagen may have contributed to the stimuli-responsive properties of the collagen-based thin films, such as the highly adaptive mechanical morphing response of MCTs. These MCTs are made of hierarchically structured collagen fibrils embedded in a softer, viscoelastic hydrogel matrix,[42] and similar highly ordered collagenous structures were observed in the AFM images of the thin films. Lastly, the structure of collagen is known to have at least two water molecules that are involved in the interchain links within the triple helix. Thus, the unique stimuli-responsive properties of the thin films can also be attributed to the adsorption/desorption of water molecules via hydrogen bond formation and destruction in the presence and absence of moisture.[43]

CONCLUSIONS

There are many sea cucumber species found in the Philippines, but only a few are studied. Among the less studied ones is the *S. cf. horrens*, which is also common in other Southeast Asian countries. In this study, the collagen from *S. horrens* was extracted and extensively characterized by using both physicochemical and biochemical techniques. The protein

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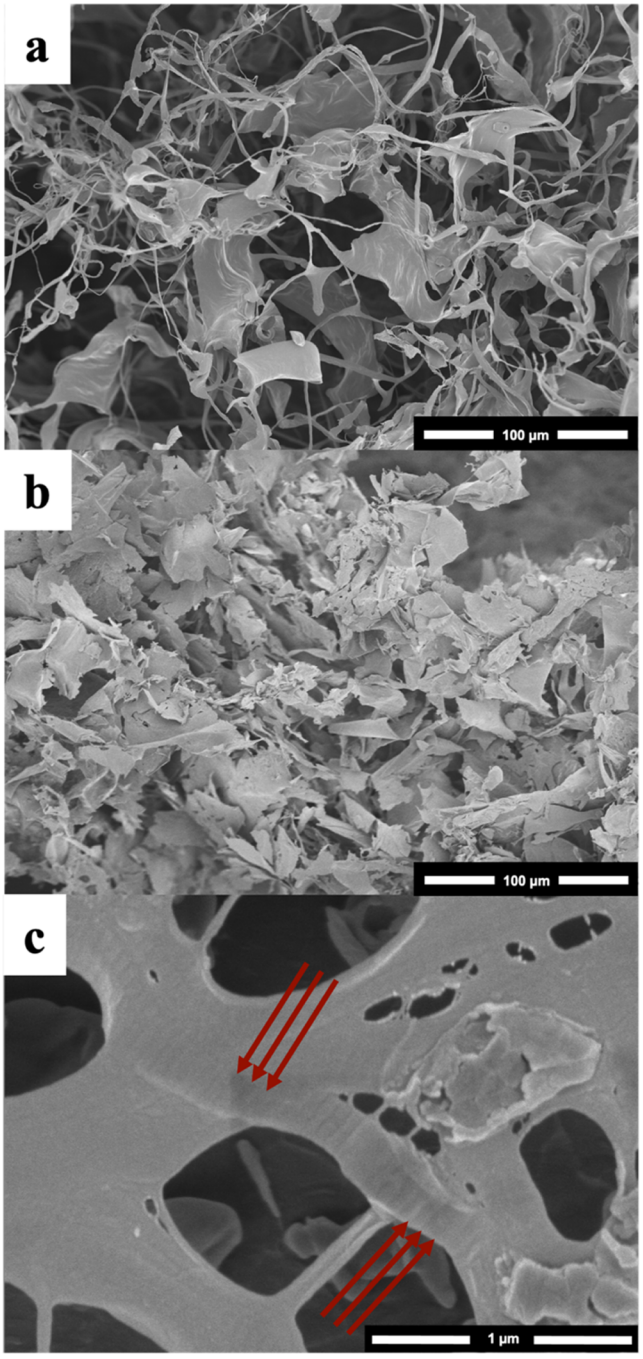

Figure 4. SEM images of the (a) rat tail collagen and (b) *S. horrens* collagen at 400× magnification and (c) visible (vis) D-banded fibrils of the *S. horrens* collagen as seen at 50 000× magnification.

identification and sequence analysis revealed a majority of fibrillar collagens α1(I) and α1(III), as well as nonfibrillar units, such as FACITS, α1(IX) unit, and type VII peptides, showing the heterotypicity of *S. horrens* collagen. A reported T_m of 56.0 ± 2 °C showed a higher stability and durability of the *S. horrens* collagen compared with other common sources of marine collagen. The collagen-based thin films displayed the characteristic peaks of type I collagen, while fibrillar networks comprising the films were observed during AFM imaging. The thermal response of the films using thermomechanical analysis highlights degradation to start at a temperature of >175 °C. At ambient temperatures, the thin films were able to revert to

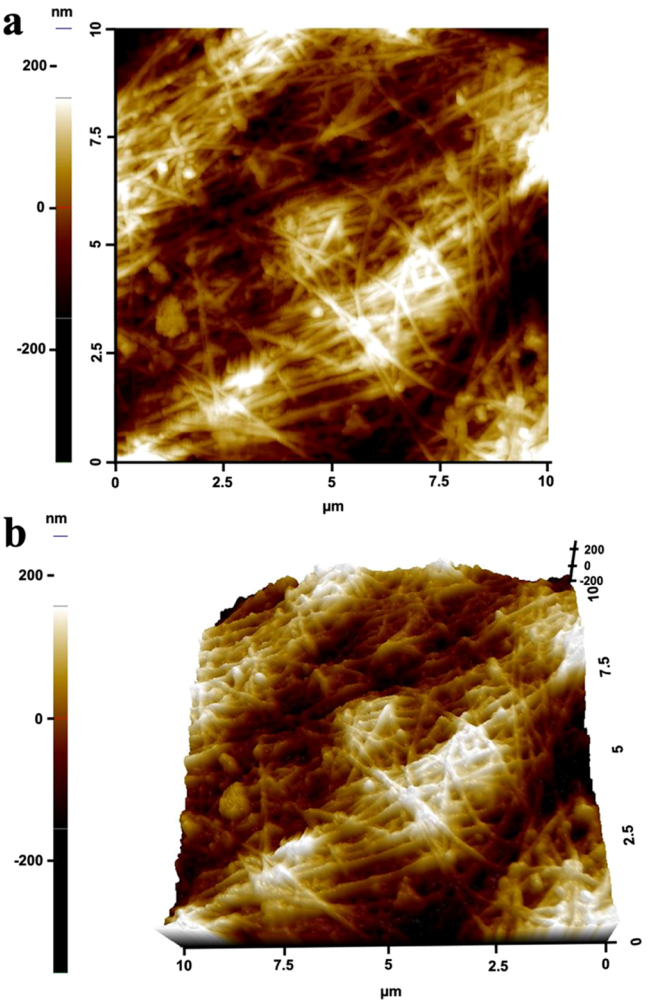

Figure 5. (a) 2D and (b) 3D AFM images of the collagen-based thin films prior to the thermomechanical analysis.

their original physical states after the analysis due to the reabsorption of moisture. These stimuli-responsive properties of the collagen-based thin films, comparable to the adaptive properties of MCTs, may be attributed to the higher thermal stability and heterotypicity of *S. horrens* collagen. Hence, *S.* cf. *horrens* collagen can be prepared into useful smart biomaterials, such as thin films stable at higher temperatures, with potential for biomedical, bio-based food packaging, and other collagen-based applications.

EXPERIMENTAL SECTION

Animal Sample Collection. Adult *S.* cf. *horrens* brood stocks with about 1 kg in weight were harvested from the wild in Bolinao, Pangasinan, Philippines. The preserved samples were transported to The Marine Science Institute, University of the Philippines Diliman, and were immediately stored at −20 °C upon arrival.

Collagen Extraction from *S. horrens* Body Wall. The body wall of *S.* cf. *horrens* was cut into small pieces, and 100 g of the tissue sample was weighed. A decomposition solution (100 mL) was added, which contains the following: 0.5 M NaCl, 50 mM ethylenediaminetetraacetic acid (EDTA), 0.2 N β-mercaptoethanol, and 0.1 N Tris−HCl. The setup was stirred for 3 days at 25 °C. The suspension was filtered via cheesecloth and centrifuged at 7500*g* for 30 min. The

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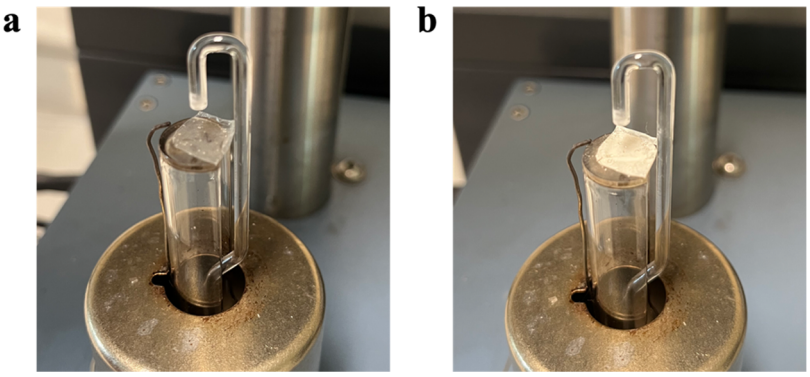

Figure 6. Physical states of the collagen-based thin films (a) before and (b) after thermomechanical analysis at 100 °C.

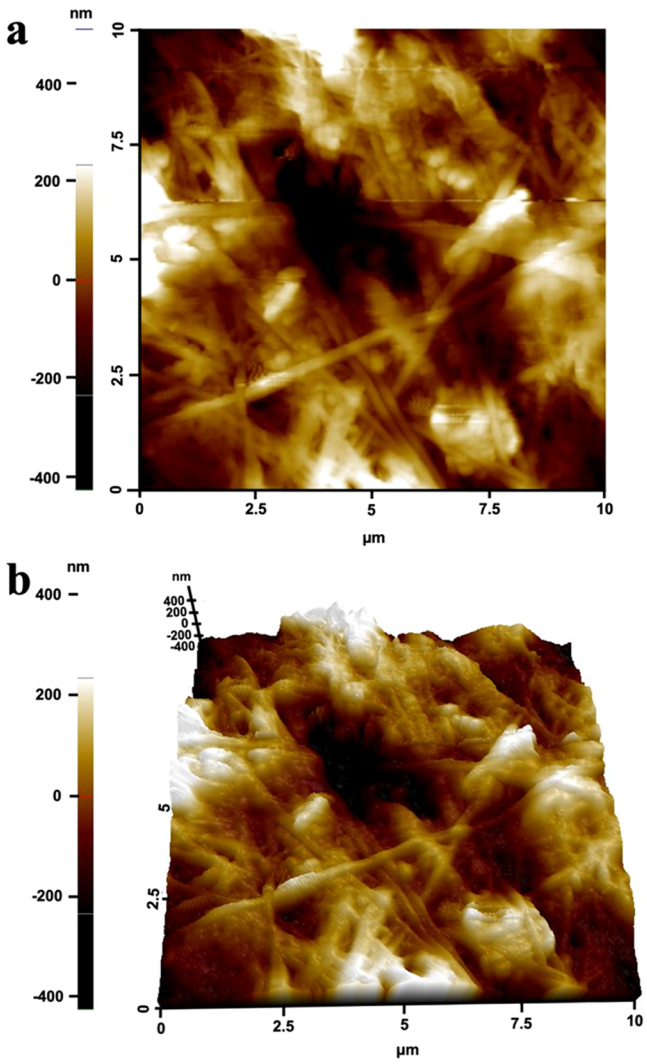

Figure 7. (a) 2D and (b) 3D AFM images of the collagen-based thin films after thermomechanical analysis at 100 °C.

precipitate was washed with distilled H_2O and then supplemented with 0.1 M NaOH and stirred for 3 days at 4 °C. The precipitate was centrifuged again at 7500*g* for 30 min, rinsed with distilled H_2O, and lyophilized using a FreeZone 4.5 L 105C Benchtop Freeze-Dryer (Kansan City, MO). The freeze-dried sample was treated with pepsin (1/100 g collagen) in 0.5 M acetic acid and stirred for 2 days at 4 °C. The solution was centrifuged at 7000*g* for 1 h and then adjusted to 0.8 M using NaCl. The precipitate was dissolved in 0.5 M acetic acid by centrifugation and dialyzed against 0.02 M Na_2HPO_4 (pH 8). The precipitate was harvested again by centrifugation, redissolved in 0.5 M acetic acid, and dialyzed against 0.1 M acetic acid. The extracted collagen was collected, lyophilized, and stored at −20 °C prior to further characterization. For every 100 g of *S. horrens* tissue, ∼24.0 mg of extracted collagen was obtained for this study.

Preparation of Collagen-Based Thin Films. The extracted collagen was left to thaw for 15 min prior to weighing. The collagen solutions (0.5, 1.0, and 1.5 mg/mL) were prepared by adding extracted collagen to 1.0 M phosphate-buffered saline (PBS) at pH 7.4 for 48 h with constant stirring. The collagen solutions (3.0 mL) were poured into glass containers and left to dry at 25 °C for 24 h to obtain collagen-based thin films. The thin films were stored at 4 °C before being used for further characterization and tests.

SDS-PAGE. Pepsin-solubilized collagen (PSC) solutions were analyzed by using SDS-PAGE. A stock PSC solution (2 $\mu g/\mu L$) and a sample buffer solution (Laemmli buffer, β-mercaptoethanol) were prepared before the run. Concentrated (1 $\mu g/\mu L$) and diluted (0.20 $\mu g/\mu L$) PSC solutions were heated at 100 °C for 1 min and run over a 12% gel (Bio-Rad Laboratories, Inc.) at a constant voltage of 120 V for 15 min and then 200 V for 30 min. The proteins in the gels were stained with Coomassie Brilliant Blue G-250 and destained using distilled H_2O with constant shaking. Bio-Rad Precision Plus Unstained Molecular Weight Standards (California) were used as a ladder with molecular weight ranging from 10 to 250 kDa to estimate the sizes of the proteins.

In-Gel Digestion of Peptides. Distinct gel bands were sliced and washed with 25 mM ammonium bicarbonate and increasing concentrations of acetonitrile in water. Washed gels were immersed in 0.02 $\mu g/\mu L$ of sequencing-grade trypsin and incubated overnight at 37 °C. The reaction was stopped using 5% formic acid and collected for LC-MS analysis.

In-Solution Digestion of Peptides. PSC samples were reduced with 10 mM dithiothreitol (DTT) at 60 °C for 1 h. 55 mM iodoacetamide was then added to the samples, followed by incubation at room temperature for 30 min in the dark. Full reduction of the proteins was achieved by adjusting the concentration of DTT to 55 mM for 1 h. Samples were then dried under a vacuum using a centrifugal vacuum concentrator at room temperature. The resulting precipitates were resuspended in 25 mM ammonium bicarbonate and digested with sequencing-grade trypsin at a protein/enzyme ratio of 40:1 at 37 °C overnight. The reaction was stopped by adjusting

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the pH of the solution to 4 using 5% formic acid and was stored at 4 °C prior to LC-MS analysis.

Protein Identification and Sequence Analysis. MS and MS/MS data generated were analyzed using MassLynx 4.1 and MASCOT using Collagen, Echinodermata, Swissprot, and Contaminants protein sequences from Uniprot, and genomes of *A. japonicus* and *Stichopus chloronotus* were downloaded from GenBank (The Uniprot Consortium, 2021). Carbamidomethylation of cysteine residues was chosen as a fixed modification for in-solution digests, and oxidation methionine residues were selected as a variable modification for both in-solution and in-gel digested samples. A mass tolerance of 0.05 Da was used, and peptides with charge states +2, + 3, and +4 were considered for the analysis. The results were then manually evaluated to check the accuracy and quality of peptide spectrum matches to account if more than half of predicted bond cleavages were observed.

UV−Visible Spectroscopy. The extracted collagen (5 mg) was dissolved in 5 mL of 0.5 M acetic acid (1 mg/mL). The light absorbance spectrum of each sample was recorded from the range of 200−700 nm using a Shimadzu UV-1700 PharmaSpec UV Spectrometer (Tokyo, Japan). The maximum absorbance of the extracted collagen and commercial rat tail tendon collagen was reported from triplicate UV−vis analyses.

Scanning Electron Microscopy. Individual images of the extracted collagen were taken using a Hitachi SU8230 field-emission scanning electron microscope (Tokyo, Japan). For each run, the samples were placed on clean aluminum foil and sputter-coated with Pt. An accelerating voltage of 2.0 kV, beam current of 30 mA, and magnification from 5000 to 50 000× were used for all test runs.

Differential Scanning Calorimetry. The extracted collagen (2 mg) was transferred into standard cells for DSC analysis using a TA Instruments DSC Q20 instrument (Delaware). The samples were heated from 20 to 200 °C at a rate of 1 °C/min to measure the difference in the endothermal transitions of collagen. The maximum transition temperature (T_m) of the extracted collagen and commercial rat tail tendon collagen was reported from triplicate analyses. DSC analyses for each of the three replicates of the collagen-based thin films (13 mg) were also performed by heating from 25 °C (ambient temperature) to 300 °C at a rate of 5 °C/min.

Fourier Transform Infrared Spectroscopy. The FT-IR spectra of the extracted collagen and collagen-based thin films were obtained using a Shimadzu IR Prestige-21 spectrometer (Tokyo, Japan) with an attenuated total reflectance (ATR) attachment. The spectra for each of the three replicates for the extracted collagen and collagen-based thin films were acquired in the range of 600−4000 cm^{-1} and processed using ATR correction.

Atomic Force Microscopy. The extracted collagen (1.5 mg/mL) was suspended in 1.0 M PBS solution (pH 7.4) under constant stirring at 4 °C. After 24 h of suspending, mica disc substrates were submerged in the collagen suspension, and the collagenous material was allowed to deposit for 30 min. The samples were rinsed with deionized water and air-dried. At least five samples of the prepared collagen-based thin films were imaged without further preparation. All of the AFM analyses were performed in tapping mode using a Park Systems NX-10 atomic force microscope (Suwon, South Korea).

Thermomechanical Analysis. Thermal measurements were performed using a TA Instruments Q400 thermomechanical analyzer (New Castle, DE). A thermally controlled AFM probe was brought into contact with the collagen-based thin films (1 in. × 1 in.) by applying a nominal load constant of 1.0 N for all samples. A typical temperature ramp of 20 °C/s was used for all thermal measurements from 25 to 100, 150, and 200 °C. After the first TMA test, the thin films were left at ambient temperature (25 °C) for 5 min until the films revert to their initial transparent color. Subsequent thermal measurements were repeated using the same parameters as in the first run. All TMA measurements were performed at least three times for 100, 150, and 200 °C.

■ ASSOCIATED CONTENT

Data Availability Statement

Converted raw data (Waters. RAW to. mzXML) can be accessed as a MassIVE Data set with Accession Number MSV000091784 (doi: 10.25345/C5WP9TH30).

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c03299.

Additional characterization of *Stichopus* cf. *horrens* collagen: UV−Vis, FT-IR, TMA, and DSC (PDF)

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Author Contributions

K.M.D.S., V.P.M.T., E.T.Y., and M.T.C. conceived and designed the experiments. K.M.D.S. and V.P.M.T. conducted experimentations, data analyses, and interpretations. K.M.D.S. and M.T.C. were mainly responsible for preparing the manuscript with further input from other authors. All authors discussed the results and commented on the manuscript.

Notes

The authors declare no competing financial interest.

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