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NOTE

Cellulose is not degraded in the tunic of the edible ascidian *Halocynthia roretzi* contracting soft tunic syndrome

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ABSTRACT: Soft tunic syndrome is a fatal disease in the edible ascidian *Halocynthia roretzi*, causing serious damage to ascidian aquaculture in Korea and Japan. In diseased individuals, the tunic, an integumentary extracellular matrix of ascidians, softens and eventually tears. This is an infectious disease caused by the kinetoplastid flagellate *Azumiobodo hoyamushi*. However, the mechanism of tunic softening remains unknown. Because cellulose fibrils are the main component of the tunic, we compared the contents and structures of cellulose in healthy and diseased tunics by means of biochemical quantification and X-ray diffractometry. Unexpectedly, the cellulose contents and structures of cellulose microfibrils were almost the same regardless of the presence or absence of the disease. Therefore, it is unlikely that thinning of the microfibrils occurred in the softened tunic, because digestion should have resulted in decreases in crystallinity index and crystallite size. Moreover, cellulase was not detected in pure cultures of *A. hoyamushi* in biochemical and expressed sequence tag analyses. These results indicate that cellulose degradation does not occur in the softened tunic.

KEY WORDS: Cellulose content · Cellulase · X-ray diffractometry · EST analysis · *Azumiobodo hoyamushi* · Ascidian aquaculture

INTRODUCTION

Soft tunic syndrome has caused serious damage to the aquaculture of the edible ascidian *Halocynthia roretzi* (Drasche) in Korea and northern Japan, where this ascidian is a popular seafood. The body of *H. roretzi* is entirely covered with a reddish, leathery matrix, called tunic, and the tunics of diseased ascidians become thinner and softer than usual. Eventually, the tunic tears and the diseased ascidians die. This is

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an infectious disease caused by the kinetoplastid flagellate *Azumiobodo hoyamushi* (Kumagai et al. 2011, Hirose et al. 2012). Although softening of the tunic is the most prominent clinical sign of the disease, the mechanism of tunic softening remains unclear.

Tunic is a leathery or gelatinous matrix covering the epidermis of ascidians and thaliaceans (Burighel & Cloney 1997). This tissue is unique among metazoans because the main component is cellulose (Belton et al. 1989, Van Daele et al. 1992, Hirose et al. 1999). The ascidian cellulose is produced by cellulose-synthesizing enzyme complexes (terminal complexes) in the apical membrane of epidermal cells (Kimura & Itoh 1996, 2004), and the cellulose synthase gene has been identified in the ascidian genome (Matthysse et al. 2004, Nakashima et al. 2004). However, there has been no evidence of cellulose degradation in the softened tunic of diseased ascidians. In the present study, we compared the cellulose contents and structures of crystalline cellulose fibrils from the tunics of healthy and diseased individuals. We also investigated cellulase activity and synthesis in pure cultures of *A. hoyamushi* by means of biochemical and expressed sequence tag (EST) analyses.

MATERIALS AND METHODS

Animals

Halocynthia roretzi were collected from an aquaculture site in Miyagi Prefecture, Japan, in 2009 and 2010. Following Kitamura et al. (2010), we selected diseased animals of Grade 3 (G3: individuals with a considerably or entirely soft tunic) and Grade 4 (G4: individuals with a soft tunic that almost tears) for the experiments. Healthy *H. roretzi* (Grade 0, G0) were collected from a farming site where the disease had never been observed.

The strain of *Azumiobodo hoyamushi* that was used in the present study was isolated from a diseased ascidian reared in the vicinity of Samenoura, Miyagi Prefecture, Japan (Kumagai et al. 2011). The flagellate was cultured in maintenance medium at 15°C and subcultured by 10-fold dilution into fresh maintenance medium at ~1 wk intervals (see Kumagai et al. 2011 for details).

Cellulose content

Tunic pieces from G0 (n = 3) and G4 individuals (n = 3) were thoroughly washed with distilled water, freeze-dried, and minced with a razor blade. For each specimen, 50 mg was mixed with 1 ml of 80% sulfuric acid and incubated for 2 h at room temperature. Then, the specimens were diluted (1:40) with distilled water, and 5 ml of diluted specimen was incubated at 100°C for 2 h in an oil bath to hydrolyze cellulose into glucose. The lysates were neutralized with calcium carbonate, and the precipitates (calcium sulfate) were removed by centrifugation. The cellulose in the specimens was determined as glucose in the supernatants using a Glucose C-II test kit (Wako Pure Chemical Industries).

X-ray diffractometry

Tunic pieces of approximately 1 g were cut from the 6 individuals (G0, n = 1; G3, n = 2; G4, n = 3). Each tunic specimen was incubated in 1 M KOH at room temperature overnight and washed with water. Then, the specimen was incubated in 0.3% $NaClO_2$ at pH 4.9 buffered with acetate buffer for 2 h at 70°C and washed with water. These KOH–$NaClO_2$ treatments were repeated until the specimens became white (Wada et al. 1993).

X-ray diffractometry of freeze-dried specimens pressed into disks was performed using a diffractometer (RINT2000; Rigaku) with monochromatic Cu Kα radiation (λ = 0.15418 nm) as previously described (Kim et al. 2010). Peak separations were carried out using the non-linear least-squares fitting program proFit (QuantumSoft), in which pseudo-Voigt functions for 3 crystalline peaks and a fifth-degree polynomial function for the background were used.

The crystallinity index (the relative amount of crystalline material in cellulose) was determined based on the ratio of the sum of the separated crystalline peak areas to the total reflection area including background. Three *d*-spacings of cellulose were calculated using Bragg's equation, and the crystallite sizes of the directions perpendicular to the 3 crystalline planes were calculated using Scherrer's equation, as described by Kim et al. (2010).

Cellulase activity

Cellulase activity in the ascidian tissues and pathogenic flagellate was measured following Rahman et al. (2014). Tunic, hepatopancreas, muscle, and digestive tract (0.22–1.05 g) from healthy and diseased individuals were minced using a knife, added to a 10× volume of 10 mM phosphate buffer (pH 7.0), and

homogenized with a hand homogenizer. Flagellates (~10^6 cells) and hemolymph (1.5–8 ml) were also homogenized in the same manner. Crude cellulase was extracted on ice for 6 h. Abalone cellulase (HdEG66) (Suzuki et al. 2003) and *Trichoderma viride* cellulase (Wako Pure Chemical Industries) were used as positive controls. A cellulase activity assay was performed with a reaction mixture containing 0.5% carboxymethyl cellulose, 10 mM sodium phosphate (pH 6.0), and 0.01–0.1 mg ml^{-1} of enzyme at 30°C. Reducing sugar released by the reaction was determined by the method of Park & Johnson (1949). One unit of cellulase activity was defined as the amount of enzyme that produces reducing sugar equivalent to 1 µmol glucose min^{-1}.

EST

Four different conditions of *A. hoyamushi* (stationary and logarithmic growth phases, and exposure to ascidian fluid and seawater) were employed for EST analysis. To obtain cells in stationary and logarithmic growth phases, the flagellates were cultured in maintenance medium. For the 2 remaining conditions, the flagellates (1.0×10^6 cells ml^{-1}) were exposed to 33% filtered-ascidian hemolymph or autoclaved artificial seawater for 6 h at 20°C. Cells from each condition were centrifuged at 2000 × *g* for 10 min at 4°C, and the supernatants were discarded. The cell pellets were suspended with TRIzol reagent (Life Technologies), and total RNA was extracted.

Extracted RNA was treated with DNase I (Qiagen) and then further purified using the RNeasy Mini Kit (Qiagen). The purified RNA was quantified by spectrophotometer, and the quality was evaluated using the Agilent RNA 6000 Nano Kit (Agilent Technologies) with a 2100 Bioanalyzer (Agilent Technologies). For RNA sequencing, double-stranded cDNA libraries were constructed using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina), and the 4 samples were independently indexed. The conditions of the obtained libraries were validated using the Agilent DNA 1000 Kit (Agilent Technologies) with a 2100 Bioanalyzer, and their concentrations were measured using the Qubit 2.0 Fluorometer (Life Technologies). The validated libraries for each sample were mixed and denatured with NaOH, and then diluted to 6 pM with the hybridization buffer included in the MiSeq Reagent Kit v2 (Illumina). The final library was subsequently loaded into a 500-cycle MiSeq reagent cartridge for sequencing on the Illumina MiSeq platform, with sequencing runs of 2 × 250 paired-end reads.

Raw sequencing data were deposited in the DNA Data Bank of Japan (accession number: DRA003472; BioSample IDs: SAMD00028958 [hoyamushi01], SAMD00028959 [hoyamushi02], SAMD00028960 [hoyamushi03], and SAMD00028961 [hoyamushi04]). The sequence reads were assembled into transcript contigs using the all-in-one package Rnnotator (Martin et al. 2010), which performs read pre-processing, de novo assembly, and contig post-processing. All transcript contigs were used for annotation against the UniProt Knowledgebase reviewed (Swiss-Prot) database (www.uniprot.org) using the BLASTX search function (Altschul et al. 1990). Enzyme Commission (EC) numbers were also annotated to the contigs that were identified as enzymes by BLAST homology search.

RESULTS AND DISCUSSION

The average cellulose content in the freeze-dried tunics was similar in healthy (G0) (22.8% [w/w], n = 3) and diseased (G4) individuals (25.4% [w/w], n = 3) (Fig. 1). These values are also comparable to the cellulose content in the tunic of *Halocynthia*

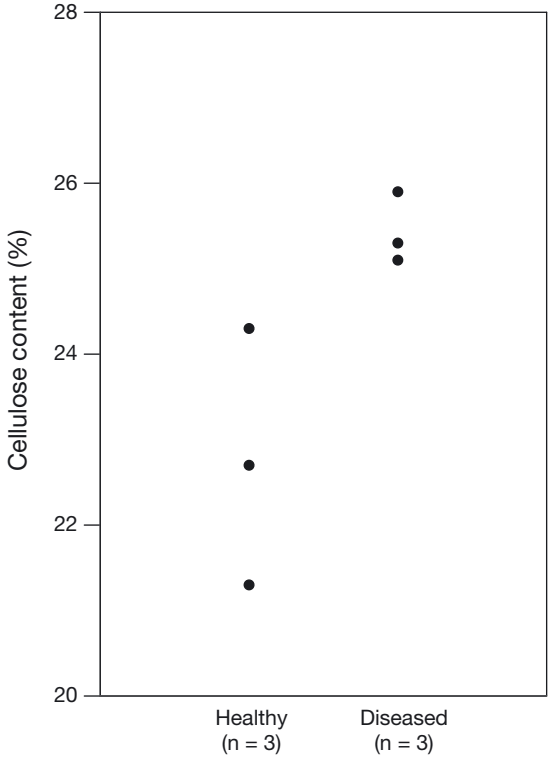

Fig. 1. Cellulose contents (%, w/w) in the freeze-dried tunics of healthy (G0, healthy tunic) and diseased individuals (G4, soft tunic almost tearing) of the edible ascidian *Halocynthia roretzi*

aurantium (~20%) (Smith & Dehnel 1970). Therefore, tunic softening is unlikely to be caused by the reduction of the cellulose content in the tunic. In contrast, the tunics of diseased individuals were always thinner than those of healthy individuals (Hirose et al. 2009, Kitamura et al. 2010), and thus the amount of cellulose in a unit area was probably decreased in the softened tunic.

There were no marked differences among the X-ray diffraction profiles of the G0 (control), G3, and G4 specimens (Fig. 2). The *d*-spacings indicated that the cellulose microfibrils consisted of only cellulose Iβ, whereas natural cellulose microfibrils produced by plants, fungi, algae, and bacteria are usually a mixture of cellulose Iα and Iβ. This is consistent with previous studies of ascidian cellulose (Wada et al. 1993, 1997). The crystallinity indices were ~80% in all specimens examined in this study, and the crystallite sizes based on the full-width at half maximum were also very similar (Table 1). These values were nearly the same as those of previous studies using healthy tunics (Wada et al. 1997, Kim et al. 2010). These results indicate that the cellulose microfibrils were not digested in the softened tunics, because digestion should have resulted in reductions in the crystallinity index and crystallite size. Moreover, cellulase activity was not detectable (<0.01 U ml^{-1}) in any samples from either ascidian tissue or *Azumiobodo hoyamushi*, while in the positive controls, the activities in abalone cellulase and *Trichoderma viride* cellulase were 6.14 and 2.12 U ml^{-1}, respectively.

Four cDNA samples prepared from independent *A. hoyamushi* cultures were sequenced using the Illumina sequencing platform. After pre-processing and

Table 1. Crystallinity indices, *d*-spacings, and crystallite sizes of cellulose from healthy and softened ascidian *Halocynthia roretzi* tunic. Disease severity is graded as follows: G0, healthy tunic; G3, entirely soft tunic; G4, soft tunic almost tearing. Values in parentheses: plane of the peaks; values in square brackets: vertical direction to the plane

Specimen no.	1	2	3	4	5	6
Grade	G0	G3	G3	G4	G4	G4
Crystallinity index (%)	79	79	79	80	78	80
d-spacing (nm)						
(1$\bar{1}$0)	0.600	0.601	0.604	0.604	0.602	0.604
(110)	0.533	0.535	0.537	0.536	0.535	0.537
(200)	0.389	0.389	0.391	0.390	0.390	0.391
Crystallite size (nm)						
[1$\bar{1}$0]	7.6	8	7.7	7.8	7.4	7.8
[110]	10.6	10.9	10.5	10.9	10.4	10.8
[200]	9.5	9.7	9.3	9.8	9.2	9.6

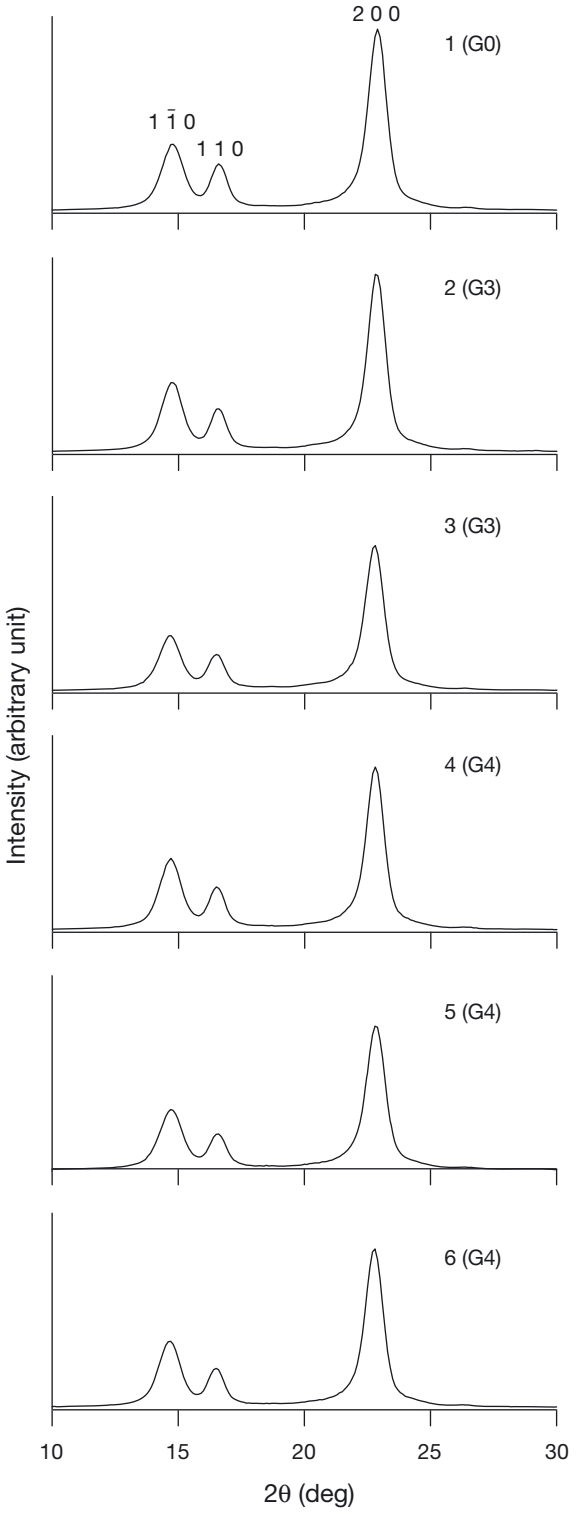

Fig. 2. X-ray diffraction profiles of tunic cellulose of the edible ascidian *Halocynthia roretzi*. The specimen number is shown in the upper right corner of each profile, and the grade of disease severity is shown in parentheses (G0, healthy tunic; G3, entirely soft tunic; G4, soft tunic almost tearing). The peaks for 1$\bar{1}$0, 110, and 200 in the profile of Specimen 1 indicate typical 3 equatorial peaks of cellulose. 2θ: scattering angle

Table 2. Summary of expressed sequence tag analysis of the flagellate *Azumiobodo hoyamushi*, which is the causative agent for soft tunic syndrome of the edible ascidian *Halocynthia roretzi*. Samples: hoyamushi01, stationary growth phase; hoyamushi02, logarithmic growth phases; hoyamushi03, exposure to ascidian fluid; hoya-mushi04, exposure to seawater. N50: weighted median statistic such that 50% of the entire assembly is contained in contigs or scaffolds equal to or larger than this value; nt: nucleotides

	hoya-mushi01	hoya-mushi02	hoya-mushi03	hoya-mushi04
Total reads	5 067 980	5 305 394	4 565 670	4 076 508
Contigs	16 257	14 260	16, 518	12, 799
≥1000 bp	1480	1285	1566	1039
500–999 bp	3619	3381	3703	2921
100–499 bp	11 158	9594	11 249	8839
N50 (nt)	627	620	622	597
Median length (nt)	333	347	340	335
Shortest contig (nt)	100	100	100	100
Longest contig (nt)	7381	4538	5913	3617

filtering, approximately 5 000 000 reads were obtained from each sample, and 12 799–16 518 contigs were generated from samples hoyamushi01–04 using Rnnotator (Table 2). BLASTX top hit analysis of all assembled contigs identified 30–36 contigs as glycosidases (EC 3.2.1.–) when BLAST results were cut off by an e-value of $<1 \times 10^{-3}$. Cellulase (EC 3.2.1.4) was not included in any of the identified *A. hoyamushi* glycosidases (see the Supplement at www.int-res.com/articles/suppl/d116p143_supp.xls). It is noteworthy that cellulase was not reported in metatranscriptomic analyses of the flagellates in the softened tunic (Jang et al. 2012).

The present study showed that cellulose is not degraded in the softened tunic. What is a potential cause(s) of tunic softening? Ascidian tunic is composed not only of cellulose but also of other polysaccharides and proteins. Some of these non-cellulosic components probably bind to or interact with cellulose microfibrils to construct the tunic matrix, although they have been poorly studied. If some cellulose-associating molecules were the nutrient source for *A. hoyamushi*, the degradation of these molecules may cause tunic softening. As for polysaccharides, whereas xylan is a major component of hemicellulose and commonly found in plant cell walls, neither xylan nor xyloglucan has been reported from any ascidians so far. In contrast, the glycosidases in the BLAST top hit analysis did not include endoxylanase but included some xylosidases that hydrolyse xylose residues from non-reducing termini (see the Supplement). These xylosidases and other glycosidases are potentially involved in the degradation of some polysaccharides associating with cellulose microfibrils. Proteins may be the principal resources in the tunic for *A. hoyamushi*: proteins compose 50% of the tunic of *H. aurantium* (Smith & Dehnel 1970). Metatranscriptomic analyses indicated that metalloproteases of the flagellates are potential virulence factors for soft tunic syndrome (Jang et al. 2012). However, it is unknown what is digested with the proteases of *A. hoyamushi* in the tunic of *H. roretzi*. A proteomic study of the softened tunic will be necessary to disclose the mechanism of tunic softening.

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