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***Cover Letter**

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Complete solubilization of cartilage using the heat-stable protease thermolysin for comprehensive GAG analysis

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Abstract

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GAGomic analysis, and is likely applicable to other collagen-rich tissues such as ligaments, skin, and blood vessels.

Key words

thermolysin, heat-stable protease, collagen, hydrogen bond, cartilage, glycosaminoglycan

Abbreviations

Hyp: hydroxyproline, **GAG:** glycosaminoglycan, **CS:** chondroitin sulfate, **KS:** keratan sulfate, **HA:** hyaluronic acid, **LC-MS/MS:** liquid chromatography-tandem mass spectrometry

1. Introduction

Articular cartilage has tough and resilient characteristics attributed to the abundance of extracellular matrix collagens, proteoglycans, and glycosaminoglycans (GAGs). The major proteoglycan aggrecan contains covalently linked GAGs chondroitin sulfate (CS) and keratan sulfate (KS), and aggregates with the GAG hyaluronic acid (HA). These GAGs are negatively charged and attract water to fill the matrix together with type II collagen meshwork [1, 2].

Changes in qualities or quantities of GAGs reportedly affect functional properties and can lead to the development of pathological conditions such as osteoarthritis [3, 4]. To confirm the clinical relevance of these changes in cartilage, comprehensive disaccharide analysis of GAGs using liquid chromatography/tandem mass spectrometry (LC-MS/MS) is required. However, the resistance of a collagen-rich tissue cartilage to proteolysis [5] has hampered complete extraction of GAGs from cartilage, precluding reproducible analyses of GAGs [6].

Collagen has triple helical structures comprising three alpha chain polypeptides with the repeating amino acid sequence Gly-X-Y, where X and Y are usually Pro and hydroxyproline (Hyp), respectively. The characteristic amino acid Hyp is crucial for the formation of stable triple helical structures through hydrogen bonding [7], and these are broken by heat treatment, leading to unfolding of collagen triple helixes and access of substrate peptide bonds to proteases [8]. Thus, at high temperatures, heat-stable proteases would efficiently solubilize collagen-rich tissues such as articular cartilage. Previous studies show the use of a heat-stable protease papain to solubilize connective tissues such as cartilage at optimal catalytic temperatures of 50–70°C [6, 9]. However, a highly heat-stable enzyme thermolysin was previously isolated from the thermophilic bacteria *Bacillus thermoproteolytics Rokko*, and was shown to have a temperature

optimum of 65–80°C [10]. Herein, we examined the efficacy of thermolysin for solubilization of cartilage at 70°C prior to extraction of GAGs and made comparisons with digestion using collagenase at 50°C. Our results warrant consideration of the heat-stable protease thermolysin to solubilize articular cartilage and other collagen-rich connective tissues for GAG analyses.

2. Materials and methods

2.1. Materials

Collagen type I from bovine skin (acid soluble) was purchased from Nippi, Inc. (Tokyo, Japan). Thermolysin (cat.no. 3504), collagenase type II (cat.no. LS004202), and Keratanase II (cat.no. FC-156) were obtained from PEPTIDE INSTITUTE, INC. (Osaka, Japan), Worthington Biochemical Corporation Inc. (NJ, USA), and GlycoSyn. (Lower Hutt, New Zealand), respectively. Chondroitinase ABC (cat.no. 100330) and standard unsaturated disaccharides of CS and HA classes (cat.no. 400571 and 400572) were purchased from Seikagaku Biobusiness (Tokyo, Japan). Standard disaccharides of the KS class were custom-synthesized by TOKYO CHEMICAL INDUSTRY CO., LTD (Tokyo, Japan). All other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Determination of thermolysin and collagenase activities with purified collagen in solution

Collagen (0.5 mg) was incubated with thermolysin at 70°C or with collagenase at 50°C in 0.2 mL aliquots of lysis buffer containing 200 mM ammonium acetate (pH 8.0) and 5 mM $CaCl_2$. Subsequently, ethanol and sodium acetate were added to final concentrations of 90% and 4.7 mM, respectively, and solutions were kept at 4°C overnight. After centrifugation at 15,000 × g for 15 min, supernatants were dried for Hyp measurements. In these experiments, single units of enzyme activity were defined as the amount of enzyme required to release 0.05 mg of Hyp per h, and corresponded with 35 and 143 ng of thermolysin and collagenase, respectively. Under these conditions, collagenase activity at 70°C was only 2.2% of that at 50°C.

2.3 Solubilization of solid articular cartilage by thermolysin or collagenase

Porcine tissues were obtained from a local slaughterhouse and articular cartilage samples from knee joints were freeze-dried. Dried cartilage samples (1 mg) were then incubated with thermolysin (0.42 unit, 15 ng) at 70°C or with collagenase (5.5 unit, 786 ng) at 50°C in 0.2 ml aliquots of lysis buffer for indicated times, and were then subjected to Hyp or GAG measurements. Further digestion was performed with thermolysin (0.42 unit) at 70°C for 24 h and total Hyp or GAG contents were determined in each sample. After the additional thermolysin

digestion, complete digestion of cartilage collagen was confirmed by the absence of Hyp in precipitated fractions.

2.4. Measurement of Hyp contents

To estimate the amount of collagen, samples were hydrolyzed in 9 N HCl at 100°C for 15 h and Hyp content was measured as described previously, with some modifications [11]. Briefly, 0.01 ml aliquots were incubated with 0.2 ml of 1.4% chloramine T, 10% *n*-propanol, and 0.5 M sodium acetate (pH 6.0) at room temperature for 25 min. Subsequently, 0.2 ml of Erlich's solution comprising 1 M *p*-dimethylaminobenzaldehyde in 70% *n*-propanol and 20% perchloric acid was added and incubated at 65°C for 20 min. Absorbance was then measured at 550 nm and Hyp contents were determined using a standard curve of Hyp solutions.

2.5. Measurement of GAG contents

After protease digestion, the cartilage solution was centrifuged at 15,000 × g for 15 min. GAGs in the supernatant were precipitated with 90% ethanol and 4.7 mM sodium acetate at 4°C overnight and collected by centrifugation at 15,000 × g for 15 min. Precipitates were

reconstituted with water and subjected to GAG measurement with 1,9-dimethylmethylene blue using the Blyscan GAG assay kit (Biocolor, Ltd.; Carrickfergus, UK) [12].

2.6. Disaccharide analysis of GAG with LC-MS/MS

Reconstituted solutions containing 5 μg of GAG were digested into disaccharides using chondroitinase ABC (5 mU) and keratanase II (2.5 mU) at 37°C for 15 h in 60 μl solutions containing 0.05% bovine serum albumin (BSA), 1 mM $CaCl_2$, and 50 mM sodium acetate (pH 7.0). Disaccharide analyses were then performed as described previously [13], with some modifications. Briefly, separation was performed on a Hypercarb column using a Shimadzu HPLC system (LC-10AD pumps, SIL-HTC auto-sampler, and DGU-14AM degasser) at a flow rate of 0.2 ml/min. The column temperature was maintained at 70°C and the mobile phases were produced using 5 mM ammonium bicarbonate (PH 11.0, solvent A) and 100% acetonitrile (solvent B). Samples were then eluted using the following gradient (the percentage of solvent B in solvent A was linearly changed as follows: 0 min, 3%; 2.5 min, 15%; 2.75 min, 95%; 3.5 min, 95%; 3.55 min, 1%; and 15 min, 1%). MS was then performed using a Shimadzu 8030 instrument with heat-block and dissolved-line temperatures of 400°C and 250°C, respectively,

and nebulizer and drying gas flow rates of 2 and 15 L/min, respectively. Nitrogen was used as the collision and nebulizer gas. Channels were defined using a combination of Q1 and Q3 mass filter transitions with other parameters (Table 1). Data acquisition and analysis was performed using Lab Solutions (version 5.60SP2 software; Shimadzu).

2.7. Statistical analysis

Data are presented as means ± standard deviations of three independent experiments. Comparisons of thermolysin- and collagenase-treated groups were performed using Welch's t-test with the free software R (version 3.3.1).

3. Results and discussion

3.1 Solubilization of articular cartilage with thermolysin

Dried porcine articular cartilage (1 mg) was incubated with thermolysin (0.42 unit, 15 ng) at 70°C. No visible pieces of solid cartilage were observed after 24 h (Fig. 1, upper right), and 110.9 ± 12.2 μg of Hyp was released (Table 2). To compare the effects of digestion on GAG extraction, a milder digestion was performed using collagenase at 50°C, as frequently used for

the collection of vital chondrocytes [14]. In contrast with thermolysin digestion, solid cartilage still remained after treatments with collagenase (5.5 unit, 786 ng), even at 13-times the activity of thermolysin (Fig. 1, lower right), and Hyp release was only 20.9 ± 1.8 μg (Table 2). Total amounts of Hyp in thermolysin- and collagenase-treated samples were 115.3 ± 16.5 and 136.5 ± 10.2 μg/mg, respectively and did not differ significantly (P = 0.1481; Table 2 and Fig. 2). Articular cartilage predominantly comprises collagen and water, and Hyp accounts for about 14% of collagen in the cartilage [15]. Thus, the expected Hyp content of 1 mg of the cartilage is 140 μg at most. In the present experiments, released Hyp amounts with thermolysin digestion as well as the total amounts shown here are similar to the estimated value. Cartilage solubilization efficiencies of thermolysin and collagenase treatments were 96.5% ± 3.4% and 15.3% ± 1.5%, respectively (P = 0.0001; Table 2 and Fig. 2). These results clearly show that thermolysin digestion at 70°C offers superior solubilization of solid cartilage.

3.2. Extraction of GAG from articular cartilage following digestion with thermolysin

273.2 ± 86.1 and 146.1 ± 13.5 μg/mg of GAG chains were extracted after treatment with thermolysin and collagenase, respectively, whereas total amounts of GAG chains in

thermolysin- and collagenase-treated samples were 275.8 ± 86.6 and 261.6 ± 29.4 μg/mg, respectively (P = 0.8114; Table 2), as shown previously [1]. GAG chain extraction efficiencies were 99.0% ± 0.2% and 56.0% ± 1.7% after solubilization of cartilage using thermolysin and collagenase, respectively (P = 0.0005; Table 2 and Fig. 2). These results indicate greater GAG extraction efficiency with thermolysin than with collagenase. After partial digestion of cartilage with less than 20% solubilization of collagen, more than 50% of GAGs were extracted.

To determine extraction efficiencies of each GAG class, we performed disaccharide analyses of GAG chains after extraction with the present proteases. Compositions of GAG classes in the total GAG (Table 3) agreed with those reported previously [1]. The extraction efficiencies of CS class, the major GAG class of the cartilage, after treatments with thermolysin and collagenase were 99.2% ± 0.2% and 59.5% ± 5.4%, respectively (P = 0.0062; Table 3 and Fig. 3). These CS extraction efficiencies are similar to those of the GAG chains shown in Table 2. Extraction efficiencies of the minor GAG components KS and HA from thermolysin-treated cartilage were 98.9% ± 0.3% and 99.4% ± 0.3%, respectively, whereas those after collagenase digestion were only 29.0% ± 5.7% and 20.7% ± 4.5%, respectively (Table 3 and Fig. 3). These efficiencies differed significantly between treatment groups, with P values for KS and HA of

0.0022 and 0.0010, respectively. Following thermolysin digestion, all three GAGs were almost completely extracted from the cartilage sample, whereas after less than 20% digestion of cartilage with collagenase, the extraction efficiencies of CS, KS, and HA were 60%, 29%, and 21%, respectively (Table 3). However, within each class, disaccharides were extracted to similar degrees. These results suggest that both amounts and compositions of GAGs, especially KS and HA, may vary depending on the degree of cartilage solubilization. Thus, for accurate and reproducible analyses of GAGs in cartilage, it is necessary to completely digest and solubilize the tissue. CS chains (~50 disaccharide repeats) are found in free C-terminal 2/3 regions of aggrecan, whereas KS chains (~30 disaccharide repeats) are abundantly present in the N-terminal small region of the protein, which binds to much longer HA chains (~10,000 disaccharide repeats) [1]. These distinct locations of GAGs may affect the accessibility of proteases and may explain differences in extraction efficiency.

The present data indicate that the heat-stable protease thermolysin efficiently digests articular cartilage at high temperatures probably due to the breaking of heat-labile hydrogen bonding between collagen peptides. Previously, we demonstrated the utility of thermolysin for extraction of GAG chains for GAGomic analyses [13]. Hence, thermolysin offers a powerful tool

for complete solubilization of tissues and may facilitate investigations of both structure–function relationships and aberrations of GAGs in disorders of collagen-rich complex biological systems, including cartilages, ligaments, skin, and blood vessels.

4. Conflict of interest

The authors declare no conflicts of interest.

5. Acknowledgments

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Figure legends

Figure 1

Solubilization of cartilage with thermolysin or collagenase

Dried cartilage samples (1 mg) were incubated for indicated times with thermolysin (0.42 unit) at 70°C (●) or collagenase (5.5 unit) at 50°C (○); *P < 0.05 compared with concomitant collagenase treatments. Images of cartilage samples after 0- and 24-h incubation are shown.

Figure 2

Efficiencies of Hyp release and GAG extraction

Dried cartilage samples were incubated with thermolysin or collagenase. Efficiencies of Hyp release (black) or GAG extraction (white) (Table 2) are shown.

Figure 3

Efficiencies of CS, KS, and HA extraction

According to disaccharide analyses of extracted GAGs from cartilages after digestion

with thermolysin or collagenase (Table 3), extraction efficiencies of each disaccharide (CS; white, KS; gray, and HA; black) are shown.

Table 1

Channels for disaccharide analysis of GAGs

Table 2

Solubilization of Hyp and GAGs from cartilage after digestion with thermolysin or collagenase

Hyp release and GAG extraction from cartilage samples were determined after digestion with thermolysin or collagenase. Total Hyp and GAG contents did not differ after digestion treatments. However, solubilization efficiencies for Hyp and GAGs differed significantly between the two treatment groups; $P < 0.001$.

Table 3

Disaccharide analysis of GAGs from cartilage after digestion with thermolysin or

collagenase

Amounts of each disaccharide in GAGs extracted from cartilages after digestion with thermolysin or collagenase (Table 2) were determined using disaccharide analyses. Extraction efficiencies and compositions of each disaccharide were calculated. Disaccharide compositions are shown in parenthesis. Total quantities of each disaccharide did not differ significantly between thermolysin- and collagenase-treated samples, whereas extraction efficiencies for all disaccharides differed significantly between the two groups; $P < 0.01$.

Figure1
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Fig.1

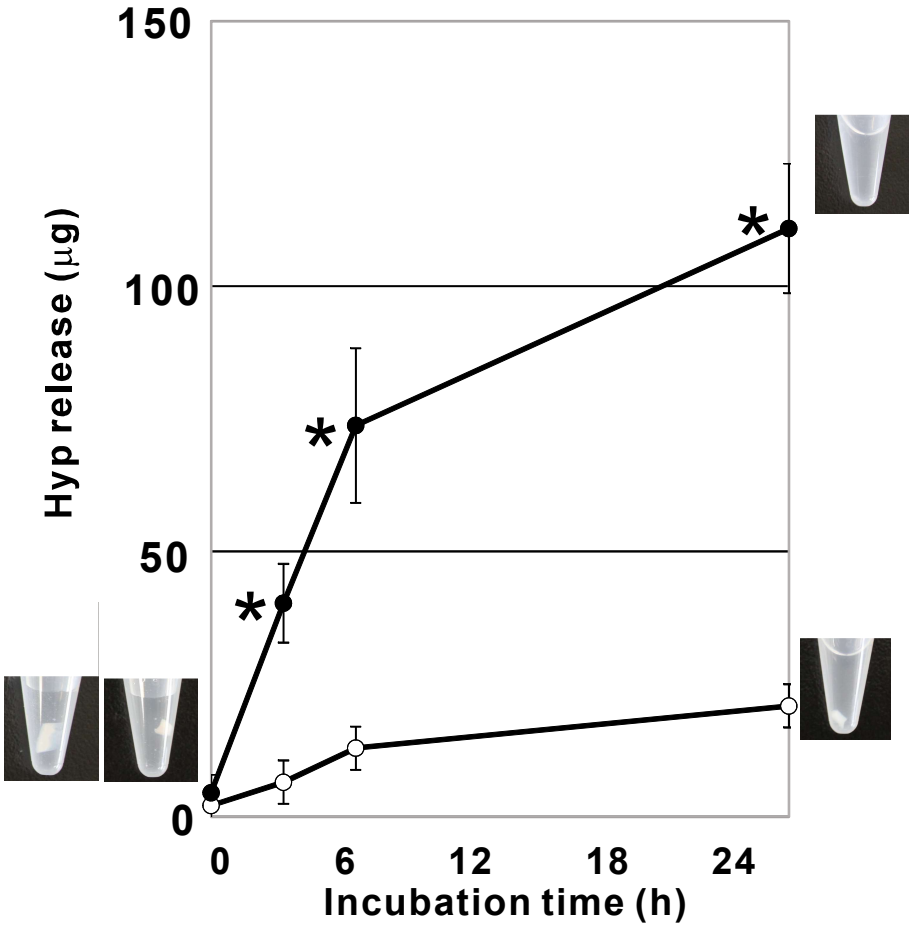

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Fig.2

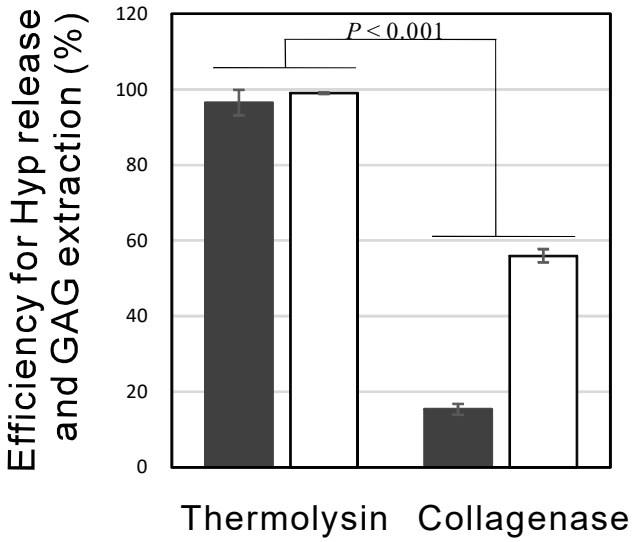

Figure3
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Fig.3

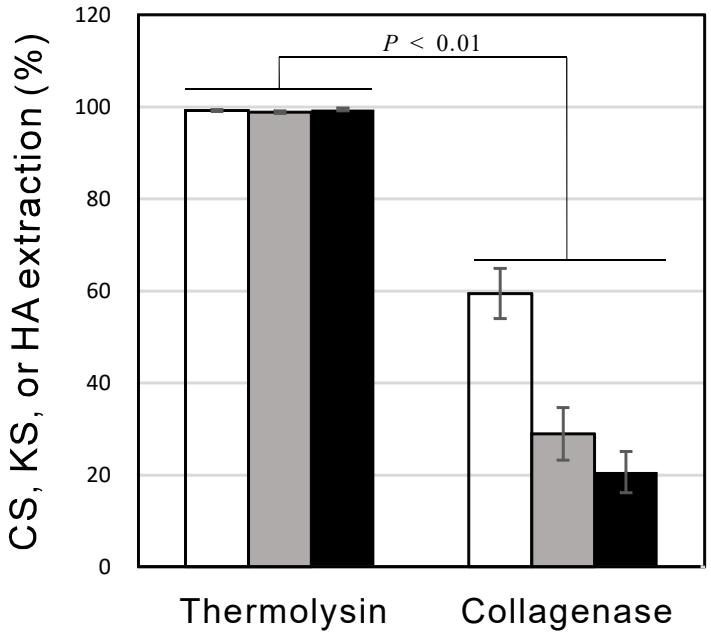

Table 1

disaccharide	transition		dwell time	CE	voltage	
	Q1	Q3			Q1	Q3
ΔCS-0S, ΔHA	378.2	175.1	100	10	27	18
ΔCS-4S	458.1	300.0	100	21	18	19
ΔCS-6S	457.9	282.0	100	24	14	30
KS-6S	542.0	462.1	100	23	15	15
KS-6S6S	462.1	97.2	100	40	18	17

Channels for disaccharide analysis of GAGs

Table 2

treatment	material	solubilized (μg)	total (μg)	efficiency (%)
thermolysin	Hyp	110.9 ± 12.2	115.3 ± 16.5	96.5 ± 3.4
	GAG	273.2 ± 86.1	275.8 ± 86.6	99.0 ± 0.2
collagenase	Hyp	20.9 ± 1.8	136.5 ± 10.2	15.3 ± 1.5
	GAG	146.1 ± 13.5	261.6 ± 29.4	56.0 ± 1.7

Solubilization of Hyp and GAGs from cartilage after digestion with thermolysin or collagenase

Hyp release and GAG extraction from cartilage samples were determined after digestion with thermolysin or collagenase. Total Hyp and GAG contents did not differ after digestion treatments. However, solubilization efficiencies for Hyp and GAGs differed significantly between the two treatment groups; $P < 0.001$.

Table3

Table 3

treatment	disaccharide	extracted (ng)	extracted (%)	total (ng)	total (%)	extraction (%)
thermolysin	0S	9940 ± 1345	(5.7 ± 1.3)	10013 ± 1345	(5.7 ± 1.3)	99.3 ± 0.2
	4S	85302 ± 37847	(45.4 ± 9.3)	86111 ± 37847	(45.5 ± 9.3)	99.1 ± 0.3
	6S	77047 ± 13506	(44.0 ± 7.7)	77509 ± 13506	(43.9 ± 7.7)	99.4 ± 0.2
	CS total	172289 ± 51424	(95.2 ± 0.7)	173632 ± 51424	(95.2 ± 0.8)	99.2 ± 0.2
	KS-6S	1567 ± 439	(0.9 ± 0.4)	1582 ± 439	(0.9 ± 0.4)	99.0 ± 0.4
	KS-6S6S	5150 ± 1372	(2.9 ± 0.4)	5211 ± 1372	(2.9 ± 0.4)	98.8 ± 0.3
	KS total	6716 ± 1626	(3.8 ± 0.7)	6794 ± 1626	(3.8 ± 0.7)	98.9 ± 0.3
	HA	1822 ± 528	(1.0 ± 0.0)	1833 ± 528	(1.0 ± 0.0)	99.4 ± 0.3
	GAG total	180827 ± 53090	(100 ± 0.0)	182259 ± 53090	(100 ± 0.0)	99.2 ± 0.2
collagenase	0S	6774 ± 2047	(6.1 ± 0.6)	10316 ± 2047	(5.5 ± 0.3)	65.7 ± 5.1
	4S	53269 ± 7116	(49.1 ± 4.8)	94449 ± 7116	(50.6 ± 3.7)	56.4 ± 6.0
	6S	47581 ± 16619	(43.0 ± 4.3)	76251 ± 16619	(40.2 ± 3.4)	62.4 ± 5.1
	CS total	107625 ± 25708	(98.3 ± 0.1)	181016 ± 25708	(96.3 ± 0.2)	59.5 ± 5.4
	KS-6S	471 ± 397	(0.4 ± 0.1)	1432 ± 397	(0.8 ± 0.1)	32.9 ± 4.4
	KS-6S6S	1017 ± 681	(0.9 ± 0.1)	3704 ± 681	(2.0 ± 0.1)	27.4 ± 6.4
	KS total	1488 ± 1078	(1.3 ± 0.1)	5135 ± 1078	(2.7 ± 0.2)	29.0 ± 5.7
	HA	385 ± 142	(0.4 ± 0.0)	1861 ± 142	(1.0 ± 0.2)	20.7 ± 4.5
	GAG total	109497 ± 26665	(100 ± 0.0)	188013 ± 26665	(100 ± 0.0)	58.2 ± 5.5

Disaccharide analysis of GAGs from cartilage after digestion with thermolysin or collagenase

Amounts of each disaccharide in GAGs extracted from cartilages after digestion with thermolysin or collagenase (Table 2) were determined using disaccharide analyses. Extraction efficiencies and compositions of each disaccharide were calculated. Disaccharide compositions are shown in parenthesis. Total quantities of each disaccharide did not differ significantly between thermolysin- and collagenase-treated samples, whereas extraction efficiencies for all disaccharides differed significantly between the two groups; $P < 0.01$.