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

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ABSTRACT

Articular cartilage comprises collagens, proteoglycans, and glycosaminoglycans (GAGs) together with water, in hyaline matrixes. Articular cartilage is resistant to proteolytic solubilization for comprehensive GAG analyses partly because of assemblies of collagen fibers with thermolabile hydrogen bonds. In this study, we used the heat-stable protease thermolysin to digest collagen in solid articular cartilage at 70 °C and compared the efficiencies of collagen digestion and GAG extraction to those with collagenase digestion at 50 °C. Overnight digestion with thermolysin completely solubilized cartilage, whereas collagenase with > 10-times higher proteolytic activity digested < 20% of collagen. Following thermolysin treatments, almost all GAGs were extracted from the cartilage, whereas only 56% of GAGs were extracted after collagenase digestion. Disaccharide analyses of extracted GAG chains revealed > 98% extraction efficiencies of several GAG classes from thermolysin-treated cartilage, compared with < 60% extraction efficiencies using collagenase, depending on GAG classes. These results indicate that thermolysin allows complete GAG extraction from solid articular cartilage and that complete solubilization is required for accurate and reproducible analyses of cartilage GAGs. Hence, thermolysin offers a tool for complete solubilization of cartilage prior to comprehensive GAGomic analysis, and is likely applicable to other collagen-rich tissues such as ligaments, skin, and blood vessels.

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.

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

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Materials and methods

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).

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.

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. To obtain total Hyp or GAG contents, further digestion was performed with thermolysin (0.42 unit) at 70 °C for 24 h in each sample. After the additional thermolysin digestion, complete digestion of cartilage collagen was confirmed by the absence of Hyp in precipitated fractions.

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.

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 in 90% ethanol, 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].

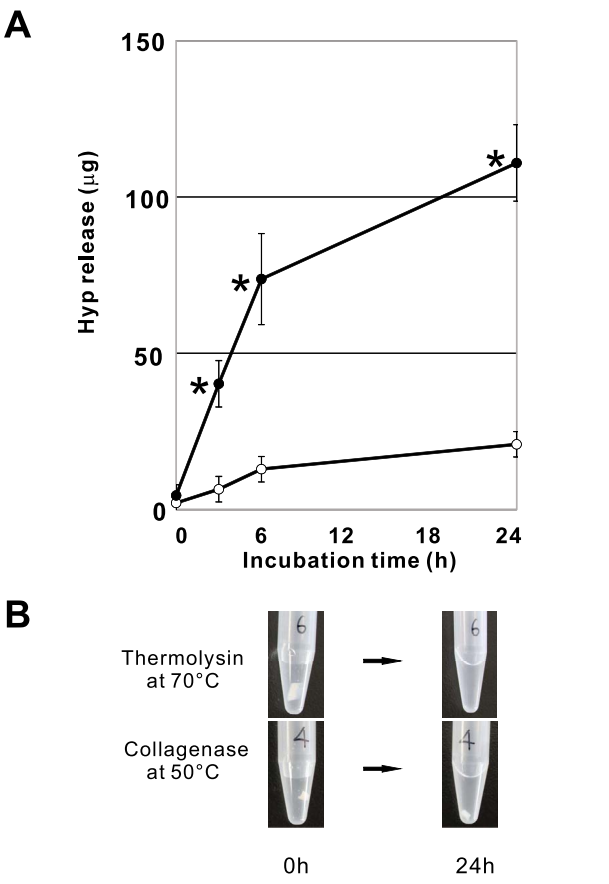

Fig. 1. Solubilization of cartilage with thermolysin or collagenase. A) Dried cartilage samples (1 mg) were incubated for indicated times with thermolysin (0.42 unit) at 70 °C (●) or collagenase (5.5 unit) at 50 °C (○); *$P < 0.05$ compared with concomitant collagenase treatments. B) Images of cartilage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

Table 1
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; $P < 0.001$.

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

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