Cleavage of the Xylosyl Serine Linkage between a Core Peptide and a Glycosaminoglycan Chain by Cellulases

Keiichi Takagaki, Mito Iwafune, Ikuko Kakizaki, Keinosuke Ishido, Yoji Kato*, and Masahiko Endo

Department of Biochemistry, Hirosaki University School of Medicine,
5 Zaifu-cho, Hirosaki 036-8562 and
Laboratory of Food Science, Faculty of Education, Hirosaki University,
1 Bunkyo-cho, Hirosaki 036-8560,
Japan

Running title: Release of Glycosaminoglycan Chains by Cellulases

Address for correspondence: Prof. Masahiko Endo, M.D.
Department of Biochemistry,
Hirosaki University School of Medicine,
5 Zaifu-cho, Hirosaki 036-8562,
Japan
Tel: +81-172-39-5015
Fax: +81-172-39-5016
SUMMARY

We previously found that endo-β-xylosidase from *Patinopecten* is an endo-type glycosidase that cleaves the xylosyl serine linkage between a glycosaminoglycan chain and its core protein (Takagaki, K., Kon, A., Kawasaki, H., Nakamura, T., Tamura, S., and Endo, M. (1990) *J. Biol. Chem.* 265, 854-860). Screening for endo-β-xylosidase activity in several cellulases detected this activity in the enzymes from *Aspergillus niger*, *Penicillium funiculosum*, *Trichoderma reesei*, *Trichoderma viride* and *Irpex lacteus*. The cellulase derived from *Aspergillus niger* was purified and its molecular weight was determined to be 26,000 by SDS-PAGE. Examination of the specificity of the cellulase revealed that 1) the enzyme acts on the linkage region (xylosyl serine) between a core peptide and a glycosaminoglycan chain; 2) enzymatic activity is greater with shorter glycosaminoglycan chains; 3) the enzyme readily hydrolyzes the linkage in glycosaminoglycan-peptides, but intact proteoglycan is cleaved only slowly; 4) the activity is unaffected by the glycosaminoglycan component (chondroitin sulfate, dermatan sulfate and heparan sulfate). Judging from these enzymatic characteristics, this cellulase is different from the endo-β-xylosidase of *Patinopecten*. We believe that this cellulase will become a useful tool in the further development of glycotechnology, because, like the endo-β-xylosidase of *Patinopecten*, it enables the release of intact glycosaminoglycans from glycosaminoglycan-peptides.
INTRODUCTION

Proteoglycans (PGs) are widely distributed in connective tissue and on the cell surface of mammalian tissues, and are functional materials influencing cell growth, differentiation and morphogenesis (1, 2). It is known that PGs consist of a core protein linked to glycosaminoglycan (GAG) chains, and that the GAG chains interact with a number of growth factors (3) and with important functional proteins such as antithrombin III (4-6) and heparin cofactor II (7). Although GAG chains are broadly divided into chondroitin sulfate (ChS), dermatan sulfate (DS), heparan sulfate (HS), heparin, hyaluronic acid and keratan sulfate by their particular combination of repeating disaccharide units, ChS, DS, HS and heparin are all attached to a serine residue of a core protein by the linkage region GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-O-Ser (8). Recently, it has been shown that the linkage region is modified by phosphate and sulfate groups (9-11). Such diversity of linkage regions is notable, because it suggests that the modification is a signal involved in the control of GAG biosynthesis (11).

The metabolism of PGs in vivo has not yet been clarified. Matsue and Endo (12) demonstrated that urinary GAG chains bear in part glucuronic acid, galactose or xylose residues at their reducing terminals. These observations demonstrate that nonterminal internal glucuronide, galactoside and xyloside linkages of GAG chains are cleaved in tissues. Indeed, Takagaki et al. (13, 14) found endo-type glycosidase activities acting on the linkage regions in rabbit liver, in the form of ChS-degrading endo-β-glucuronidase, endo-β-galactosidase and endo-β-xylosidase activities. These enzymes appear to act in the initial catabolism of PGs in animal tissues. One of them, endo-β-xylosidase, was purified from the mid-gut gland of the mollusk Patinopecten (15). It specifically hydrolyzes the internal xylosyl serine (Xyl-Ser) linkage between a core protein and a GAG chain (ChS, DS and HS) and is therefore a useful tool for isolating native GAG chains, including the linkage region, from GAG-peptides.

Cellulases endolytically hydrolyze 1,4-β-glucoside linkages, and are widely distributed in plants, microorganisms and in particular, fungi. Furthermore, the presence of endogenous
cellulases in animals has been suggested recently (16-21). There is evidence that exo-type enzymes recognize both xyloside and glucoside linkages. An exo-xylosidase acting on p-nitrophenyl β-xylose and p-nitrophenyl β-glucose has been purified from Charonia lampas (22, 23). Additionally, it was suggested that β-glucosidase purified from Aspergillus sojae has β-xylosidase activity (24), and a single protein in pig kidney has both β-xylosidase activity and β-glucosidase activity (25). Xylose and glucose have very similar configurations, with the C-5 of xylose having a hydrogen atom rather than the hydroxymethyl group of glucose. Therefore, it is likely that enzymes that recognize glucose will also recognize xylose. Additionally, it is of interest to determine whether the endo-β-xylosidase derived from Patinopecten was originally a cellulase, or whether it is a cellulase with endo-β-xylosidase activity, or whether the two activities represent independent enzymes.

In this paper, we demonstrate that a fungal cellulase is able to hydrolyze the Xyl-Ser linkage between the core peptide and the GAG chain of GAG-peptides. Although this cellulase has endo-β-xylosidase activity, it is different from the endo-β-xylosidase of Patinopecten.
EXPERIMENTAL PROCEDURES

Materials — Cellulase preparations (EC 3.2.1.4) derived from *Aspergillus niger* (1.6 units/mg, 1 unit defined as the amount of enzyme that liberates 1 µmol of reducing sugar as glucose in 1 h at pH 4.5 at 37°C), *Penicillium funiculosum* (5.1 units/mg, 1 unit defined as the amount of enzyme that liberates 1 µmol of reducing sugar as glucose in 1 h at pH 5.0 at 37°C), *Trichoderma reesei* (6.3 units/mg, 1 unit defined as the amount of enzyme that liberates 1 µmol of reducing sugar as glucose in 1 h at pH 5.0 at 37°C), and *Trichoderma viride* (4.0 units/mg, 1 unit defined as the amount of enzyme that liberates 1 µmol of reducing sugar as glucose in 1 h at pH 5.0 at 37°C) were purchased from Sigma Chem. Co., St. Louis, MO. A cellulase preparation derived from *Eupenicillium* sp. (1.0 units/mg, 1 unit defined as the amount of enzyme that liberates 1 µmol of 4-methylumbelliferone from 4-methylumbelliferyl-β-lactoside in 1 h at 37°C) was a gift from Dr. Yasushi Mitsuishi, National Institute of Advanced Industrial Science and Technology, Japan. Cellulase from *Irpex lacteus* (1.07 units/mg, 1,000 units defined as the amount of enzyme that completely destroys 2 sheets of quantitative filter paper 1 cm x 1 cm in 1 min at pH 5.0 at 37°C) was a gift from Kyowa Hakko Kogyo Co., Tokyo, Japan. Cellulases from *Pisum sativum* L. and *Populus nigra* (2 units/ml and 1 unit/ml, respectively, 1 unit defined as the amount of enzyme that causes a 1% decrease in viscosity of carboxymethyl-cellulose in 2 h at pH 6.2 at 35°C) were gifts from Dr. Takahisa Hayashi, Wood Research Institute of Kyoto University, Japan.

Endo-β-xylosidase was purified from *Patinopecten* as described previously (15). 4-Methylumbelliferyl GAG (GAG-MU), which is an artificial substrate for endo-β-xylosidase, was prepared from cultured medium of human skin fibroblasts by the method previously reported (26). After digestion of GAG-MU with hyaluronidase from *Streptomyces hyalurolyticus* (Seikagaku Kogyo Co., Tokyo, Japan), Galβ1-4Xylβ1-MU and Galβ1-3Galβ1-4Xylβ1-MU were purified by Bio-Gel P-4 (Bio-Rad, Richmond, CA) column chromatography as described previously (27, 28). To obtain ΔGlcAβ1-3GalNAcβ1-3GlcAβ1-
3Galβ1-3Galβ1-4Xylβ1-MU and ΔGlcAβ1-3Galβ1-3Galβ1-4Xylβ1-MU, GAG-MU was digested with chondroitinase ABC and chondroitinase ACII (Seikagaku Kogyo Co.) as described by Saito et al. (29), and then purified by Sephadex G-50 (Amersham Pharmacia Biotec AB, Uppsala, Sweden) column chromatography. 4-Methylumbelliferyl-β-cellobioside (cellobiose-MU) and 4-methylumbelliferyl-β-xylose (Xyl-MU) were purchased from Sigma Chem. Co.

Chondroitin sulfate proteoglycan (ChS-PG), dermatan sulfate proteoglycan (DS-PG) and heparan sulfate proteoglycan (HS-PG) were extracted from salmon cartilage, pig skin and bovine lung, and purified by standard procedures as described by Heinegård and Hascall (30). These PGs were digested with actinase E (Kaken Pharmaceutical Co., Tokyo, Japan) in 0.1 M Tris-HCl buffer, pH 8.0, containing 10 mM CaCl₂ at 50°C for 24 h. After digestion, the GAG-peptides (ChS-peptides, DS-peptides and HS-peptides) were separated by DEAE-cellulose (Whatman Chemical Separation Ltd., Maidstone, United Kingdom) column chromatography and then purified by Sephacryl S-200 HR (Amersham Pharmacia Biotec AB) column chromatography. The molecular weights of the peptide moieties of the ChS-peptides, DS-peptides and HS-peptides were all about 1,000 as estimated from gel-filtration chromatography based on the method of Ishido et al. (31). The linkage regions of the GAG-peptides were analyzed for sulfate and phosphate contents based on the method of Takagaki et al. (28). Sulfate or phosphate were not detected; therefore, it was considered that the GAG-peptides used in this study did not contain phosphorylated xylose or sulfated galactose. All other chemicals were obtained from commercial sources.

Fluorescence labeling with 2-aminopyridine — Fluorescence labeling of the reducing terminal of the oligosaccharides with 2-aminopyridine (PA; Wako Pure Chemical Ind. Co., Tokyo, Japan) was carried out as described previously (32).

Assay of endo-β-xylosidase activity and cellulase activity — Endo-β-xylosidase activity was
assayed as described previously (15). A complete incubation mixture (200 µl), which contained 2 µM GAG-MU as a substrate, 0.1 M glucono-1,5-lactone (Wako Pure Chem. Co.) as an inhibitor of exo-glucosidase, 0.1 M sodium acetate buffer, pH 5.0, and enzyme solution, was incubated at 37°C for 1 h. The reaction was stopped and fluorescence developed by adding 1 ml of 0.5 M glycine-NaOH buffer, pH 10.4. The fluorescence was measured on a spectrofluorometer (Hitachi F-4500; Hitachi, Tokyo, Japan) at excitation and emission wavelengths of 350 and 450 nm.

Cellulase activity was assayed with 2 µM cellobiose-MU as substrate based on the method of Chernoglazov et al. (33).

One unit was defined as the amount of enzyme that liberated 1 µmol/min of MU from GAG-MU or cellobiose-MU.

Another assay for cellulase activity using CM-cellulose as a substrate was performed as described by Hurst et al. (34).

Assay of hydrolysis of natural substrates — Hydrolysis of natural substrates, GAG-peptides (10 nmol) or intact ChS-PG (10 nmol; the molar amount of ChS-PG was calculated as the molar amount of the GAG sugar chains), was assayed as described previously (35). Each GAG-peptide was incubated with the purified cellulase from A. niger at 37°C for 12 h, and the reducing terminals of the liberated GAG chains were labeled with PA. Then, each GAG-PA chain was hydrolyzed in 2 M HCl at 100°C for 2 h. PA-xylose was detected by HPLC with an Ultrasphere ODS column (4.6 mm x 250 mm; Beckman Coulter Inc., Fullerton, CA). The molar amounts of the GAG-peptides were determined as the molar amount of PA-xylose (Takara Shuzo, Kyoto, Japan).

Purification of cellulase— The following procedures were conducted at 4°C. First, commercial cellulase (30 mg of protein) from A. niger was applied to a column of Sephacryl S-100 HR (1.5 cm x 102 cm; Amersham Pharmacia Biotec AB) equilibrated previously with
20 mM Tris-HCl buffer, pH 7.0, at a flow rate of 15 ml/h, and the fractions with cellulase activity and endo-β-xylosidase activity were pooled. Next, the active fractions were applied to a column of POROS PI (4.6 mm x 100 mm; Applied Biosystems Japan, Tokyo, Japan) equilibrated previously with 20 mM Tris-HCl buffer, pH 7.0. The column was eluted with a linear gradient of NaCl from 0 to 1 M containing the above buffer at a flow rate of 10 ml/min. The fractions with both activities were pooled and, lastly, the pool was subjected to isoelectric focusing using a Rotofor IEF Cell (Bio-Rad) with ampholyte (Bio-Rad) ranging from pH 3.0 to 10.0 for 4 h.

**High-performance liquid chromatography (HPLC)** — A high-performance liquid chromatograph (Hitachi L-6200) connected to a fluorescence detector (Hitachi F-1050) was used. MU-derivatives and MU were analyzed on an Ultrasphere ODS column (4.6 mm x 250 mm; Beckman Coulter Inc.) with a linear gradient of acetonitrile from 0% to 30% for 50 min at a flow rate of 1.0 ml/min at 30°C. For detection, eluates were monitored at excitation and emission wavelengths of 325 and 380 nm. PA monosaccharides were analyzed on an Ultrasphere ODS column (4.6 mm x 250 mm; Beckman Coulter Inc.) with 0.25 M sodium citrate and 1% acetonitrile at a flow rate of 0.5 ml/min at 30°C, as described previously (26). For detection, eluates were monitored at excitation and emission wavelengths of 320 and 400 nm. PA-glucose, PA-galactose and PA-xylose (Takara Shuzo) were used as standards.

**Electrophoresis** — Native polyacrylamide gel electrophoresis (PAGE) was done in 7.5% polyacrylamide gel at 4°C. Protein was stained with Coomassie Brilliant Blue R-250, and duplicate gels were cut into 5-mm segments. Each segment was extracted with 10 mM Tris-HCl buffer, pH 7.0, at 4°C for 24 h, and the activity was assayed. SDS-PAGE was carried out in 10% polyacrylamide gel according to the methods of Weber and Osborn (36). Protein was stained with Coomassie Brilliant Blue R-250.
**Ion-spray mass spectrometry** — Mass spectra were obtained on an API-100 triple-quadrupole mass spectrometer (PE SCIEX, Ontario, Canada) equipped with an atmospheric pressure ionization source, as described previously (27, 37). The samples were dissolved in 50% methanol and injected at 2 µl/min with a micro-HPLC syringe pump. In positive-ion mode, scanning was done from m/z 30 to 250 during the 1-min scan (ten cycles).

**Protein assay** — Protein was determined by the method of Bradford (38), with bovine serum albumin as a standard.
RESULTS

Screening of endo-β-xylosidase activity in cellulases — Endo-β-xylosidase activity, which hydrolyzed the Xyl-MU linkage of GAG-MU as a substrate, and cellulase activity, which hydrolyzed cellobiose-MU as a substrate, were assayed in eight cellulase preparations from five kinds of ascomycetes, one basidiomycetes and two plants, and in endo-β-xylosidase from Patinopecten mid-gut gland (Fig. 1). Endo-β-xylosidase activity was found in the five cellulase preparations from Aspergillus niger, Penicillium funiculosum, Trichoderma reesei, Trichoderma viride and Irpex lacteus. Particularly high endo-β-xylosidase activities were detected in the celllases from A. niger and P. funiculosum. These results indicated that some cellulases have endo-β-xylosidase activity in addition to cellulase activity. Fig. 1-I shows that the endo-β-xylosidase from Patinopecten appeared to be different from the cellulases, having much higher endo-β-xylosidase activity and very little cellulase activity.

To study the purity of each cellulase preparation, electrophoresis in 7.5% polyacrylamide gel was performed and then the gels were stained with Coomassie Brilliant Blue. As a result, several protein bands were observed in the cellulase preparations, except for that from Eupenicillium sp. Duplicate gels were cut into 5-mm segments, extracted individually for 24 h with 10 mM Tris-HCl buffer, pH 7.0, at 4°C, and endo-β-xylosidase activity and cellulase activity were measured in each fraction. These results with native PAGE are summarized in Fig. 2. In the cellulase preparation from A. niger, endo-β-xylosidase activity was detected in the same fractions in which cellulase activity was detected (Fig. 2-A). Similar results were obtained with the preparations from T. reesei, T. viride, and I. lacteus (Fig. 2-C, D and F). In contrast, the cellulase preparation from Eupenicillium sp. had only cellulase activity but not endo-β-xylosidase activity (Fig. 2-E). In P. funiculosum, both activities were detected in segment numbers 4 - 6 and only cellulase activity was detected in segment numbers 7 - 9 (Fig. 2-B). These results suggested that there are two types of cellulase, one having both endo-β-xylosidase and cellulase activities and one having only cellulase activity.
Purification of the cellulase from *A. niger* — In order to verify that a single enzyme protein has both cellulase and endo-β-xylosidase activities, we performed the following experiment. Although the β-xylosidase activity in *P. funiculosum* was higher than that in *A. niger* (Fig. 1), the cellulase from *A. niger* is more widely available commercially. Thus, from the commercial cellulase preparation the enzyme was purified 5-fold at 11% yield in endo-β-xylosidase activity and cellulase activity by the sequential use of Sephacryl S-100 HR column chromatography, POROS PI column chromatography and isoelectric focusing electrophoresis (Table I). The cellulase exhibited endo-β-xylosidase activity during the course of purification, and the ratio of each enzyme activity was almost constant. Fig. 3 shows that native PAGE of this purified enzyme yielded a single band, with an estimated molecular weight of 26,000 by SDS-PAGE. These results showed that the single protein band has the activities of endo-β-xylosidase and cellulase. Additionally, the optimal pH for the enzyme toward CM-cellulose was 3.8 (data not shown). The molecular weight of this enzyme and its optimal pH toward CM-cellulose corresponded with the properties of cellulase from *A. niger*, which has been purified previously (34, 39).

Action of the purified cellulase from *A. niger* on artificial substrates — To examine the action of the purified cellulase from *A. niger* on the artificial substrates GAG-MU and cellobiose-MU, the enzymatic products were subjected to reverse-phase HPLC (Fig. 4). After incubation with the purified cellulase, the peaks of GAG-MU and cellobiose-MU had disappeared and new fluorogenic components were found to be eluted at the position of authentic MU (Fig. 4). The new fluorogenic components were collected and ion-spray mass spectrometry analysis was carried out. The spectra of both new fluorogenic components showed two peaks of \( m/z \) [M+H]+ ion and [M+Na]+ ion at \( m/z \) 177 [M+H]+ and 199 [M+Na]+, respectively (Fig. 5). The combined results of HPLC analysis and ion-spray mass spectrometry indicated that this fluorogenic component was MU, presumably released from the corresponding reducing ends.
of GAG-MU and cellobiose-MU.

To identify the newly exposed reducing terminal sugars of the other incubation products, they were fluorescence-labeled with PA and hydrolyzed with 2 M HCl at 100°C for 2 h. After \(N\)-acetylation with acetic anhydride, PA-sugars were subjected to reverse-phase HPLC on an Ultrasphere ODS column as described previously (35). An elution profile is shown in Fig. 6. The PA-sugar derived from GAG-MU gave a single peak at the position of standard PA-Xyl (Fig. 6-A). On the other hand, that from cellobiose-MU gave a single peak at the position of standard PA-glucose (Fig. 6-B). These data proved that xylose and glucose were exposed at the reducing terminal ends of the sugar chains after digestion of GAG-MU and cellobiose-MU with the cellulase. Thus, these results showed that the purified cellulase from \(A.\ niger\) was able to hydrolyze endolytically the 4-methylumbelliferyl-\(\beta\)-xyloside linkage and the 4-methylumbelliferyl-\(\beta\)-glucoside linkage of GAG-MU and cellobiose-MU, respectively.

Comparison of endo-\(\beta\)-xylosidase activity and cellulase activity of purified enzyme from \(A.\ niger\) – The optimal pH values, time courses, and effects of metal ions were compared for both hydrolysis reactions. For both activities, optimal pH was around 5.0 and the activity curves were similar (Fig. 7-A). In the time course studies, hydrolysis by endo-\(\beta\)-xylosidase was slower than that by cellulase (data not shown). Neither activity was affected by monovalent cations or EDTA, with little difference discernible between endo-\(\beta\)-xylosidase activity and cellulase activity. However endo-\(\beta\)-xylosidase activity was slightly decreased by bivalent cations compared with cellulase activity (Table II).

Effect of GAG size on the endo-\(\beta\)-xylosidase activity from \(A.\ niger\) — To investigate the effect of GAG size on the purified cellulase, several MU derivatives were incubated with the purified cellulase. After incubation, liberated MU was measured by HPLC. Hydrolysis activity was greater with shorter GAG chains, but Xyl-MU was not hydrolyzed (Table III). Hydrolysis by the endo-\(\beta\)-xylosidase from \(Patinpecten\), obtained from a previous report (15),
is also shown. Endo-β-xylosidase from *Patinopecten* hydrolyzed GAG-MU efficiently, but oligosaccharides were unable to serve as substrates. We concluded that substrate specificity for GAG chain length is different between the purified cellulase from *A. niger* and the endo-β-xylosidase from *Patinopecten*.

*Activity of the endo-β-xylosidase from A. niger toward natural substrates* — The properties of the endo-β-xylosidase activity from *A. niger* toward natural substrates were examined.

First, in order to investigate whether the enzyme can act on intact PG, the time course of hydrolysis of ChS-PG was compared with that of ChS-peptide (Fig. 8). The results showed that the hydrolysis rate of ChS-PG was small compared with that of ChS-peptide. Therefore, it is difficult for the enzyme to cleave the Xyl-Ser linkage of PG.

Secondly, the effects of pH and metal ions on the activity toward ChS-peptide were examined to determine optimal assay conditions. With ChS-peptide as substrate, the optimal pH was around 5.0 (Fig. 7-B), which corresponded with the optimal pH values toward cellobiose-MU and GAG-MU (Fig. 7-A). In addition, the effect of metal ions on endo-β-xylosidase activity toward ChS-peptide was similar to that on activity toward GAG-MU (Table II).

Thirdly, the effect of GAG components on hydrolysis activity was examined at pH 5.0 without any metal ions. The purified cellulase hydrolyzed the Xylβ1-O-Ser linkage of ChS-peptide, DS-peptide and HS-peptide. Hydrolysis rates of the GAG-peptides are shown in Table IV. The Km value for ChS-peptide was estimated to be 0.84 mM by Lineweaver-Burk plot (data not shown).
DISCUSSION

PGs are composed of a core protein and a GAG chain whose molecular weight is several tens of thousands (1, 2). The GAG chains are attached to serine residues of the core protein via a linkage region, GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-O-Ser, which is common to ChS, DS, HS and heparin (8). In this paper, we showed that the cellulase purified from *A. niger* has endo-β-xylosidase activity, which cleaves the Xyl-Ser linkage between the core protein and the GAG chain of GAG-peptides, in addition to containing endo-β-glucosidase. An endo-β-xylosidase with similar catalytic activity has been purified from *Patinopecten* and characterized (15). Examination of the specificity of the cellulase from *A. niger* revealed that 1) the enzyme acts on the linkage region (Xyl-Ser) between a core peptide and GAG chains; 2) enzymatic activity is greater with shorter GAG chains; 3) the enzyme readily hydrolyzes the linkage in GAG-peptides, but intact PG is cleaved only slowly; 4) the activity is unaffected by the GAG component (ChS, DS and HS). This enzyme was similar to endo-β-xylosidase from *Patinopecten* in 1), 3) and 4). However, *Patinopecten* endo-β-xylosidase catalyzes hydrolysis of long GAG chains, but short GAG chains are hardly cleaved. Additionally, the amino acid sequence of endo-β-xylosidase from *Patinopecten* showed no homology to that of the cellulases (unpublished data). Taken together with the fact that the endo-β-xylosidase from *Patinopecten* had little cellulase activity, these observations show that the *Patinopecten* enzyme is different from the cellulase purified in this study.

It is known that some glycosidases recognize both xyloside linkages and glucoside linkages as a result of the similarity of the configurations of these molecules. An exo-type glycosidase acting on both *p*-nitrophenyl β-glucoside and *p*-nitrophenyl β-xyloside has been purified from *Charonia lampas* (22, 23). Moreover, it has been suggested that β-glucosidase purified from *Aspergillus sojae* has β-xylosidase activity (24), and a single protein in pig kidney has both β-xylosidase activity and β-glucosidase activity (25). Also, it has been reported that addition of Xyl-MU to cultured human skin fibroblasts or Chinese hamster...
ovary cells induces SAα2-3Galβ1-4Xylβ1-MU, which is initiated by β-xyloside as a primer, rather than the expected SAα2-3Galβ1-4Glcβ1-MU, which is initiated by β-glucose as a primer (40, 41), suggesting that the galactosyltransferases involved in biosynthesis cannot distinguish between xylose and glucose. Similarly, cellulase, which is a glucosidase, seems also to show endo-type β-xylosidase activity.

Endo-β-xylosidase activity was compared with endo-β-glucosidase activity in the purified cellulase from *A. niger*. The optimal pH was around 5.0 and the activity curves were similar for both activities. Hydrolysis by endo-β-xylosidase was slower than that by endo-β-glucosidase (data not shown). The inhibitory effects of metal ions were similar for endo-β-xylosidase activity and endo-β-glucosidase activity across the range of 11 reagents examined. Endo-β-xylosidase activity was slightly inhibited by bivalent cations compared with cellulase activity. GAG-MU was used as a substrate for assay of endo-β-xylosidase activity. GAG contains a large number of negative charges such as sulfate groups and carboxyl groups (42). It is known that bivalent cations, for example Ca^{2+}, can form chelates between the negative charges of GAG (43). Thus, the configuration of the entire GAG may be altered by binding of cation, which may have affected enzymatic activity.

The properties of endo-β-xylosidase activity toward natural substrates were investigated. The optimal pH for, and the effect of metal ions on, hydrolysis of ChS-peptide were similar to those of hydrolysis of GAG-MU. The enzyme hydrolyzed the Xyl-Ser linkages of ChS-peptide, DS-peptide and HS-peptide, but not ChS-PG. In addition, the hydrolysis of HS-peptide was faster than that of ChS-peptide and DS-peptide.

Although GAG chains are attached to serine residues of the core protein via the common linkage region (8), it has been shown recently that the linkage region is modified by phosphate and sulfate groups (9-11). Additionally, it has been suggested that the linkage region can act as a recognition signal for sorting in the biosynthesis of different GAG chains (11). The linkage regions of the GAG-peptides used in this study did not contain phosphorylated xylose or sulfated galactose. Therefore, it is unclear whether the enzyme can
act on substrates containing phosphorylated xylose and/or sulfated galactose, and this requires further investigation.

Endo-β-xylosidase activity was found in cellulase preparations from *A. niger*, *P. funiculosum*, *T. reesei*, *T. viride* and *I. lacteus*, although not in the preparations from *Eupenicillium* sp., *P. nigra* and *P. sativum*. Consequently, this activity is not specific for the cellulase from *A. niger*. Although the presence or absence of endo-β-xylosidase activity may be related to the different action patterns of cellulases, for example endolytic or exolytic cleavage, this is not clear at the present time.

The structural diversity of GAG chains, in terms of sugar chain length, sugar chain composition and position, and degree of sulfation, is well established. However, all GAG chains are attached to a xylose residue, which forms an *O*-glycoside linkage (9). A chemical method, β-elimination (44), is known to release GAG chains, but may cause their decomposition by the peeling reaction. On the other hand, endo-β-xylosidase is useful for analysis of GAG chains, since it specifically releases intact GAG chains from GAG-peptides. Using this enzyme, it was possible to analyze GAG chains from 50 mg wet weight of animal tissues with the combined use of PA-labeling at the reducing terminal of the released sugar chains and HPLC (45). Furthermore, this enzyme is useful in the molar quantification of GAG chains (45), in giving directivity to GAG chains (46), and in preparing artificial substrates for novel endo-type enzymes (35). Therefore, we believe that cellulases with endo-β-xylosidase activity will become useful tools for structural and functional analysis.
REFERENCES


Footnotes:

This work was supported by Grants-in-Aid (nos. 09358013, 11121203, 11470029, 12680603 and 12793010) for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and grants from the Aomori Support Center for Industrial Promotion.

The abbreviations used are: PG, proteoglycan; GAG, glycosaminoglycan; ChS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; Ser, serine; MU, 4-methylumbelliferone; PA, 2-aminopyridine; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; GlcA, glucuronic acid; Gal, galactose; Xyl, xylose; ΔGlcA, D-gluco-4-enepyranosyluronic acid; GalNAc, N-acetylgalactosamine; SA, sialic acid; IdoA, iduronic acid. All sugars mentioned in this paper are of D configuration except for iduronic acid.
FIGURE LEGENDS

Fig. 1. **Comparison between endo-β-xylosidase activity and cellulase activity in cellulase preparations from various origins and in endo-β-xylosidase**. Each activity was measured using 1 unit of each enzyme as defined in *Materials* under “EXPERIMENTAL PROCEDURES”. Endo-β-xylosidase activity was measured with 2 µM GAG-MU as substrate (solid bars), and cellulase activity was measured with 2 µM cellobiose-MU as a substrate (open bars). The cellulase preparations were derived from A, *A. niger*; B, *P. funiculosum*; C, *T. reesei*; D, *T. viride*; E, *Eupenicillium* sp.; F, *I. lacteus*; G, *P. sativum* L.; H, *P. nigra*; and I, endo-β-xylosidase from *Patinopecten*.

Fig. 2. **Native PAGE of various cellulase preparations**. Each cellulase preparation (A, *A. niger*; B, *P. funiculosum*; C, *T. reesei*; D, *T. viride*; E, *Eupenicillium* sp.; F, *I. lacteus*) was separated in 7.5% polyacrylamide gel and stained with Coomassie Brilliant Blue. Duplicate gels were cut into 5-mm segments, and extracted individually for 24 h with 10 mM Tris-HCl buffer, pH 7.0, at 4°C, and endo-β-xylosidase activity (solid bars) and cellulase activity (open bars) were measured in each fraction.

Fig. 3. **Native PAGE (A) and SDS-PAGE (B) of the purified cellulase from *A. niger***. PAGE and SDS-PAGE were performed in 7.5% and 10% polyacrylamide gel, respectively, at a constant 20 mA. Proteins were stained with Coomassie Brilliant Blue.

Fig. 4. **Analysis by HPLC of GAG-MU (A and B) and cellobiose-MU (C and D) before and after digestion with the purified cellulase from *A. niger***. MU derivatives were analyzed by HPLC before digestion (A and C) and after digestion (B and D) with the purified cellulase from *A. niger*. An Ultrasphere ODS column (4.6 mm x 250 mm) was used with a linear gradient of acetonitrile from 0% to 30% for 50 min at a flow rate of 1.0 ml/min at 30°C.
For detection, eluates were monitored at excitation and emission wavelengths of 325 and 380 nm. The arrow indicates the elution position of authentic MU.

**Fig. 5.** Ion spray mass spectra of new fluorogenic products obtained from GAG-MU and cellobiose-MU by cellulase digestion. GAG-MU (A) and cellobiose-MU (B) were digested with the purified cellulase from *A. niger*, and positive-mode mass spectra of the hydrolysates were analyzed. The spectra were acquired after injection of the samples dissolved in a mobile phase of 50% methanol.

**Fig. 6.** HPLC chromatograms of the reducing terminal sugars in the hydrolysates obtained from GAG-MU and cellobiose-MU by cellulase digestion. GAG-MU (A) and cellobiose-MU (B) were digested with the purified cellulase from *A. niger*. The hydrolysates were fluorescence-labeled with PA and hydrolyzed with 2 M HCl at 100°C for 2 h. After N-acetylation with acetic anhydride, PA-sugars were subjected to reverse-phase HPLC. An UltraspHERE ODS column (4.6 mm x 250 mm) was used with 0.25 M sodium citrate and 1% acetonitrile at a flow rate of 0.5 ml/min at 30°C. For detection, eluates were monitored at excitation and emission wavelengths of 320 and 400 nm. The arrows indicate the elution positions of the PA-mono- and disaccharide standards: 1, PA-glucose; 2, PA-galactose; 3, PA-xylose.

**Fig. 7.** Effect of pH on the purified cellulase from *A. niger*. GAG-MU (solid symbols) and cellobiose-MU (open symbols) were incubated with the purified enzyme (A). ChS-peptide was incubated with the enzyme (B). The assay method for enzyme activity followed that described in “EXPERIMENTAL PROCEDURES” except for the composition of the buffer. The following buffers were used: circles, 0.1 M glycine-HCl buffer; triangles, 0.1 M sodium acetate buffer; diamonds, 0.1 M sodium phosphate buffer; squares, 0.1 M Tris-HCl buffer.
Fig. 8.  Time courses of the hydrolysis of natural substrates by the purified cellulase from *A. niger*. ChS-PG (open circles) and ChS-peptide (solid circles) were incubated with the purified enzyme as described under “EXPERIMENTAL PROCEDURES”.
Fig. 1

Activity of cellulase (unit, □)

Activity of endo-β-xylosidase (unit, ■)

6.7
Fig. 2
Fig. 4
Fig. 5
Fig. 7
<table>
<thead>
<tr>
<th></th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Recovery</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>units</td>
<td>units/mg</td>
<td>%</td>
<td>-fold</td>
</tr>
<tr>
<td>Commercial enzyme</td>
<td>30.0</td>
<td>29.7</td>
<td>55.9</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Sephacryl S-100 HR</td>
<td>7.6</td>
<td>9.8</td>
<td>23.1</td>
<td>33</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.3</td>
<td>3.0</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>POROS PI</td>
<td>1.2</td>
<td>3.5</td>
<td>10.6</td>
<td>12</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.0</td>
<td>8.8</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Rotofor</td>
<td>0.6</td>
<td>3.3</td>
<td>6.0</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.5</td>
<td>10.0</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.3</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE I**

Purification of cellulase from *A. niger*

A, endo-β-xylosidase activity; B, cellulase activity.
TABLE II

Effects of various agents on cellulase from A. niger

The enzyme was assayed under normal conditions described in "EXPERIMENTAL PROCEDURES" except for the presence of various agents.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cellobiose-MU</td>
</tr>
<tr>
<td>10 mM</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>74</td>
</tr>
<tr>
<td>FeSO₄</td>
<td>3</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>99</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>94</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>95</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>97</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>95</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>104</td>
</tr>
<tr>
<td>NaCl</td>
<td>97</td>
</tr>
<tr>
<td>KCl</td>
<td>99</td>
</tr>
<tr>
<td>EDTA</td>
<td>102</td>
</tr>
</tbody>
</table>
TABLE III
Comparative activity of cellulase purified from A. niger

After 1 μM substrates were incubated with purified cellulase from A. niger, the liberated MU was assayed by HPLC. The preparation of substrates is described in "EXPERIMENTAL PROCEDURES". An Ultrasphere ODS column (4.6 mm x 250 mm) was used with a linear gradient of acetonitrile from 0% to 30% for 50 min at a flow rate of 1.0 ml/min at 30°C. For detection, eluates were monitored at excitation and emission wavelengths of 350 and 450 nm. The activities were determined using the peak area of authentic MU.

| MU derivative                      | Relative activity  
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Purified cellulase from A. niger</td>
</tr>
<tr>
<td>-(GlcA/IdoA-GalNAc(SO₃))₆-GlcA-Gal-Gal-Xyl-MU</td>
<td>100</td>
</tr>
<tr>
<td>ΔGlcA-GalNAc-GlcA-Gal-Gal-Xyl-MU</td>
<td>134</td>
</tr>
<tr>
<td>ΔGlcA-Gal-Gal-Xyl-MU</td>
<td>148</td>
</tr>
<tr>
<td>Gal-Gal-Xyl-MU</td>
<td>173</td>
</tr>
<tr>
<td>Gal-Xyl-MU</td>
<td>264</td>
</tr>
<tr>
<td>Xyl-MU</td>
<td>2</td>
</tr>
</tbody>
</table>

a The activity shows the relative rate when the activity of hydrolysis using GAG-MU as a substrate is 100.

b The data is obtained from Takagaki et al. (15).
TABLE IV

Effect of hydrolysis activity toward natural substrates
Each substrate was incubated with the purified enzyme under the typical conditions detailed in "EXPERIMENTAL PROCEDURES".

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ChS-peptide</td>
<td>100</td>
</tr>
<tr>
<td>DS-peptide</td>
<td>97</td>
</tr>
<tr>
<td>HS-peptide</td>
<td>124</td>
</tr>
</tbody>
</table>

$^a$ The activity shows the relative activity of hydrolysis using ChS-peptide as a substrate is 100.
The cleavage of the xylosyl serine linkage between a core peptide and a glycosaminoglycan chain by cellulases
Keiichi Takagaki, Mito Iwafune, Ikuko Kakizaki, Keinosuke Ishido, Yoji Kato and Masahiko Endo

J. Biol. Chem. published online February 27, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M111985200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts