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PRESENCE OF AN ENDO-β-GALACTOSIDASE DEGRADING THE LINKAGE REGION BETWEEN THE CHONDROITIN SULFATE CHAIN AND CORE PEPTIDE OF PROTEOGLYCAN

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Pyridylamino chondroitin sulfate, of which the reducing terminal xylose was coupled with a fluorescent 2-aminopyridine, was incubated at pH 4.0 with an extract from the mid-gut gland of Patnopecten. The high- and low-molecular-weight products were separated by ethanol precipitation, and identified by high-performance liquid chromatography analysis. The enzyme was found to expose a galactose residue at the reducing terminus of chondroitin sulfate, and also released the pyridylamino disaccharide, galactosylxylose, from the reducing terminal site of pyridylamino chondroitin sulfate. These results suggest that endo-β-galactosidase activity, which hydrolyzes the galactosylgalactose linkage of peptidochondroitin sulfate, is present in the mid-gut gland of Patnopecten.

Proteoglycans are a family of complex macromolecules that consist of a core protein and covalently attached glycosaminoglycan (GAG) chains. The linkage region between the core protein and the GAG chains is assumed to be GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-O-Ser, in common with chondroitin sulfate, dermatan sulfate, heparin, and heparan sulfate (1-3). Endo-β-glucuronidase and endo-β-xylosidase, which act on the linkage region of the peptidoglycan, have been isolated and characterized recently (4,

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Abbreviations : PA, 2-aminopyridine; ChS, chondroitin sulfate; GAG, glycosaminoglycan; Gal, galactose; Xyl, xylose; GlcA, glucuronic acid; GalNAc, N-acetylgalactosamine. All sugars mentioned in this paper have a D configuration.

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5). It is likely that these enzymes play an important role in the catabolic degradation of proteoglycan in tissue. Endo-β-galactosidases which cleave the galactosyl linkage of N-acetyllactosamine have been isolated from several sources (6-8). However, none of these endo-β-galactosidases hydrolyze the internal galactosyl linkage of the peptidoglycan linkage region. In 1988, we found evidence for the presence of a new endo-β-galactosidase in rabbit liver, but have not yet succeeded in characterizing it (9).

The present paper describes a peptidoglycan linkage region-degrading endo-β-galactosidase that was found in the mid-gut gland of Patnopecten using a pyridylaminated fluorescent chondroitin sulfate chain as a substrate.

MATERIALS AND METHODS

Preparation of Pyridylamino Chondroitin Sulfate: Pyridylamino chondroitin sulfate (ChS-PA) was prepared as described previously (10). To obtain a ChS chain bearing a xylose residue at the reducing terminus, proteochondtroitin sulfate from bovine cartilage was digested with pronase followed by digestion with endo-β-xylosidase (5) obtained from the mid-gut gland of the mollusc Patnopecten at 37°C, pH 4.0, for 2 h. The xylose residue at the reducing terminus of ChS was coupled with a fluorescent compound, 2-aminopyridine (PA) (11, 12). Two milligrams of ChS was dissolved in 50 μl of PA solution (prepared by mixing 1.0 g of PA, 0.76 ml of conc. HCl, and 2.2 ml of water, final pH 6.2). After sealing and heating at 100°C for 15 min, the tube was opened and 4 μl of the reducing reagent (prepared by mixing 10 mg of sodium cyanoborohydride and 0.5 ml of water) was added. The tube was resealed and heated at 90°C for 15 h. The reaction mixture was then diluted with 0.5 ml of water and chromatographed on a Sephadex G-15 column to remove the excess reagents. The ChS-PA was collected. Potential aldehyde groups of the xylose residue at the reducing termini of ChS was reductively aminated with PA as shown in Fig. 1. Pyridylamino oligosaccharides, Gal-Gal-Xyl-PA and Gal-Xyl-PA, were prepared from culture medium of human skin fibroblasts (5), and pyridylamino monosaccharides were prepared as reported previously (10).

Enzyme Source: The preparation of a crude enzyme fraction was carried out as follows (5). Acetone-treated powder of the mid-gut gland of Patnopecten was stirred with 10 mM Tris-HCl buffer, pH 7.0, containing 5 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 0.1 M ε-amino-n-caproic acid, 5 mM benzamidine-HCl, 10 mM N-ethylmaleimide and 0.36 mM pepstatin and then centrifuged at 24,000 x g for 30 min. Protamine sulfate was added to the resulting supernatant to a final concentration of 0.2%. After centrifugation, the fraction emerging within the range of 50-70% saturation of ammonium sulfate was collected from the supernatant,

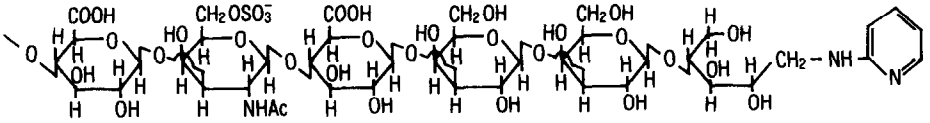

Fig. 1. Structure of ChS-PA.

dialyzed against the same buffer as mentioned above, and used as a crude enzyme fraction.

Incubation Studies: Two hundred microliters of a complete incubation mixture, which contained 1 μM ChS-PA as a substrate, 50 mM D-galactal, 50 mM saccharo-1,4-lactone, 0.1 M sodium acetate buffer, pH 4.0, and enzyme solution, was incubated at 37°C for 0-12 h. To terminate the incubation, the mixture were boiled for 5 min. D-Galactal and saccharo-1,4-lactone were included in this series of experiments in order to inhibit exo-type β-galactosidase and β-glucuronidase, respectively (13, 14). As a control, ChS-PA was incubated with heat-inactivated enzyme.

High-perfomance Liquid Chromatography (HPCL): A liquid chromatograph (Hitachi L-6200) equipped with a Hitachi F-1050 fluorescence spectrophotometer was used. For detection of PA-sugars, an excitation wavelength of 320 nm and an emission wavelength of 400 nm were used. Reverse-phase HPLC for analysis of PA-monosaccharides was carried out with an Ultrasphere-ODS columm (4.6 x 250 mm) using 0.25 M sodium citrate buffer, pH 4.0, containing 1% acetonitrile (10). Size-fractionation of PA-sugar was done with a Shodex OHpak KB-803 column (8 x 300 mm) using 0.2 M NaCl. The flow rate was 0.5 ml/min and the column temperature was 30°C.

RESULTS

Newly Exposed Reducing Terminus of Reaction Product: ChS-PA was incubated with the enzyme fraction at pH 4.0 for 12 h in order to separate high- and low-molecular-weight products. After incubation, four volumes of ethanol saturated with NaCl were added to the reaction mixture. After centrifugation, the resulting precipitates were pooled as high-molecular-weight products. The recovered products were once more coupled with PA in order to determine the newly exposed reducing termini of the ChS chain, which had been liberated by the endo-type glycosidases, producing the corresponding PA-sugar (10). After acid hydrolysis with 2 N HCl at 100°C for 2 h, PA-sugars were subjected to reverse-phase HPLC. A typical elution profile is shown in Fig. 2. In the control experiment (Fig. 2-A), peak I was observed and identified as Xyl-PA according to the standard elution profile

(10). This Xyl-PA was derived from the original ChS-PA. However, after prolonged incubation with the enzyme fraction, peak I was decreased and a new peak (peak II) appeared at the position of the standard Gal-PA in turn (Fig. 2). These data proved that galactose was the reducing terminal sugar of ChS after incubation with the enzyme fraction.

Analysis of the Oligosaccharide Released from ChS-PA by the Enzyme Fraction: The oligosaccharide released from the reducing terminal site of ChS-PA by the enzyme was characterized as follows. After incubation, four volumes of ethanol saturated with NaCl were added to the reaction mixture and low-molecular-weight products in the supernatant were pooled by centrifugation. An aliquot of the product was subjected to size-fractionation HPLC analysis (Fig. 3). After incubation, peak III appeared at the position of Gal-Xyl-PA, which was disaccharide-PA, and the area of this peak increased following incubation. In the absence of D-galactal, peak III was decreased and peak IV appeared at the position of Xyl-PA (Fig. 3-F). These results suggest that peak IV was formed from peak III by digestion with the exo-β-galactosidase contained in the enzyme fractions. To confirm this, peak III was pooled and digested with exo-β-galactosidase from Aspergillus niger (SIGMA) (15). After digestion of peak III with exo-β-galactosidase, peak IV appeared at the position of Xyl-PA on reverse-phase HPLC (Fig. 4). This indicated that the non-reducing terminal sugar of peak III was galactose. These data proved that the oligosaccharide-PA was a disaccharide-PA , Gal-Xyl-PA, and that this was released from the corresponding reducing site of ChS-PA (16).

These results confirmed the presence in the mid-gut gland of Patnopecten of an endo-β-galactosidase which hydrolyses the galactosylgalactose linkage of the linkage region between the ChS chain and peptide core of peptidoglycan.

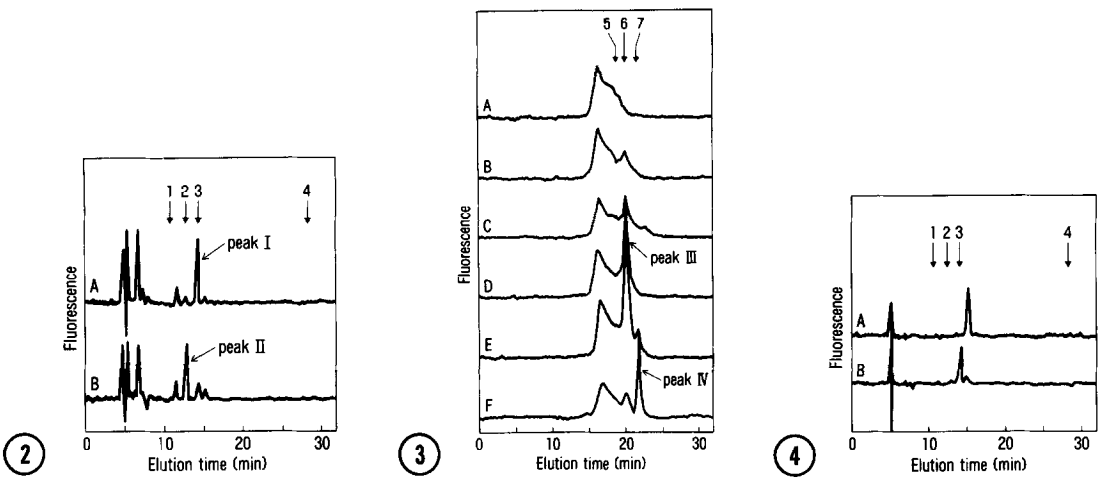

Fig. 2. Reverse-phase HPLC elution profiles of PA-sugar residues at the reducing termini of ChS-PA. ChS-PA was incubated with heat-inactivated enzyme (A) and enzyme (B) for 12 h. The high-molecular-weight products recovered by ethanol precipitation were recoupled with PA followed by hydrolysis, and then analyzed by reverse-phase HPLC. The column was an Ultrasphere-ODS (4.6 x 250 mm) and the buffer was 0.25 M sodium citrate buffer, pH 4.0, containing 1.0% acetonitrile with a flow rate of 0.5 ml/min. Arrows indicate the elution positions: 1, GlcA-PA; 2, Gal-PA; 3, Xyl-PA; 4, GalNAc-PA.

Fig. 3. Size-fractionation HPLC elution profiles of the low-molecular-weight products. ChS-PA was incubated with enzyme for 0 h (A), 0.5 h (B), 1.5 h (C), 5 h (D) and 12 h (E), and incubated with the enzyme in the absence of D-galactal for 12 h (F). After incubation, four volumes of ethanol, saturated with NaCl, were added to the reaction mixture. An aliquot of the supernatant was subjected to size-fractionation HPLC. The column was Shodex OHpak KB-803 (8 x 300 mm) and the solution was 0.2 M NaCl with a flow rate of 0.5 ml/min. Arrows indicate the elution positions: 5, Gal-Gal-Xyl-PA; 6, Gal-Xyl-PA; 7, Xyl-PA.

Fig. 4. Reverse-phase HPLC elution profiles of peak III before (A) and after (B) exo-β-galactosidase digestion. For numbering, see legend to Fig. 2.

DISCUSSION

Endo-β-galactosidases, which are active against poly-lactosaminoglycans, have been found in several sources (6-8). However, the presence of an endo-β-galactosidase acting specifically on the galactosylgalactose linkage region of peptidoglycans has not hitherto been reported. Furthermore, such an enzyme in the midgut gland of Patnopecten has been unknown up to now. This enzyme exposes the galactose residue at the reducing terminus of ChS, and also releases Gal-Xyl-PA from the reducing terminal site.

Therefore, the present findings strongly suggest that this endo-β-galactosidase specifically hydrolyzes the galactosylgalactose linkage in the linkage region of ChS. However, it is likely that this enzyme does not hydrolyze the adjacent galactosylxylose linkage because Xyl-PA was not liberated from ChS-PA in the presence of D-galactal, which is an inhibitor of exo-β-galactosidase. Recently, Takagaki et al. (4,5) demonstrated that the linkage region-degrading endo-β-glycosidases, endo-β-glucuronidase and endo-β-xylosidase, played important roles in the catabolism of proteoglycans. On the other hand, it has also been reported that urinary chondroitin sulfate as a final catabolic product bears glucuronic acid, galactose and xylose residues at the reducing terminus (17). The biological function and substrate specificity of the present endo-β-galactosidase are not yet completely clear. However, judging from the results of this study, it is likely that the enzyme is related to the catabolism of proteoglycans, together with other endo- and exo-glycosidases.

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