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Demonstration of *N*-acetylchondrosine-degrading β-glucuronidase in rabbit liver

Toshiya Nakamura, Keiichi Takagaki, Mitsuo Majima* and Masahiko Endo

Department of Biochemistry, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036, Japan

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N-Acetylchondrosine was incubated at pH 4.0 with a rabbit-liver crude enzyme extract. Gel filtration of the reaction products on Sephadex G-15 revealed the presence of monosaccharide liberated from the disaccharide. The monosaccharide fraction was analyzed by gas-liquid chromatography, and identified as a mixture of glucuronic acid and *N*-acetylgalactosamine. These results indicate the presence of β-glucuronidase, which degrades *N*-acetylchondrosine, in rabbit liver. The discovery of the presence of this enzyme may help to establish the complete degradation process of chondroitin sulfates.

N-Acetylchondrosine; β-Glucuronidase

1. INTRODUCTION

It seems likely that the process of catabolism of glycosaminoglycans, such as hyaluronic acid or chondroitin sulfates, occurs through cleavage of the internal bonds of glycosaminoglycans by a hyaluronidase-like enzyme (endo-β-*N*-acetylhexosaminidase), the resulting oligosaccharides then being degraded by exo-β-glucuronidase, exo-β-*N*-acetylhexosaminidase and sulfatase stepwise from the non-reducing terminals [1]. However, the two disaccharides derived from hyaluronic acid and chondroitin sulfates, *N*-acetylhyalobiuronic acid (GlcUAβ(1→3)GlcNAc) and *N*-acetylchondrosine (GlcUAβ(1→3)GalNAc), are resistant to β-glucuronidase [2,3]. Therefore, the complete process of glycosaminoglycan degradation remains unsolved.

Recently, the presence of endo-β-glucuronidase in rabbit liver was demonstrated in our laboratory [4], and the enzyme was isolated and characterized [5]. Its activity was determined using the chondroitin sulfate chain as a substrate, because this enzyme did not act on any artificial substrates. This suggests that some enzymes may remain undetected if artificial substrates are employed.

Therefore, in order to investigate the presence of the β-glucuronidase activity that was able to degrade the disaccharides mentioned above, *N*-acetylchondrosine was incubated with a rabbit-liver crude enzyme extract. After incubation, gel chromatography of the reaction mixture revealed the presence of glucuronic acid liberated from the disaccharide.

The present paper describes the demonstration of this disaccharide-degrading exo-β-glucuronidase activity in rabbit liver.

2. MATERIALS AND METHODS

2.1. *Chemicals*

N-Acetylchondrosine and *N*-acetylhyalobiuronic acid were

Correspondence address: M. Endo, Department of Biochemistry, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki 036, Japan

* *Present address:* Kushiro Junior College, Kushiro 085, Japan

Abbreviations: GlcUA, D-glucuronic acid; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; GLC, gas-liquid chromatography

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the same as those described in [6]. Glucuronic acid and gulonolactone were the same as those described in [4]. *p*-Nitrophenyl-β-D-glucuronide was purchased from Boehringer (Mannheim). Phenylmethylsulfonyl fluoride (PMSF) and saccharo-1,4-lactone were purchased from Sigma (St. Louis, MO). Sephadex G-15 and G-25 were purchased from Pharmacia (Uppsala). Other reagents and chemicals were obtained from commercial sources.

2.2. *Analytical methods*

Total uronic acid was determined by the method of Bitter and Muir [7]. The reducing power of free uronic acid was determined by a modification [6] of the method of Milner and Avigad [8]. Protein was determined by the method of Lowry et al. [9] using bovine serum albumin as a standard.

2.3. *Preparation of crude enzyme*

A 10% homogenate of fresh rabbit liver (12-month-old male, Japanese white rabbit, JW, supplied by the Institute for Animal Experiments, Hirosaki University School of Medicine) was prepared in 10 mM phosphate buffer (pH 7.3) containing 0.1% Triton X-100 and 0.1 mM PMSF, followed by centrifugation at 15000 × *g* for 30 min. The fraction emerging within the range from 30 to 50% saturation of ammonium sulfate was collected from the supernatant, dialyzed against 10 mM phosphate buffer (pH 7.3) containing 0.1 mM PMSF, and used as a crude enzyme extract.

2.4. *Enzyme assay*

2.4.1. β-Glucuronidase activity with *N*-acetylchondrosine

An incubation mixture (500 μl) containing 5 mM *N*-acetylchondrosine, 100 mM acetate buffer (pH 4.0), 0.1 mM PMSF and enzyme protein was sealed after the addition of one drop of toluene as a preservative. Incubation was carried out at 37°C for 12 h and then stopped by heating the reaction mixture at 100°C for 3 min. After centrifugation, the free glucuronic acid in the supernatant was determined by the method of Milner and Avigad [8].

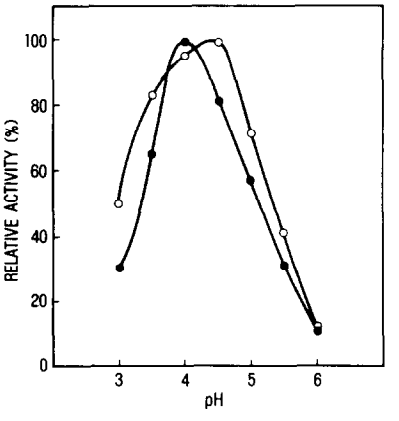

Fig.1. Effects of pH on the rates of hydrolysis of *N*-acetylchondrosine and *p*-nitrophenyl-β-D-glucuronide by rabbit-liver crude enzyme extract. (●) *N*-Acetylchondrosine; (○) *p*-nitrophenyl-β-D-glucuronide.

2.4.2. β-Glucuronidase activity with *p*-nitrophenyl-β-D-glucuronide

An incubation mixture (500 μl) containing 2 mM *p*-nitrophenyl-β-D-glucuronide, 100 mM acetate buffer (pH 4.5) and enzyme protein was incubated at 37°C for 20 min. The enzyme reaction was stopped by the addition of 1.0 ml of 0.1 N NaOH and the absorbance at 420 nm was measured.

2.5. *Identification of reaction products*

Identification of the reaction products was carried out by GLC. The enzyme reaction was stopped by heating the reaction mixture at 100°C for 3 min. After centrifugation, the supernatant was passed through columns of Sephadex G-25 and G-15. The monosaccharide fraction was pooled, evaporated to dryness, and reduced with sodium borohydride using the method of Ögren and Lindahl [10]. Acetyl or butaneboronic acid ester derivatives of the borohydride-reduced sugars were

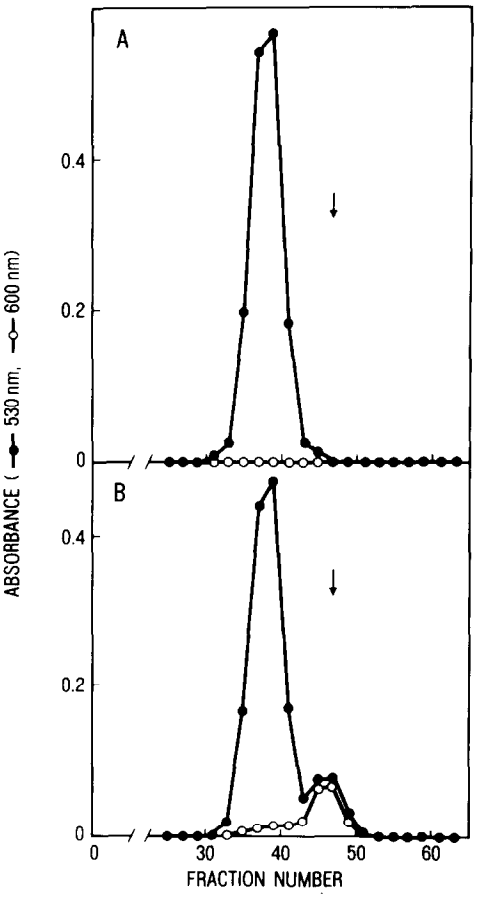

Fig.2. Sephadex G-15 chromatography of *N*-acetylchondrosine before (A) and after (B) incubation with rabbit-liver crude enzyme extract. The column (1.5 × 130 cm) was eluted with 0.1 M acetic acid. Volume of each fraction was 3.5 ml. Arrows indicate the elution positions of glucuronic acid. (●) Carbazole reaction; (○) Milner-Avigad reaction.

prepared as described [11]. Then GLC analysis using a JEOL JGC-20K equipped with a flame ionization detector was carried out employing a glass column (0.3 × 100 cm) packed with either 3% Silicone OV-17 (for glucuronic acid) or 1% ECNSS-M (for *N*-acetylgalactosamine) on Gas-Chrom Q.

3. RESULTS

3.1. *Incubation of enzyme*

N-Acetylchondrosine was incubated with rabbit-liver crude enzyme extract at various pH values. It was found that the liberation of glucuronic acid from the disaccharide, evaluated by the Milner-Avigad method, was maximal at pH 4.0 (fig.1). On the other hand, β-glucuronidase activity with *p*-nitrophenyl-β-D-glucuronide was maximal at pH 4.5, being comparable to the result reported by Dean [12]. The amount of glucuronic acid liberated from the disaccharide at pH 4.0 showed a linear increase for at least 18 h, and was correlated with the concentration of protein, indicating that the reaction was protein-dependent. Furthermore, the effect of saccharo-1,4-lactone, a known inhibitor of β-glucuronidase, upon the reaction was examined, and it was found that 0.1 mM saccharo-1,4-lactone produced 90% inhibition.

3.2. *Identification of reaction products*

N-Acetylchondrosine was incubated with crude enzyme at pH 4.0 for 12 h, and the reaction products were passed through a column of Sephadex G-25. The uronic acid-containing fraction was pooled and then chromatographed on a column of Sephadex G-15 (fig.2). The chromatogram showed Milner-Avigad reaction-positive material at an elution position corresponding to that of glucuronic acid (fig.2B). The monosaccharide fraction was pooled and analyzed by GLC. As shown in fig.3, gulonolactone (fig.3A), derived from glucuronic acid, and *N*-acetylgalactosaminitol (fig.3B) were identified. Similarly, when *N*-acetylhyalobiuronic acid was used as a substrate, glucuronic acid and *N*-acetylglucosamine were detected in the monosaccharide fraction of the reaction products (not shown). These results indicated the presence of β-glucuronidase activities, degrading *N*-acetylchondrosine and *N*-acetylhyalobiuronic acid in rabbit liver.

4. DISCUSSION

In the process of glycosaminoglycan catabolism, β-glucuronidase cleaves glucuronic acid residues

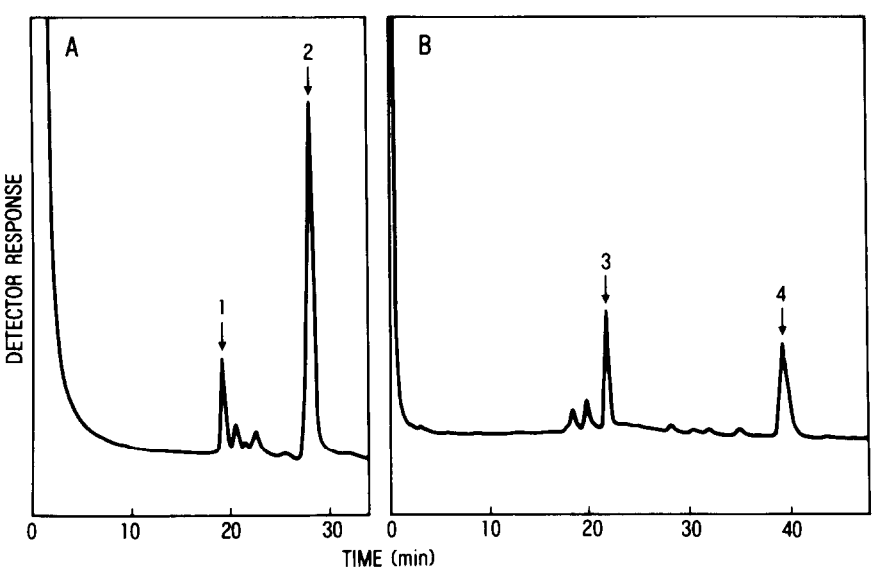

Fig.3. GLC of the monosaccharide fraction of the reaction products. After incubation of *N*-acetylchondrosine with rabbit-liver crude enzyme extract, the monosaccharide fraction of the reaction products was subjected to GLC. Conditions were as described in section 2. (A) Butaneboronic acid ester derivatives; (B) alditol acetate derivatives. (1) Mannitol (internal standard); (2) gulonolactone; (3) inositol (internal standard); (4) *N*-acetylgalactosaminitol.

from the non-reducing terminals of glycosaminoglycan-oligosaccharides [13,14]. Some disaccharides (GlcUAβ(1→3)galactose [1], GlcUAβ(1→4)2,5-anhydromannitol and GlcUAβ(1→3)GalNAc(4-sulfate) [3]) are known to be susceptible to cleavage by β-glucuronidase, whereas the two disaccharides derived from hyaluronic acid and chondroitin sulfates, GlcUAβ(1→3)GlcNAc and GlcUAβ(1→3)GalNAc, have been concluded to be resistant to the enzyme [2,3].

In this study, the presence of β-glucuronidase activities acting on these disaccharides were investigated for the following two reasons. (i) Only artificial substrates have been used previously in the purification of β-glucuronidases. (ii) Some enzymes may be undetectable if artificial substrates are employed; this was confirmed by the present authors, who previously purified such an enzyme [5]. *N*-Acetylchondrosine was therefore incubated with a rabbit-liver crude enzyme extract, yielding glucuronic acid and *N*-acetylgalactosamine as reaction products. As shown in fig.2, the amount of glucuronic acid liberated from the disaccharide seemed to be small. In our experiment, the rate of hydrolysis was correspondingly low upon incubation under standard conditions. This may have been due to the use of crude enzyme extract. As the amount of enzyme was increased, however, further liberation of glucuronic acid was observed. The enzyme showed maximum activity at pH 4.0, and was inhibited by saccharo-1,4-lactone, a known β-glucuronidase inhibitor.

These results thus confirm the presence in rabbit liver of β-glucuronidase activities that are capable of degrading *N*-acetylchondrosine and *N*-acetylhyalobiuronic acid. It is not clear whether the *N*-acetylchondrosine-degrading enzyme is the same as the enzyme acting on *N*-acetylhyalobiuronic acid. However, it is considered that the discovery of these enzyme activities will contribute to the elucidation of the complete degradation process of glycosaminoglycans such as hyaluronic acid and chondroitin sulfates.

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