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Degradation of Acharan Sulfate and Heparin by *Bacteroides stercoris* HJ-15, a Human Intestinal Bacterium

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When glycosaminoglycan (GAG)-degrading enzymes were measured in normal human stool suspensions, all 5 tested different stools degraded titrable heparin and acharan sulfate. GAG-degrading bacteria were screened from the isolates of human stools. Among them, HJ-15 had the most potent activities of heparinases (GAGs-degrading enzymes). However, HJ-15 produced the enzyme even if in the media without heparin. Acharan sulfate lyase was induced by acharan sulfate and heparin. Heparinase production was also induced by these GAGs. These enzymes, acharan sulfate lyase and heparinase, were produced in exponential and stationary phase of HJ-15 growth, respectively. Optimal pHs of the acharan sulfate lyase and heparinase activities were 7.2 and 7.5, respectively. The biochemical properties of HJ-15 was similar to those of *B. stercoris*. However, difference from *B. stercoris* was utilization of raffinose. This HJ-15 also degraded chondroitin sulfates A and C.

Key words : Acharan sulfate, Heparin, Intestinal microflora, Acharan sulfate lyase, *Bacteroides stercoris* HJ-15.

INTRODUCTION

Glycosaminoglycans (GAGs) are a family of linear anionic polysaccharides that are typically isolated as proteoglycans covalently bound to the core protein (Ernst *et al.*, 1995). Heparin and acharan sulfate are the representative GAGs.

Heparin is a GAG having repeating disaccharide structure of ->4)-2amino, 2deoxy-α-D-glucopyranose (1->4)-α-L-ido (or β-D-gluco)pyranosyl uronic acid. Its structure is complicated by variable N-, O-sulfation and N-acetylation (Ernst *et al.*, 1995; Linhardt *et al.*, 1986). Acharan sulfate is a glycoaminoglycans isolated from the giant African snail Achatina fulica, which are orally administered as foods (Kim *et al.*, 1996). This polysaccharide has an uncomplicated repeating disaccharide structure of ->4)-2-amino, 2-deoxy-α-D-glucopyranose (1->4)-2-sulfo-β-L-idopyranosyl uronic acid.

These GAGs produced from the intestinal wall or contained in foods were inevitablely in contact with intestinal microflora in alimentary tract. Since Gesner and Jenkin (1961) had studied the production of heparinases by *Bacteroides*, the bacteria degrading GAGs, *Bacteroides heparinolyticus, Bacteroides fragilis* and *Bacteroides thetaitaomicron*, have been isolated from intestinal microflora and GAGs-metabolizing enzymes, heparinases and chondroitinases, have been purified from them (Ernst *et al.*, 1995; Galliher *et al.*, 1981; Gu *et al.*, 1995; Lohse *et al.*, 1992; Nakamura *et al.*, 1988; Sayers *et al.*, 1977; Salyers *et al.*, 1977; Salyers and Kotarski, 1980; Salyer and O'Brien, 1980). These GAGs degrading enzymes are usually dividied into lyases and hydrolases (Ernst *et al.*, 1995). The polysaccharide lyases are divided into heparinases, chondroitinases and hyaluronidases. However, studies on heparinases and chondroitinases from human intestinal bacteria are not complete, although the heparinases from *Flavobacterium heparinum* which was isolated from soil has been studied in detail (Gu *et al.*, 1995).

Therefore, we investigated the metabolism of acharan sulfate and heparin, and isolated *Bacteroides stercoris* HJ-15 different from previous ones degrading GAGs in taxonomical classification.

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MATERIALS AND METHODS

Materials

Heparin, chitosan, chondroitin sulfate A and C, toluidine blue, sodium thioglycolate and ascorbic acid were purchased from Sigma Chem. Co. (U.S.A.). Brain heart

infusion (BHI), Muller-Hinton (MH), Tryptic soy (TS), bactopeptone, yeast extract and agar were from Difco Co. (U.S.A.). General anaerobic medium (GAM) was from Nissui Pharm. Co., ltd., (Japan). Acharan sulfate was prepared according to our previous method (Kim *et al.*, 1996).

Screening of acharan sulfate-degrading bacteria

Aliquot (0.2 ml) of 10^3 to 10^7-diluted human feces was inoculated in plate media (GAM). The plate was anaerobically incubated at 37°C for 4 days. More than 200 colonies isolated from several plates were cultured in 10 ml GAM broth and for the screening of GAG degrading enzymes, the cultured cells were collected at 5000 g for 20 min and washed twice with saline. For the collected cells, GAGs-degrading enzyme activities were measured. The potently positive bacterium was HJ-15. The taxonomic properties of the GAG-degrading strain, HJ-15, were examined in accordance with methods described in Bergey's Manual of Determinative Bacteriology (Hensyl, 1994; Johnson *et al.*, 1986) (Table I).

Assay of GAGs-degrading enzymes

A simple assay of GAGs-degrading enzyme activity developed by Gesner and Jenkins (1961) was used here. The assay mixture (total 2 ml) contained 0.2 ml of 2 mM acharan sulfate (or heparin), 0.2 ml of the enzyme and 1.6 ml of 20 mM phosphate buffer, pH 7.0. The mixture was incubated at 37°C for 1 h and was centrifuged at 3000 g for 20 min. The supernatant was filtered through a 0.22 μm membrane filter, and 0.5 ml of supernatant was added to 2 ml of 0.002% toluidine blue (Riley, 1987). An aqueous solution of toluidine blue mixed with 0.5 ml of a 4 unit/ml heparin solution had a distinct pink-purple color, which was easily distinguished from the blue color when heparin was degraded.

In additon, the supernatants of the reaction mixture were assayed by PAGE and UV absorbance at 232 nm.

Reducing groups were estimated after the method of Meyer and Gibbons (1951) in which 0.5 ml of the test samples was subjected to 0.5 ml of 1.5% 3,5-dinitrosalicylic acid and 0.5 ml of 6N NaOH for 30 min at 60°C. The mixture was diluted with 10 ml of water and optical density was read at 510 nm using maltose as the standard.

Table I. Characteristics of HJ-15 isolated from human intestinal microflora

	HJ-15	*B. stercoris*
Colony diameter	<0.5 mm	<0.5 mm
Gram stain	negative (rod)	negative (rod)
Methyl red test	-	
Voges-Prokauer test	-	
Indole produced	+	+
Catalase produced	-	-
Gelation digested	-	-
Esculin hydrolyzed	+	+
Starch hydrolyzed	+	+
H2S produced	-	-
Gas produced	-	-
Urease	-	-
α-Rhamnosidase	+	+
β-Glucuronidase	-	-
Acid produced from		
Arabinose	-	-/+
Ribose	+	+
Xylose	+	+
Rhamnose	+	+
Glycerol	-	-
Glucose	+	+
Cellobiose	-	-/+
Lactose	+	+
Maltose	+	+
Mellibiose	-	-/+
Melezitose	-	-
Sucrose	+	+
Trehalse	-	-
Raffinose	-	+
Salicin	-	-

Culture of HJ-15 in TS media containing several kinds of carbohydrates

Several kinds of carbohydrates (0.25%) were used in instead of glucose of tryptic soy broth containing 0.1% ascorbic acid and 0.01% sodium thioglycolate. The previously isolated *Bacteroides* HJ-15 was incubated in these media and cultured at 37°C for 10 h.

To investigate whether the productivity of GAG-degrading enzymes was constitutive or inducible, HJ-15 was cultured in 10ml GAM containing various concentrations of glycosaminoglycans. After 15 h incubation at 37°C, the cultured cells were collected at 5000 g for 20 min. Activity of the GAG-degrading enzymes were measured according to the above standard method.

Partial purification of GAG-degrading enzyme

Bacteroides HJ-15 was cultured at 37°C for 10 h in tryptic soy broth containing 0.1% ascorbic acid and 0.01% sodium thioglycolate. The cultured cells were centrifuged at 3,000 g for 30 min and washed twice with cold saline. The resulting pellet was suspended in 20 mM phosphate buffer (pH 7.0), distrupted by sonication (High Intensity Ultrasonic Processor VC-501, Japan) and centrifuged at 10,000 g for 60 min. Fractional precipitation of the supernatant was performed by varying the concentration of ammonium sulfate and the precipitate (saturated 40~70%) was resuspended in 20 mM phosphate buffer containing 1 M ammonium sulfate. The suspended solution was applied to CM-

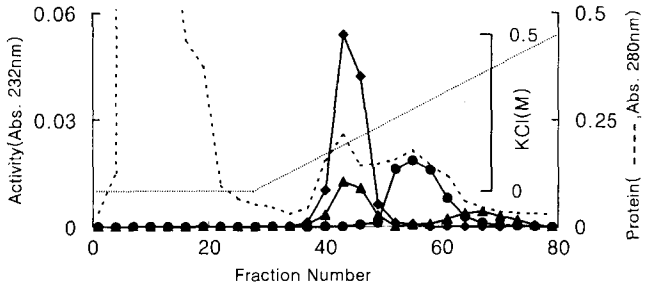

Fig. 1. Elution pattern of GAG lyases from HJ-15 on CM-Sephadex C-25. CM-Sephadex column chromatography (2.6× 20 cm) equlibrated with 50 mM sodium phosphate buffer, pH 7.0, was applied to the crude enzyme and then washed with 160 ml of 50 mM sodium phosphate buffer, pH 7.0 (Fraction No. 1-28). The column was eluted with a leanear gradient between 150 ml of 50 mM phosphate buffer buffer, pH 7.0, and 150 ml of the same buffer containing 0.5 M KCl (Fraction No. 29-79): Fraction volume, 75.9 ml; Flow rate, 0.5 ml/min. ◆, Acharan sulfate lyase; ▲, Heparinase; ●, chondrotin AC lyase; −, Abs 280 nm.

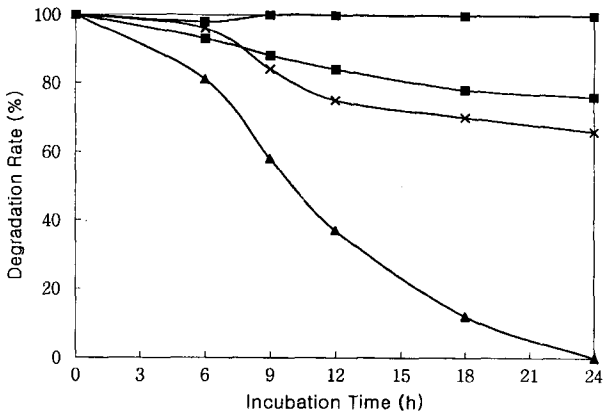

Fig. 2. Degradation of acharan sulfate by bacteria derived from human feces. In all of these experiments, the fresh intestinal microflora (5 mg of wet feces) were added into 15 ml of reaction mixture (3 ml of 0.5 mg/ml acharan sulfate in 50 mM phosphate buffer, pH 7.0) and incubated at 37°C. ×, human intestinal microflora; ◆, *E. coli*; ■, *Bacteroides* JY-6; ▲, *Bacteroides* HJ-15.

Sephadex C-25 column chromatography (Fig. 1). The active fractions were used as the partially purified enzyme.

Protein determination

Protein was determined by the method of Bradford's protein assay kit, using bovine serum albumin as a standard (Bradford, 1976).

RESULTS AND DISCUSSION

Isolation of GAGs-degrading bacteria from human intestinal bacteria

Normal human stool suspensions were incubated with heparin or acharan sulfate and were taken for the assay of GAG-degrading enzymes at 6 h intervals. In every case of 5 different stools tested, all the titrable heparin and acharan sulfate disappeared within 24 h. A typical rate of degradation by a crude stool sample is shown in Fig. 2. Fresh fecal suspension of human degraded acharan sulfate according to the lapse of the incubation time. This suspension also degraded heparin like the previous report (data not shown) (Gesner and Jenkins, 1951). More than two hundred colonies were isolated from the human fresh feces degrading heparin as well as acharan sulfate. GAG degrading activities for these isolated bacteria were measured. Several strains degraded acharan sulfate and heparin. Among them, HJ-15 belonging to *Bacteroides* spp. was found to be the most potent. The production of heparinase by *Bacteroides* spp. was first described by Gesner and Jenkins (1951). Compared with *Bacteroides* spp. isolated by Gesner and Jenkins or *Bacteroides* spp. isolated by Riley and Mee (1984), HJ-15 had most potent activities of heparinases (GAGs-degrading enzymes). The previously isolated bacteria degrading heparin did not produce the heparinase in the media without heparin (Galliher *et al.*, 1981; Riley and Mee, 1984). Howezver, HJ-15 produced the enzyme even if the media did not contain heparin. The other biochemical properties of HJ-15 were different from those of *Bacteroides fragilis, B. heparinolyticus* and *B. thetaitaomicron,* which were previously isolated as the bacteria producing heparinases. HJ-15 was strictly anaerobic, gram-negative rod-shape and did not have flagella (Hensyl, 1994). The strain of HJ-15 were different from the above *Bacteroides* spp. in carbohydrate utilization and biochemical properties (Table I). HJ-15 did not use trehalose, mellobiose and raffinose as a carbon source. However, the other *Bacteroides* species used them. It was able to reduce nitrate and contained α-rhamnosidase activity. These properties of HJ-15 were similar to those of *B. stercoris* which has not been studied about heparinase and acharan sulfate lyase (Johnson *et al.*, 1986). However, the difference of HJ-15 from *B. stercoris* was utilization of raffinose. *Bacteroides stercoris* HJ-15 was a novel intestinal bacterium degrading GAGs. If heparin is adiminstered orally into human, its pharmacological activity will be disappeared because these species will degrade it. To administer orally heparin, intestinal bacteria must be considered.

Growth and GAGs-degrading enzyme activities of HJ-15

HJ-15 was not cultured in commerical media (BHI, MH, TS, NB, LB) except GAM (Data not shown). However, HJ-15 was cultured, when 0.01% sodium thioglycolate and 0.1% ascorbic acid were added in

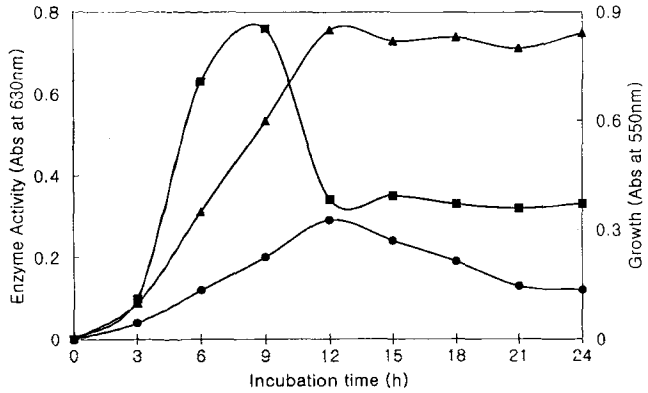

Fig. 3. The growth and enzyme activity of *Bacteroides* HJ-15. The bacterium HJ-15 was inoculated on 500 ml GAM broth and anaerobically cultured at 37°C for 24 h. ■, acharan sulfate lyase; ●, heparinase; ▲, growth.

the TS, and produced heparinases and acharan sulfate lyase well. Therefore, our experiments used tryptic soy broth containing 0.01% sodium thioglycolate and 0.1% ascorbic acid in an anaerobic box as the media culturing HJ-15. The growth of HJ-15 in a 1 liter of the complex medium is shown in Fig. 3. After inoculation, HJ-15 gradually grew and a stationary phase was reached 12 h after incubation. The acharan sulfate lyase activity was dramatically increased at the initial growth of HJ-15 and was the highest 9 h after incubation. However, heparinase production was the highest 12 h after incubation. This HJ-15 produced potently heparinases in the media without heparin. This property of HJ-15 was also different from that of the previously reported *Bacteroides* spp. To develop better media than TS with sodium thioglycolate and ascorbic acid, glucose of TS with sodium thioglycolate and ascorbic acid were replaced by several carbon sources and the GAGs-degrading activities (heparinase and acharan sulfate lyase) were assayed (Fig. 4). The lyase activities of heparin and acharan sulfate was the highest in the media containing maltose as a carbon source, followed by inulin, sucrose, fructose and glucose. However, most of sugars did not increase significantly the activities of heparinase and acharan sulfate lyase.

Induction of GAGs-degrading enzymes by polysaccharides

To investigate the inducibility of GAG-degrading enzymes of HJ-15 by GAGs, HJ-15 was cultured in the media containing natural polysaccharides and the activities of their enzymes were assayed (Fig. 5).

Acharan sulfate lyase activity was induced by acharan sulfate, followed by heparin. At 0.01~0.02 mg/ml acharan sulfate, acharan sulfate lyase activity was effectviely induced 5.4-fold, although HJ-15 did not grow at more than 0.5 mg/ml acharan sulfate (Fig.

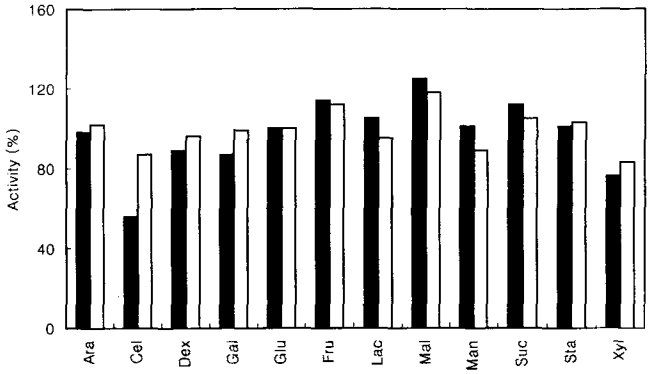

Fig. 4. Effect of carbohydrate on the productivity of heparinase and acharan sulfate lyase of HJ-15. Activity of HJ-15 cultured in GAM without D-glucose was taken as 100 %: ■, acharna sulfate lyase; □, heparinase. Ara, arabinose; Cel, cellulose; Dex, dextran; Gal, galactose; Glu, glucose; Fru, fructose; Lac, lactose; Mal, maltose; Man, mannose; Suc. sucrose; Sta, starch; Xyl, xylose.

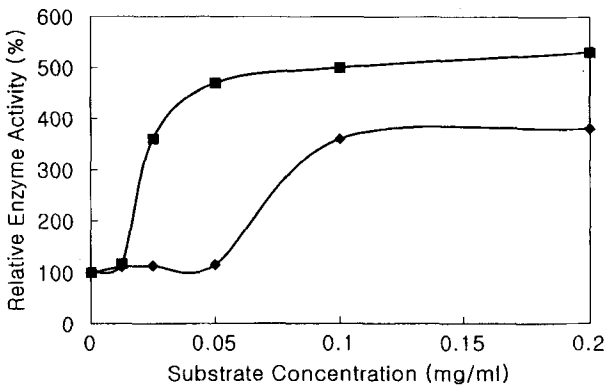

Fig. 5. Induction of heparin lyase and acharan sulfate lyase of HJ-15 by polysaccharides. Activity of HJ-15 cultured with polysaccharides was taken as 100 %: ■, acharan sulfate lyase; □, heparinase.

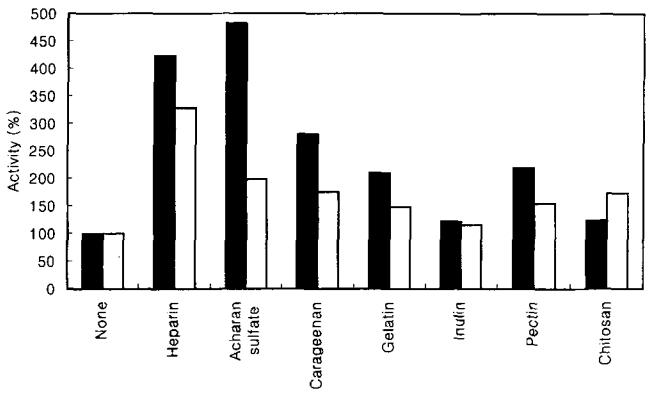

Fig. 6. Induction of heparin lyase and acharan lyase of *Bacteroides* HJ-15 by heparin and acharan sulfate. ■, Acharan sulfate lyase; ◆, Heparinase.

6). Heparinase production was potently induced by heparin, followed by acharan sulfate, carageenan and chitosan. At more than 0.05 mg/ml heparin, heparinase production was induced 3.9-fold.

Partial purification of enzymes

The enzyme was partially purified by sonication, ammonium sulfate fractionation (saturated 30~70%), dialysis and CM-Sephadex column chromatography. GAGs-degrading activities of the partially purified enzymes were determined by the decrease in metachromasia, the increase in 232 nm and the release of reducing group. The decrease in metachromasia, the increase in 232 nm and the release of reducing group were approximately proportinal to the concentration of heparin or acharan sulfate over a range of about 0.1 to 5 mg/ml. The release of reducing groups was found to be proportional to the enzyme concentration and incubation time in 1 ml of reaction mixture. Other glycosaminoglycans (chondroitin sulfate A and C) in 20 mM sodium phosphate buffer (pH 7.0) were also degraded with the partially purified enzymes. Optimal pH of acharan sulfate lyase was weak alkaline (pH 7.2). Optimal pH of heparinase was also weak alkaline (pH 7.5). Particularly, *B. stercoris* producing acharan sulfate lyase was first isolated from intestinal bacteria.

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