Characterization of $\beta$-Glucosidase and $\beta$-Glucuronidase of Alkalotolerant Intestinal Bacteria

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The number of alkalotolerant intestinal bacteria was 1% of the total flora in humans and 0.8% of those in rats. The $\beta$-glucosidase and $\beta$-glucuronidase activity of these intestinal bacteria was induced by elevating the pH of the medium, but the growth was not changed. The enzyme activity in a medium of pH 7 was 5- to 10-fold higher than that in a medium of pH 6. Isolated bacteria from human and rat feces were cultured in a pH 5 general anaerobic medium (GAM) broth to reach a stationary phase, then the pH of the media was changed from 5 to 8. Both $\beta$-glucosidase and $\beta$-glucuronidase were increased 9.2–12.1-fold. The activity of these enzymes was also increased 2–16-fold by adding substrates ($p$-nitrophenyl-$\beta$-$d$-glucopyranoside or $p$-nitrophenyl-$\beta$-$d$-glucuronide). $\beta$-Glucuronidase(s) was inhibited by saccharic acid 1,4-lactone or $\alpha$-glucuronic acid. However, when lactulose was added to the medium, and then intestinal microflora were inoculated in the medium, the productivity of these enzymes dramatically decreased. We thus contend that the induction of the $\beta$-glucosidase and $\beta$-glucuronidase of intestinal bacteria by a high pH can cause colorectal cancer.

Keywords $\beta$-glucosidase; $\beta$-glucuronidase; alkalotolerant intestinal bacteria; lactulose

Geographic variations in the incidence of colon cancer indicate that environmental factors such as diet are important to the etiology and pathogenesis of colon cancer.1)2) The incidence of intestinal cancer is apparently related to economic development.3)4) Some theories have implicated that a high dietary intake of meat can cause colon cancer, although other influences have not been excluded.5) Such carcinogenicity is due to the induction of some enzymes, such as $\beta$-glucuronidase, azoreductase, nitroreductase and steroid 7-$\alpha$-dehydrogenase, of intestinal bacteria by meat.6)7)

Goldin et al. demonstrated that a diet containing Lactobacillus acidophilus more significantly lowered the activity of fecal $\beta$-glucuronidase, nitroreductase and azoreductase in rats and humans than a diet without it.6)8)

Recently, we discovered that $\beta$-glucosidase and $\beta$-glucuronidase were pH-inducible.9) These enzymes of intestinal bacteria were induced by high pH, but the optimal pH of the enzymes was 6–7.

Here, the objective of the present study is to isolate alkalotolerant bacteria from intestinal microflora and to investigate physiological inducers and inhibitors of the $\beta$-glucosidase and $\beta$-glucuronidase of alkalotolerant intestinal bacteria.

MATERIALS AND METHODS

Materials 2,4-Dimethylhydrazine, benzo[a]pyrene, lactulose, saccharic acid 1,4-lactone, $\alpha$-glucuronic acid, $p$-nitrophenyl-$\beta$-$d$-glucopyranoside and $p$-nitrophenyl-$\beta$-$d$-glucuronide were purchased from Sigma Chem. Co. (U.S.A.). General anaerobic medium (GAM) and tryptic soy broth (TS) media were from Nissui Seiyaku Co., Ltd. (Japan). The antibiotics were from Dong-A Pharm. Co., Ltd. (Korea).

Culture of Alkalotolerant Intestinal Bacteria and Screening of Bacteria Producing the Enzymes An aliquot of 0.2 ml of the $10^3$, $10^4$, $10^5$-diluted human and rat feces was inoculated in GAM and TS (containing 5% sheep blood) agar plate, which had pH value of 5, 6, 7, 8, 9, 10 and 11: these pH were adjusted with HCl or NaOH. Next, GAM agar plates were anaerobically incubated in a gas pack jar at 37°C for 2–5 d and TS agar plates were incubated aerobically at 37°C for 1–2 d. Each colony cultured at the plate of pH 10 and 11 was incubated in 5 ml of GAM broth, and the productivity of the $\beta$-glucuronidase and $\beta$-glucosidase was measured. The bacteria producing $\beta$-glucosidase or $\beta$-glucuronidase are judged as enzyme-positive. The isolated bacteria were identified according to Bergey's manual.10)

The Preparation of a Fecal Sample for Assay of Enzyme Activity Fresh feces were collected from 3 healthy men (twenties, 60–70 kg) and pooled. Male rats (SDD Wistar, 180–200 g) were maintained on pelleted food (Samyang, Korea) and tap water ad lib. Each group consisted of 3 rats. In the case of rats treated with antibiotics, the antibiotic mixtures11) (175 mg chloramphenicol, 500 units nystatin, 20 mg streptomycin, 10 mg erythromycin and 200 units penicillin per rat) were administered orally once a day for 3 d before the experiments. Fresh feces of these rats were collected and pooled. Each feces was immediately suspended in 10 ml of 25 mm phosphate buffer at pH 7.0. The suspended samples were centrifuged at 30 × g for 5 min and the supernatants were used for the enzyme solution. The above enzyme-positive bacteria were cultured in GAM broth (for the experiment involving enzyme induction by substrates, in GAM broth with additional $p$-nitrophenyl-$\beta$-$d$-glucopyranoside for $\beta$-glucosidase and $p$-nitrophenyl-$\beta$-$d$-glucuronide for $\beta$-glucuronidase: final concentrations of the substrate were 0, 0.1, 0.5 and 1.0 mM), centrifuged at 6000 × g for 20 min and suspended in 10 ml of 25 mm phosphate buffer at pH 7.0. This was used as the enzyme solution. Sample suspension and subsequent manipulation were performed at 4°C.
Enzyme Assay For the assay of β-glucosidase, the reaction mixture consisting of 0.4 ml of 2 mM \( p \)-nitrophenyl-\( \beta \)-D-glucopyranoside, 0.6 ml of 0.1 M phosphate buffer, pH 7.0, and 0.2 ml of enzyme solution was prepared. For the assay of β-glucuronidase, a reaction mixture consisting of 40 μl of 10 mM \( p \)-nitrophenyl-\( \beta \)-D-glucuronide, 0.76 ml of 0.1 M phosphate buffer, pH 7.0, and 0.2 ml of the enzyme solution was prepared. Each reaction mixture was incubated at 37°C for 30 min and the reaction was stopped by adding 0.25 N NaOH. The stopped reaction mixture was centrifuged at 1000 \( \times \) g for 10 min. Then, the absorbance of the reaction mixture was measured at 405 nm within 10 min (Shimadzu UV 120-02, Japan).

RESULTS

Enzyme Activity of the Fecal Suspension The fecal β-glucosidase activity was 1.69 for rat and 3.94 μmol/min/g wet feces for human. The β-glucuronidase activity of these feces was 1.54 and 2.92, μmol/min/g wet feces, respectively. After oral administration of antibiotics, 80 to 90% of the enzyme activity was reduced in rats. After stopping the administration of antibiotics, the enzyme activity was restored within 2 weeks. That is, the majority of the enzymes, β-glucosidase and β-glucuronidase, were originated from intestinal bacteria, not the intestinal membrane. The pH-profile of these enzyme activities is shown in Fig. 1. The optimal pH of β-glucosidase and β-glucuronidase was 6—7 and 7, respectively.

A Profile of Alkalotolerant Intestinal Bacteria When the intestinal microflora were cultured in an agar medium of a different pH, they grew well in a a wide pH range of the medium. About \( 8 \times 10^{10} \) bacteria per g wet feces of humans and rats were observed in the GAM agar medium of pH 7, and \( 2-6 \times 10^{8} \) bacteria per g wet feces of both species were in the TS agar medium of pH 7. However, by the changing pH of the GAM medium to 11, the bacteria were dramatically decreased to 0.8% of the total bacteria in pH 7, and in the case of the TS medium, no colony was observed at pH 11.

Optimal pH of the Enzymes of the Alkalotolerant Intestinal Bacteria The productivity of β-glucosidase or β-glucuronidase was investigated for each intestinal bacteria grown in GAM and TS agar plates of pH 10 and 11. From the feces of humans and rats, 6 and 5 kinds of β-glucosidase producing bacteria were isolated, respectively. Among them, two bacteria isolated from human feces and three from rat feces were Streptococcus faecalis. The other bacteria were not identified. The pH profiles of the enzyme activities of these alkalotolerant bacteria are shown in Fig. 2. The optimal pH of the enzyme fell into two groups, 6—8 (neutral) and 8—10 (alkaline). The enzymes of some bacteria worked only in one group of optimal pH, but the other did so in both groups of the optimal pH.

When these alkalotolerant bacteria were cultured in GAM broth having 6 different pH values (5 to 10), the enzyme activity was increased by elevating the pH of the

![Fig. 1. pH Profile of β-Glucosidase (A) and β-Glucuronidase (B) Activities of the Feces of Humans (▲) and Rats (●)](image_url)

![Fig. 2. Induction and Optimal pH of β-Glucosidase of the Isolated Bacteria](image_url)
medium to 8—9, but the growth of the alkalotolerant bacteria was unchanged at the different medium pH. Particularly, the enzyme of the alkalotolerant bacteria, whose optimal pH was alkaline, were induced well in the alkaline medium.

From the feces of humans and rats, 11 and 5 kinds of bacteria producing β-glucuronidase were isolated, respectively. Among them, three bacteria isolated from human feces and two from rat feces were E. coli. The other bacteria were not identified. The pH profiles of the β-glucuronidase activity of these alkalotolerant bacteria are shown in Fig. 3 (all were not shown). The optimal pH fell into one group, pH 6—7. When these isolated bacteria were cultured in a GAM broth having 6 different pH values, the enzyme activity in the medium of pH 6 was increased 4.5-fold by elevating the pH of the medium to 8—9, but the growth was unchanged at a different medium pH.

**Effect of pH on Enzyme Activity during the Growth of the Intestinal Bacteria**

Alkalotolerant bacteria (β-glucosidase producing bacterium isolated from human total flora (HGO)-7 and β-glucuronidase producing bacterium isolated from human total flora (HGU)-3) isolated from human feces were cultured in a pH 5 GAM broth to reach a stationary phase, then the pH of these cultured media was adjusted to 8. By changing the pH of the media from 5 to 8, HGO-7 β-glucosidase activity increased 9.2-fold. HGU-3 β-glucuronidase activity was also dramatically increased 11.5-fold.

The bacteria (β-glucosidase producing bacterium isolated from rat total flora (RGO)-3 and β-glucuronidase producing bacterium isolated from rat total flora (RGU)-2), isolated from rat feces, were also cultured in a pH 5 GAM broth to reach a stationary phase, then the medium changed from pH 5 to 8. By changing the pH of the medium from 5 to 8, β-glucosidase and β-glucuronidase were induced 10.3- and 12.1-fold, respectively.

**Induction and Inhibition of the Enzyme**

To investigate whether or not the enzyme of the intestinal bacteria was induced by its substrate, p-nitrophenyl-β-D-glucopyranoside and p-nitrophenyl-β-D-glucuronide were used as substrates of β-glucosidase and β-glucuronidase, respectively. β-Glucosidase and β-glucuronidase of the intestinal microflora of humans and rats faeces were significantly induced, 4- and 7-fold, by adding 0.1 mm of the substrates to the medium, respectively. However, these enzymes were not induced by increasing the substrate level in the medium from 0.1 to 1 mm. β-Glucosidase and β-glucuronidase of the alkalotolerant intestinal bacteria were also induced 3 to 15-fold and 2 to 16-fold by adding 0.1 to 1.0 mm of the substrates to the medium, respectively.

β-Glucuronidase(s) of total intestinal bacteria and alkalotolerant bacteria was inhibited by saccharic acid 1,4-lactone. The 50% inhibitory concentrations were 0.5 mm
for the total intestinal bacteria and 0.4 mm for the alkalo-tolerant bacteria, respectively. d-Glucuronic acid weakly inhibited β-glucuronidase (IC₅₀ 40 mm).

**Productivity of the Enzyme by Lactulose** The inhibitory effect of lactulose on the enzymes, β-glucosidase and β-glucuronidase, was investigated (Fig. 4). When β-glucuronidase or β-glucosidase were incubated with lactulose, the enzyme activities of these enzymes were not inhibited by lactulose. However, when the lactulose was added in the medium and intestinal microflora was inoculated in the medium, the productivities of these enzymes were dramatically decreased. When human intestinal microflora were cultured in the medium containing 1% (w/v) lactulose, the activities of β-glucosidase and β-glucuronidase were decreased to 27% and 14%, of the control, respectively. At 5% (w/v) lactulose containing medium, the activities of β-glucosidase and β-glucuronidase were decreased to 18% and 11%, respectively. After intestinal microflora were cultured in the medium containing 1% (w/v) lactulose, the pH of the medium was decreased compared to that of control. The pH of the medium containing lactulose was 1.1 lower than that of the control. The pH of the medium containing 5% (w/v) lactulose was 0.15 lower than that of the medium containing 1% (w/v) lactulose.

**DISCUSSION**

β-Glucosidase and β-glucuronidase of the intestinal bacteria are associated with the conversion of a pro-carcinogen to a carcinogen: β-glucosidase hydrolyzes natural glycosides, such as cycasin and amygdalin, and β-glucuronidase does glucuronic acid conjugates of endogenous and exogenous compounds, bilirubin and benzof[b]pyrene. The induction of these enzymes of intestinal bacteria are related to the incidence of colon cancer. Also, a high pH stool is related to the incidence of colon cancer. Populations with alkaline fecal pH have a greater risk for colon cancer than those with acid faecal pH. Thornton insisted that a high pH colon promotes colorectal cancer. However, we insist that the high pH induces the enzymes of intestinal bacteria, and the induced enzymes are what promote colon cancer. Some diets are bacterically degraded, and colonic pH may become alkaline. High colonic pH induces the activity of β-glucosidase and β-glucuronidase. Therefore, these enzymes promote colon cancer. The enzyme activity in a media of 7 was about 10-fold higher than that of pH 6.

This kind of situation can be occured anytime in the intestine. *Lactobacillus*-like beverages may also change the pH of the intestine, and thus decrease the incidence of colon cancer. Furthermore, β-glucuronidase and β-glucosidase were induced by their substrates. These results suggest that some toxic compounds were excreted into the intestine via the biliary duct, the conjugates induce β-glucuronidase(s), and the metabolites become carcino-gens in the colon. The productivity of these enzymes, β-glucosidase and β-glucuronidase, were inhibited by culturing bacteria obtained from intestinal microflora in the medium containing lactulose, but lactulose did not directly inhibit the enzyme. However, saccharic acid 1,4-lactone inhibited the β-glucuronidase of alkalo-tolerant intestinal bacteria. That is, when intestinal bacteria were inoculated into a medium containing lactulose, some bacteria which utilized lactulose grew well, and the pH of the medium was decreased. By significantly decreasing the medium, the productivity of the enzyme was, we thought, decreased. These results were supported by Samelson's insistence that rats with an acid stool pH produced by the consumption of lactulose had significantly fewer colon tumors.

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**REFERENCES**